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## A Cardiovascular Drug Rescues Mice from Lethal Sepsis by Selectively Attenuating a Late-Acting Proinflammatory Mediator, High Mobility Group Box 1<sup>1</sup>

Wei Li<sup>\*</sup>, Jianhua Li<sup>†</sup>, Mala Ashok<sup>\*</sup>, Rongqian Wu<sup>†</sup>, Dazhi Chen<sup>†</sup>, Lihong Yang<sup>†</sup>, Huan Yang<sup>†</sup>, Kevin J. Tracey<sup>†</sup>, Ping Wang<sup>†</sup>, Andrew E. Sama<sup>\*</sup>, and Haichao Wang<sup>2,\*†</sup>

<sup>\*</sup>Department of Emergency Medicine, North Shore University Hospital, New York University School of Medicine, Manhasset, NY 11030

<sup>†</sup>Feinstein Institute for Medical Research, Manhasset, NY 11030

### Abstract

The pathogenesis of sepsis is mediated in part by bacterial endotoxin, which stimulates macrophages/monocytes to sequentially release early (e.g., TNF, IL-1, and IFN- $\gamma$ ) and late (e.g., high mobility group box 1 (HMGB1) protein) proinflammatory cytokines. The recent discovery of HMGB1 as a late mediator of lethal sepsis has prompted investigation for development of new experimental therapeutics. We found that many steroidal drugs (such as dexamethasone and cortisone) and nonsteroidal anti-inflammatory drugs (such as aspirin, ibuprofen, and indomethacin) failed to influence endotoxin-induced HMGB1 release even at superpharmacological concentrations (up to 10–25  $\mu$ M). However, several steroid-like pigments (tanshinone I, tanshinone IIA, and cryptotanshinone) of a popular Chinese herb, Danshen (*Salvia miltiorrhiza*), dose dependently attenuated endotoxin-induced HMGB1 release in macrophage/monocyte cultures. A water-soluble tanshinone IIA sodium sulfonate derivative (TSNIIA-SS), which has been widely used as a Chinese medicine for patients with cardiovascular disorders, selectively abrogated endotoxin-induced HMGB1 cytoplasmic translocation and release in a glucocorticoid receptor-independent manner. Administration of TSNIIA-SS significantly protected mice against lethal endotoxemia and rescued mice from lethal sepsis even when the first dose was given 24 h after the onset of sepsis. The therapeutic effects were partly attributable to attenuation of systemic accumulation of HMGB1 (but not TNF and NO) and improvement of cardiovascular physiologic parameters (e.g., decrease in total peripheral vascular resistance and increase in cardiac stroke volume) in septic animals. Taken together, these data re-enforce the pathogenic role of HMGB1 in lethal sepsis, and support a therapeutic potential for TSNIIA-SS in the treatment of human sepsis.

Sepsis is defined as a systemic inflammatory response syndrome resulting from a microbial infection. As a continuum of increasing clinical severity, severe sepsis is defined as sepsis associated with one or more acute organ dysfunctions (1). Despite recent advances in antibiotic therapy and intensive care, sepsis is still the most common cause of death in intensive care units, claiming ~225,000 victims annually in the United States alone. The pathogenesis of sepsis is attributable, at least in part, to dysregulated systemic inflammatory responses

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<sup>2</sup>Address correspondence and reprint requests to Dr. Haichao Wang, Department of Emergency Medicine, North Shore University Hospital, New York University School of Medicine, 350 Community Drive, Manhasset, NY 11030. E-mail address: hwang@nshs.edu

#### Disclosures

H. Wang, A. E. Sama, and D. Chen are coinventors of a patent application with the title “Inhibition of inflammatory cytokine production with tanshinones.” This patent application has been filed by their employer, The Feinstein Institute for Medical Research, which is designated as the sole owner of the patent application.

characterized by excessive accumulation of various proinflammatory mediators such as TNF or IL-1 (2), IFN- $\gamma$  (3), NO (4), and macrophage migration inhibitory factor (5-7).

We recently discovered that a ubiquitous protein, high mobility group box 1 (HMGB1),<sup>3</sup> is released by activated macrophages/monocytes (8-11) and functions as a late mediator of lethal endotoxemia and sepsis (8,12-14). Circulating HMGB1 levels are elevated in a delayed fashion (after 16–32 h) in endotoxemic and septic mice (8,12) and in patients with sepsis (8). Administration of recombinant HMGB1 to mice recapitulates many clinical signs of sepsis, including fever (15), derangement of intestinal barrier function (16), tissue injury (17), and multiple organ failure (8). Administration of anti-HMGB1 Abs or inhibitors (e.g., ethyl pyruvate, nicotine, or stearyl lysophosphatidylcholine) significantly protects mice against LPS-induced acute tissue injury (17,18) and lethal endotoxemia (8,13,19,20). Notably, these anti-HMGB1 reagents are capable of rescuing mice from lethal experimental sepsis even when the first doses are given 24 h after the onset of sepsis (12,13,21,22), indicating a wider window for HMGB1-targeted therapeutic strategies. Therefore, agents proven clinically safe, and yet still capable of attenuating HMGB1 release, may hold potential in the prevention and treatment of inflammatory diseases.

Throughout human history, herbal medicine has formed the basis of folk remedies for various inflammatory ailments. The use of willow bark extract to reduce pain and fever was documented by a Greek physician (Hippocrates) in the 5th century B.C. The subsequent discovery of salicylic acid as a pain or fever relief active component gave rise to the first synthetic nonsteroidal anti-inflammatory drug (NSAID), aspirin, and the birth of the pharmaceutical industry. Among thousands of Chinese medicinal herbs, only a few have been entitled Shen (e.g., Ren Shen (ginseng), Dan Shen (*Salvia miltiorrhiza*)). Danshen refers to a medicinal herb (termed shen) containing substance of premier medicinal value (termed Dan, cinnabar), and has been widely used in China for patients with cardiovascular disorders (23). Its beneficial effects are attributable to several red pigments including tanshinones I, II, IV, and cryptotanshinone (24,25), which exhibit various anti-inflammatory properties (26-28). In this study, we evaluated their capacity in inhibiting endotoxin-induced HMGB1 release, and explored their therapeutic potential in animal models of lethal endotoxemia and sepsis.

## Materials and Methods

### Cell culture

Murine macrophage-like RAW 264.7 cells were obtained from the American Type Culture Collection, and primary peritoneal macrophages were isolated from BALB/c mice (male, 7–8 wk, 20–25 g) at 2–3 days after i.p. injection of 2 ml thioglycolate broth (4%) as previously described (9,10). Murine macrophages were precultured in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10% FBS and 2 mM glutamine. Human blood was obtained from anonymous healthy donors recruited by the Long Island Blood Bank. The study was considered to be exempt by the North Shore-Long Island Jewish Health System, Office of the Institutional Review Board, in accordance with the guidelines for human subject protection. Human PBMC were isolated from the blood of healthy donors by density gradient centrifugation through Ficoll (Ficoll-Paque PLUS; Amersham Biosciences), and cultured in RPMI 1640 supplemented with 10% heat-inactivated human serum/2 mM L-glutamine as previously described (9).

<sup>3</sup>Abbreviations used in this paper: HMGB1, high mobility group box 1; CLP, cecal ligation and puncture; NSAID, nonsteroidal anti-inflammatory drug; TSNIIA-SS, tanshinone IIA sodium sulfonate.

## LPS stimulation

Adherent macrophages or monocytes were gently washed with, and cultured in, serum-free OptiMEM I medium 2 h before stimulation with bacterial endotoxin (LPS, *Escherichia coli* O111:B4; Sigma-Aldrich). At 16 h after LPS stimulation, levels of HMGB1 in the culture medium were determined as previously described (8-10).

## Preparation of herbal extract

Various Chinese herbs were obtained from New York Tongrentang and extracted in water (85°C) for 4 h. The water-soluble fraction was cleared sequentially by centrifugation (3300 × *g* for 20 min, 4°C) and filtration (through a 0.2- $\mu$ m filter), and the filtrate fraction was examined for HMGB1-suppressing activities.

## Chemical sources and stock solutions

Dexamethasone, cortisone, and RU486 were obtained from Sigma-Aldrich, and 10 mM stock solutions were prepared in 100% ethanol. Aspirin (catalog no. A0819), ibuprofen (catalog no. I0481), indomethacin (catalog no. I5315) were obtained from the LKT Laboratories, and 10 mM stock solution were prepared in water (20 mM). Tanshinone I (catalog no. T0153, 98.6% HPLC purity), tanshinone IIA (catalog no. T0154, 93.0% HPLC purity), and cryptotanshinone (catalog no. C7097, 98.8% HPLC purity) were obtained from the LKT Laboratories, and 20 mM stock solutions were prepared in DMSO. Tanshinone IIA sodium sulfonate (TSNIIA-SS) was obtained from the Shanghai No. 1 Biochemical & Pharmaceutical, and its purity was determined by HPLC using a Nova-Pak C18 column (3.9 × 150 mm) and 0.065% trifluoroacetic acid (v/v, in water) as the mobile phase. The sample was eluted by a linear gradient of 0–59% acetonitrile (v/v, in 0.065% trifluoroacetic acid) over 12 min at a flow rate of 1.0 ml/min, and monitored at a wavelength of 452.2 nm. Each HPLC peak was analyzed using a Liquid Chromatography-Mass Spectrometry (LCQ DECA XP<sup>PLUS</sup>; Thermo Electron Corporation). The major peak (at a retention time of 8.3 min) accounted for >80% of the total area under the HPLC peaks and contained a red pigment with a mass-to-charge ratio of  $m/z = 373.07$  (100.0%), corresponding to an empirical formula of  $C_{19}H_{17}O_6S^-$  (TSNIIA-SS).

## Animal models of endotoxemia and sepsis

This study was approved and performed in accordance with the Guidelines for the Care and Use of Laboratory Animals at the Feinstein Institute for Medical Research (Manhasset, NY). Endotoxemia was induced in BALB/c mice (male, 7–8 wk) by i.p. injection of bacterial endotoxin (LPS, 15 mg/kg) as previously described (8,19,29). Sepsis was induced in male BALB/c mice (7–8 wk, 20–25 g) or Sprague-Dawley rats (290–310 g) by cecal ligation and puncture (CLP) as previously described (29,30). Herbal components were administered i.p. into mice at indicated doses and time points, and mice were monitored for survival for up to 2 wk. In parallel experiments, mice were euthanized to collect blood at 28 h (following two doses of herbal components at –0.5 and +24 h) after endotoxemia and at 52 h (following two doses of herbal components at +24 and +48 h) after CLP.

## TNF ELISA

The levels of TNF in the culture medium or serum were determined using commercial ELISA kits (catalog no. MTA00; R&D Systems) with reference to standard curves of purified recombinant TNF at various dilutions as previously described (9,10,29).

## NO assay

The levels of NO in the culture medium were determined indirectly by measuring the  $\text{NO}_2^-$  production with a colorimetric assay based on the Griess reaction (9).  $\text{NO}_2^-$  concentrations were determined with reference to a standard curve generated with sodium nitrite at various dilutions.

## HMGB1 Western blotting analysis

The levels of HMGB1 in the culture medium or serum were determined by Western blot analysis as previously described (8-10). The relative band intensity was quantified by using the NIH Image 1.59 software to determine HMGB1 levels with reference to standard curves generated with purified HMGB1.

## Cytokine Ab array

Murine cytokine Ab array (catalog no. M0308003; RayBiotech), which detects 62 cytokines on one membrane, was used to determine the profile of cytokines in the culture medium following the manufacturer's instructions. Briefly, the membranes were sequentially incubated with equal volume of cell-conditioned culture medium, primary biotin-conjugated Ab, and HRP-conjugated streptavidin. After exposing to x-ray film, the relative signal intensity was determined using NIH Image 1.59 software with reference to the positive controls on the membrane.

## Immunocytochemistry and cell fractionation or Western blot

At 16 h after LPS stimulation, cellular HMGB1 was immunostained with anti-HMGB1 polyclonal Abs, and images were acquired using fluorescent microscope (Carl Zeiss Microimaging) as previously described (9,10). Alternatively, localization of HMGB1 was examined by a cell fractionation (Western blotting) technique as previously described (11). Cell fractionation is based on differential lysis of plasma and nuclear membranes by nonionic detergent (Nonidet P-40). Briefly, after selective lysis of the plasma membrane in low salt buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, and 1% Nonidet P-40), the intact nuclei was collected by a quick centrifugation step ( $7000 \times g$  for 1 min at  $4^\circ\text{C}$ ), leaving the cytoplasmic fraction in the supernatant. The nuclei pellet was resuspended in Nonidet P-40 high salt buffer (20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, and 1% Nonidet P-40), and briefly sonicated to generate the nuclear fraction. After fractionation, the protein content of different fractions was determined by a Bradford method, and each fraction was assayed for levels of various protein by Western blot analysis using primary Abs specific for HMGB1, a cytoplasmic protein ( $\beta$ -actin; Santa Cruz Biotechnology), and a proliferating cell nuclear Ag protein (BD Biosciences).

## Cell viability assays

Cell viability was assessed by trypan blue exclusion assays as previously described (9). Briefly, trypan blue was added to cell cultures at a final concentration of 0.08%. After incubation for 5 min at  $25^\circ\text{C}$ , cell viability was assessed by the percentage of dye-excluding cells in five  $\times 40$  microscope fields.

## Measurement of cardiac output

We used a radiolabeled microsphere technique to measure cardiac output at 20 h after CLP or sham operation as previously described (30). Briefly, a bolus of  $^{141}\text{Ce}$ -labeled microspheres ( $4.0 \mu\text{Ci}$ ; DuPont NEN) was injected into the left ventricle, where they were mixed uniformly

with the oxygenated blood at the root of the aorta and subsequently distributed via aortic blood flow to the capillary beds within each organ. The reference blood sample was withdrawn from the femoral arterial catheter (at a rate of 0.7 ml/min) with a pump (Harvard Apparatus), after which isotonic sodium chloride solution was infused manually to replace the volume of blood lost. At 20 h after CLP, animals were sacrificed to harvest various organs for measurement of radioactivity with an automatic gamma counter (1470 Wizard; Wallac). Cardiac output and organ blood flow were calculated according to the following equations, respectively:  $((RBF \times C_T)/C_r) \times 1/100$  and  $((RBF \times C_i)/C_r) \times 1/100$ , where *RBF* is the reference blood sample withdrawal rate (0.7 ml/min), *C<sub>T</sub>* is cpm of total injected dose, *C<sub>i</sub>* is cpm per gram of tissue, and *C<sub>r</sub>* is cpm in the reference blood sample. Stroke volume and total peripheral resistance were calculated as previously described (30).

### Statistical analysis

Data are expressed as mean  $\pm$  SD of two independent experiments in triplicate. One-way ANOVA was used for comparison among all different groups. When the ANOVA was significant, posthoc testing of differences between groups was performed using Tukey's test. The Kaplan-Meier method was used to compare the differences in mortality rates between groups. A value of  $p < 0.05$  was considered statistically significant.

## Results

### Danshen (*Salvia miltiorrhiza*) extract and components attenuated endotoxin-induced HMGB1 release

The successful identification of salicylic acid as the active principle of willow bark to reduce fever and pain gave rise to the first synthetic NSAID, aspirin. Many NSAIDs (such as aspirin, ibuprofen, and indomethacin) can inhibit cyclooxygenases, but are unable to protect animals against lethal sepsis (31-33). In this study, we found that none of these cyclooxygenase inhibitors (e.g., aspirin, ibuprofen, and indomethacin) significantly affected LPS-induced HMGB1 release even at superpharmacological concentrations (1–25  $\mu$ M, data not shown). Conversely, we also screened 25 medicinal herbs, and found that Danshen (*Salvia miltiorrhiza*) extract dose dependently attenuated endotoxin-induced HMGB1 release (Fig. 1A).

To determine Danshen's active principle(s), we examined its anti-inflammatory components for HMGB1-inhibiting activities. One potential anti-inflammatory component, ferulic acid, effectively attenuated LPS-induced NO production, but failed to affect LPS-induced HMGB1 release (29). Danshen also contains abundant red pigments (termed tanshinone I, tanshinone IIA, and cryptotanshinone) (Fig. 1B), a group of substances with medicinal value for patients with cardiovascular abnormalities (23). Interestingly, all three tanshinones (I, IIA, and cryptotanshinone) effectively attenuated LPS-induced HMGB1 release, with estimated  $IC_{50} < 25 \mu$ M (Fig. 1C).

### Water-soluble TSNIIA-SS selectively attenuated endotoxin-induced HMGB1 release

Most tanshinones are barely water-soluble at physiological temperature, and this poor solubility may adversely affect their bioavailability in vivo. We thus examined a water-soluble derivative TSNIIA-SS (Fig. 1B) for HMGB1-inhibiting activities. TSNIIA-SS is a widely used Chinese medicine for patients with cardiovascular disorders (23), and was obtained from the Shanghai No. 1 Biochemical & Pharmaceutical. HPLC analysis revealed a major peak (>80%, at a retention time of 8.3 min) containing a red pigment with an  $m/z = 373.07$ , corresponding to an empirical formula of  $C_{19}H_{17}O_6S^-$  (tanshinone IIA sulfonate). The minor HPLC peak (<20%, at a retention time of 6.8 min) contained a red pigment with an  $m/z = 389.11$ , corresponding to an empirical formula of  $C_{19}H_{17}O_7S^-$  (an unknown analog of tanshinone IIA

sulfonate). The moderately purified TSNIIA-SS preparation (containing both TSNIIA-SS ( $C_{19}H_{17}O_6S^-$ , >80%) and TSNIIA-SS analog ( $C_{19}H_{17}O_7S^-$ , <20%)) dose dependently inhibited HMGB1 release, with an estimated  $IC_{50} < 5 \mu M$  (Fig. 2A). Similarly, it effectively inhibited LPS-induced HMGB1 release in cultures of primary human PBMC (Fig. 2B) and murine peritoneal macrophages (data not shown).

To identify the active principles of the moderately purified TSNIIA-SS preparation, we screened each HPLC fractions for potential activities to inhibit endotoxin-induced HMGB1 release. The HMGB1-inhibiting activities were coeluted specifically with two HPLC peaks (monitored at a wavelength of 452.2 nm, at a retention time of 6.8 and 8.3 min, respectively) corresponding to TSNIIA-SS ( $C_{19}H_{17}O_6S^-$ ) or TSNIIA-SS analog ( $C_{19}H_{17}O_7S^-$ ) (data not shown). In light of the observations that all tanshinones tested (including I, IIA, cryptotanshinone, TSNIIA-SS, and TSNIIA-SS analog) were capable of inhibiting endotoxin-induced HMGB1 release, we performed most studies using the moderately purified TSNIIA-SS preparation (containing >80% TSNIIA and <20% TSNIIA analog).

To further evaluate its anti-inflammatory properties, we determined the potential effects of TSNIIA-SS on LPS-induced release of other proinflammatory mediators. At concentrations (100  $\mu M$ ) that completely abrogated LPS-induced HMGB1 release, TSNIIA-SS did not completely block LPS-induced release of NO (Fig. 2A, *top*) or TNF (Fig. 2B, *bottom*). Furthermore, its inhibitory effects on TNF (but not HMGB1) release were often lost when macrophages were stimulated with LPS at higher concentrations (>200 ng/ml), indicating selectivity for HMGB1 suppression. To evaluate this specificity, we determined its effects on the release of 62 cytokines using a cytokine Ab array. At concentrations that completely abrogated LPS-induced HMGB1 release (100  $\mu M$ , data not shown), TSNIIA-SS did not suppress the release of most cytokines (such as IL-6, IL-12p40/p70, KC, MCP-1, MIP-1 $\alpha$ , MIP-1 $\gamma$ , MIP-2, or TNF) in RAW 264.7 cells (Fig. 2C, *top panels*) or primary peritoneal macrophages (Fig. 2C, *bottom panels*). Consistent with a previous report (27), TSNIIA-SS only partially attenuated LPS-induced release of IL-12p70 (by  $45 \pm 5\%$ ), along with a few other cytokines (such as IL-1 $\alpha$  (by  $50 \pm 7\%$ ), platelet factor 4 (by  $35 \pm 6\%$ ), and MCP-5 (by  $25 \pm 5\%$ )). Taking together, these data indicate that TSNIIA-SS selectively attenuates LPS-induced release of HMGB1 as opposed to other proinflammatory cytokines.

### **Delayed administration of TSNIIA-SS still significantly attenuated endotoxin-induced HMGB1 release**

As compared with early proinflammatory cytokines (such as TNF), HMGB1 is released in a delayed fashion following endotoxin stimulation. It is intriguing to consider whether TSNIIA-SS can inhibit HMGB1 release if added after LPS stimulation. Whereas concurrent administration of TSNIIA-SS with LPS was maximally effective in suppressing HMGB1 release, significant inhibition was retained when it was added 2–6 h after LPS (Fig. 3). It may thus be feasible to pharmacologically attenuate late-acting proinflammatory mediators (such as HMGB1) by strategically administering TSNIIA-SS in a delayed fashion.

### **TSNIIA-SS inhibited endotoxin-induced HMGB1 release by blocking its cytoplasmic translocation**

To investigate a mechanism of tanshinone-mediated suppression of HMGB1 release, we determined its effect on endotoxin-induced HMGB1 cytoplasmic translocation, an essential step for HMGB1 release (9,10,34). Consistent with previous reports (10), quiescent macrophages constitutively expressed HMGB1 and maintained an intracellular “pool” of HMGB1 predominantly in the nucleus (Fig. 4A, *left column*). At 16 h after LPS stimulation, we observed significant HMGB1 staining in cytoplasmic vesicles (Fig. 4A, *middle column*), confirming that LPS stimulates macrophages to actively translocate nuclear HMGB1 to the

cytoplasm before releasing it into the extracellular milieu. Although the TSNIIA-SS did not affect the nuclear localization of HMGB1 in resting cells (data not shown), it almost completely abrogated LPS-induced HMGB1 cytoplasmic translocation in most endotoxin-stimulated cells (Fig. 4A, *right column*), indicating that TSNIIA-SS attenuates HMGB1 release by interfering with its cytoplasmic translocation.

To further validate the above hypothesis, cytoplasmic and nuclear fractions were isolated, and immunoblotted with Abs specific for HMGB1, a proliferating cell nuclear Ag protein, or  $\beta$ -actin (a cytoplasmic protein), respectively. Consistently, levels of HMGB1 in the cytoplasmic fractions were dramatically increased after LPS stimulation (data not shown), but were dramatically reduced by TSNIIA-SS treatment (Fig. 4B), confirming that TSNIIA-SS attenuates HMGB1 release by interfering with its cytoplasmic translocation.

### **TSNIIA-SS inhibited endotoxin-induced HMGB1 release in a glucocorticoid receptor-independent mechanism**

In light of the structural resemblance (i.e., the presence of a four-fused ring structure) between tanshinones and steroidal anti-inflammatory drugs (Fig. 5A), we evaluated: 1) whether corticosteroids similarly attenuated LPS-induced HMGB1 release and 2) whether TSNIIA-SS abrogates HMGB1 release in a glucocorticoid receptor-dependent mechanism. Even at concentrations up to 10  $\mu$ M, dexamethasone and cortisone failed to reduce LPS-induced HMGB1 release (Fig. 5B), although they effectively attenuated LPS-mediated TNF secretion (Fig. 5C). Steroidal anti-inflammatory drugs inhibit cytokines through binding to intracellular glucocorticoid receptor (35), which can be over-ridden by specific glucocorticoid receptor antagonists (such as RU486). Indeed, RU486 almost completely abrogated dexamethasone-mediated suppression of TNF secretion (Fig. 5C), but did not affect TSNIIA-SS-mediated inhibition of TNF (Fig. 5C), or HMGB1 release (data not shown). Taken together, these data indicate that TSNIIA-SS and dexamethasone use distinct mechanisms to suppress endotoxin-induced cytokine release.

### **TSNIIA-SS protected against lethal endotoxemia**

In light of the capacity of TSNIIA-SS in attenuating LPS-induced HMGB1 release, we explored its efficacy in an animal model of lethal endotoxemia. Administration with a single dose of TSNIIA-SS 30 min after an LD<sub>75</sub> dose of LPS did not significantly improve animal survival rate (25% for control receiving saline ( $n = 20$  mice/group) vs 37.5% for experimental group receiving TSNIIA-SS at 15 mg/kg ( $n = 20$  mice/group);  $p > 0.05$ ). By treating animals with three additional doses of TSNIIA-SS (+24, +48, and +72 h), we observed a dose-dependent improvement in animal survival (from 20% to 80%) (Fig. 6A). Furthermore, administration of TSNIIA-SS dose dependently attenuated circulating HMGB1 levels ( $140 \pm 20$  ng/ml, LPS plus vehicle vs  $40 \pm 25$  ng/ml ( $n = 10$  mice);  $p < 0.01$ ), suggesting that TSNIIA-SS protects animals against lethal endotoxemia partly through attenuating systemic HMGB1 accumulation.

### **TSNIIA-SS rescues mice from lethal sepsis**

Although endotoxemia is useful to investigate the complex cytokine cascades, more clinically relevant animal models are necessary to explore therapeutic agents for the treatment of human sepsis. One well-characterized, standardized animal model of sepsis is induced by CLP. In light of the late and prolonged kinetics of HMGB1 accumulation in experimental sepsis (12), we reasoned that it might be possible to rescue mice from lethal sepsis even if TSNIIA-SS is administered after the onset of sepsis. The first dose of TSNIIA-SS was given 24 h after the onset of sepsis, a time point at which mice developed clear signs of sepsis (including lethargy, diarrhea, piloerection). An i.p. administration with a single dose of TSNIIA-SS 24 h after the onset of sepsis failed to improve survival rate (33% for control receiving saline ( $n = 24$  mice/group) vs 50% for experimental group receiving TSNIIA-SS at 15 mg/kg ( $n = 24$  mice/group);

$p > 0.05$ ). However, repeated administration of TSNIIA-SS beginning 24 h after the onset of sepsis (followed by additional doses at 48, 72, and 96 h after sepsis) conferred a dose-dependent protection against lethal sepsis ( $n = 30$  mice/group) (Fig. 6B), significantly increasing animal survival rate from 33% to 73% ( $p < 0.05$ ).

### **TSNIIA-SS attenuates sepsis-induced systemic HMGB1 accumulation**

To gain insight into its protective mechanism, we evaluated the effects of TSNIIA-SS on the systemic accumulation of TNF, NO, and HMGB1. Consistent with an early report (31), systemic TNF was barely detectable at late stage of sepsis. Delayed administration of TSNIIA-SS did not attenuate circulating TNF levels at 52 h after the onset of sepsis (TNF =  $65 \pm 15$  pg/ml, vehicle control group ( $n = 10$  mice/group) vs TNF =  $85 \pm 23$  pg/ml, TSNIIA-SS group ( $n = 10$  mice/group);  $p > 0.05$ ). Similarly, delayed administration of TSNIIA-SS did not attenuate circulating NO levels at 52 h after the onset of sepsis ( $18.0 \pm 4.5$   $\mu$ M, vehicle control group vs  $15.5 \pm 3.3$   $\mu$ M, TSNIIA-SS group ( $n = 3$  mice);  $p > 0.05$ ). In contrast, repeated administration of TSNIIA-SS dose dependently and significantly attenuated ( $p < 0.05$ ) circulating HMGB1 levels in septic mice (Fig. 7), indicating that TSNIIA-SS confers protection against lethal sepsis partly by attenuating systemic HMGB1 accumulation.

### **TSNIIA-SS protected against sepsis-induced cardiac dysfunction**

Because TSNIIA-SS has been successfully used for patients with cardiovascular disorders (23), we also evaluated whether it improves cardiovascular function in septic animals. Despite the lack of myocardial injury (36), there is a hypodynamic change in cardiovascular function manifested by a significant decrease in cardiac output in the late stage (e.g., 20 h after the onset) of sepsis (30). Administration of TSNIIA-SS did not significantly affect the mean arterial blood pressure ( $106.1 \pm 4.7$  mm Hg, CLP group vs  $96.4 \pm 8.7$  mm Hg, CLP with TSNIIA-SS ( $n = 6$  mice);  $p > 0.05$ ), but slightly reduced the heart rate ( $378.3 \pm 25.1$  beats/minutes, CLP group vs  $334.1 \pm 25.8$  beats/minutes, CLP with TSNIIA-SS, 15 mg/kg ( $n = 6$  mice);  $p < 0.05$ ). More importantly, it dose dependently reduced total peripheral vascular resistance (Fig. 8A), and yet significantly increased cardiac stroke volume (Fig. 8B) and cardiac output (Fig. 8C). Taken together, these data indicate that TSNIIA-SS, an effective pharmacologic agent used for patients with cardiovascular disorders in China, appears to be protective against sepsis-induced cardiovascular dysfunction in an animal model of sepsis.

## **Discussion**

The pathogenesis of lethal sepsis remains obscure, but is associated with dysregulated inflammatory response, tissue injury, and multiple organ dysfunction. The inflammatory response is mediated in part by bacterial endotoxin (37), which stimulates macrophages/monocytes to sequentially release early (e.g., TNF and IL-1) and late (e.g., HMGB1) proinflammatory cytokines. Although early cytokines may be protective against infection (38), dysregulated inflammatory response sustained by late-acting mediators (such as HMGB1) may contribute to the development of tissue injury and organ dysfunction at the late stage of lethal sepsis. Therefore, agents capable of selectively attenuating systemic HMGB1 accumulation may hold potential in the treatment of lethal sepsis.

Many NSAIDs (e.g., aspirin, ibuprofen, and indomethacin) fail to protect against lethal sepsis (31), and consistently fail to significantly inhibit LPS-induced HMGB1 release (32,33). In contrast, the Chinese herb Danshen (*Salvia miltiorrhiza*) contains medicinal substances (such as tanshinone I, tanshinone IIA, and cryptotanshinone) that effectively attenuate endotoxin-induced HMGB1 release in macrophage/monocyte cultures. However, due to poor solubility and bioavailability, tanshinones I and IIA failed to rescue mice from lethal sepsis, even after repeated administration at 24, 48, 72, and 96 h after the onset of sepsis (survival rate = 50%,



control vehicle group vs survival rate = 57%, tanshinone I group, 12 mg/kg; survival rate = 61%, tanshinone IIA group, 12 mg/kg ( $n = 14$  mice/group);  $p > 0.05$ ), forcing us to explore other water-soluble derivatives as potential therapeutic agents.

TSNIIA-SS, a clinically approved drug for patients with cardiovascular disorders, completely abrogates endotoxin-induced HMGB1 release in macrophage/monocyte cultures. The mechanism by which TSNIIA-SS inhibits endotoxin-induced HMGB1 release remains elusive, but is partly attributable to its ability to interfere with LPS-induced HMGB1 cytoplasmic translocation. Although containing similar backbone chemical structure with glucocorticoids, TSNIIA-SS does not appear to use the glucocorticoid receptor to inhibit HMGB1 release because specific glucocorticoid receptor antagonist fails to abolish TSNIIA-SS-mediated inhibition of HMGB1 release. Interestingly, tanshinone I can inhibit phospholipase A<sub>2</sub> (26), an enzyme that enhances endotoxin-induced HMGB1 release by generating lysophosphatidylcholine (19). Similarly, cryptotanshinone can inhibit acetylcholinesterase (34), an enzyme that eliminates the HMGB1-inhibiting neuro-transmitter, acetylcholine (13, 39,40). It will thus be important to determine whether TSNIIA-SS inhibits HMGB1 release by inhibiting secretory phospholipase A<sub>2</sub> or acetylcholinesterase in future studies.

At concentrations that completely abrogated LPS-induced HMGB1 release, TSNIIA-SS does not affect the release of most (58 of 62) other cytokines, indicating selectivity for inhibiting HMGB1 over most other cytokines. Even when given several hours after LPS stimulation, TSNIIA-SS is still effective in blocking HMGB1 release, distinguishing itself from all previously known HMGB1 inhibitors (including ethyl pyruvate, nicotine, and stearyl lysophosphatidylcholine) (13,19,21). These unique properties enable us to strategically administer TSNIIA-SS in a delayed fashion to selectively attenuate systemic HMGB1 accumulation at late stage of sepsis. Indeed, delayed administration of TSNIIA-SS beginning at 24 h after CLP, a time point when all mice developed clear signs of sepsis (and some mice started to die), significantly rescued mice from lethal sepsis.

The observations that TSNIIA-SS failed to attenuate systemic NO accumulation, and failed to increase the mean arterial pressure at a late stage of sepsis, argue against a NO-dependent protective mechanism. Although TSNIIA-SS can somewhat reduce LPS-induced TNF secretion in monocytes (but not macrophages), its TNF-suppression activity may not account for its protective effects against lethal sepsis. First, TNF accumulates systemically within few hours following CLP (31), long before our strategic, delayed administration of TSNIIA-SS (at 24 h after CLP). Consistently, we observed that delayed administration of TSNIIA-SS did not significantly attenuate systemic accumulation of TNF at a late stage of sepsis. Second, TNF may play a protective role in sepsis because suppression of TNF activities with neutralizing Abs did not improve, but actually worsened survival in animal model of sepsis (38). It remains to be determined, however, whether TNF-suppression activity of TSNIIA-SS is attributable to its protective effects against lethal endotoxemia.

In a sharp contrast, delayed administration of TSNIIA-SS significantly attenuates systemic HMGB1 accumulation, suggesting that TSNIIA-SS rescues mice from lethal sepsis partly through attenuation of systemic accumulation of late-acting proinflammatory mediators. Nevertheless, the present study cannot eliminate the possibility that TSNIIA-SS confers protection against lethal endotoxemia or sepsis through additional mechanisms (such as inhibition of HMGB1-mediated inflammatory response). Indeed, our preliminary experimental data indicated that TSNIIA-SS, at concentrations up to 100  $\mu$ M, effectively attenuated HMGB1-induced release of TNF (by 50–60%) and NO (by 90–95%) in murine macrophage cultures, implicating that TSNIIA-SS may improve animal survival by suppression of HMGB1 release and cytokine activities.

In response to septic insult, rodents develop an early, hyperdynamic cardiovascular response (characterized by an increase in cardiac output and a decrease in total peripheral resistance) 5 h after CLP, which is followed by a late, hypodynamic cardiovascular response (manifested by a decrease in cardiac output and an increase in total peripheral resistance) at 20–24 h after CLP (41). As an effective pharmacologic agent used for patients with cardiovascular disorders in China, TSNIIA-SS dramatically reduces total peripheral vascular resistance, but significantly increases cardiac stroke volume and cardiac output in septic animals. Clinically, some patients with severe sepsis have normal or high cardiac output, which fuels an ongoing debate regarding the necessity to raise cardiac output (to improve oxygen delivery) and reduce systemic vascular resistance (to improve tissue oxygenation) for patients with severe sepsis (42,43). It will thus be important to investigate in future clinical studies how TSNIIA-SS affects the cardiovascular function of patients with severe sepsis, and whether TSNIIA-SS improves outcome.

### Acknowledgments

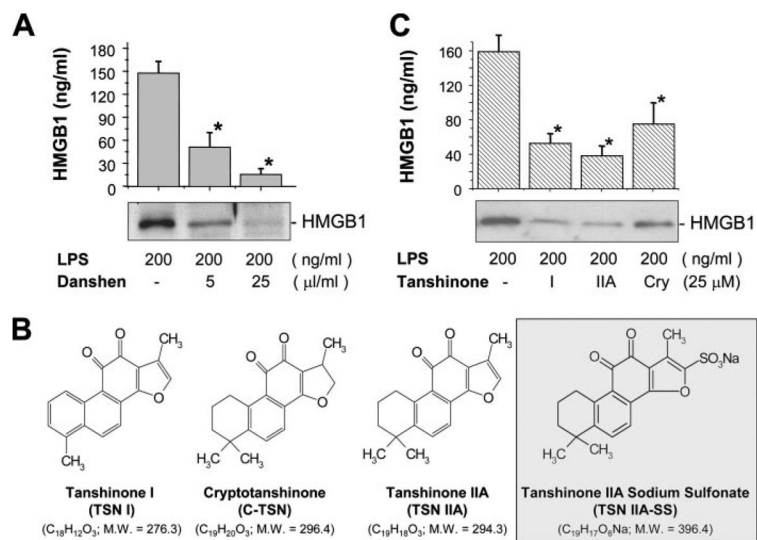
We thank Drs. Guoqian Chen, Weifeng Dong, and Mahendar Ochani for help with animal studies, and Dr. Bela Ruzsicska for expert help with the Liquid Chromatography-Mass Spectrometry analysis of tanshinones.

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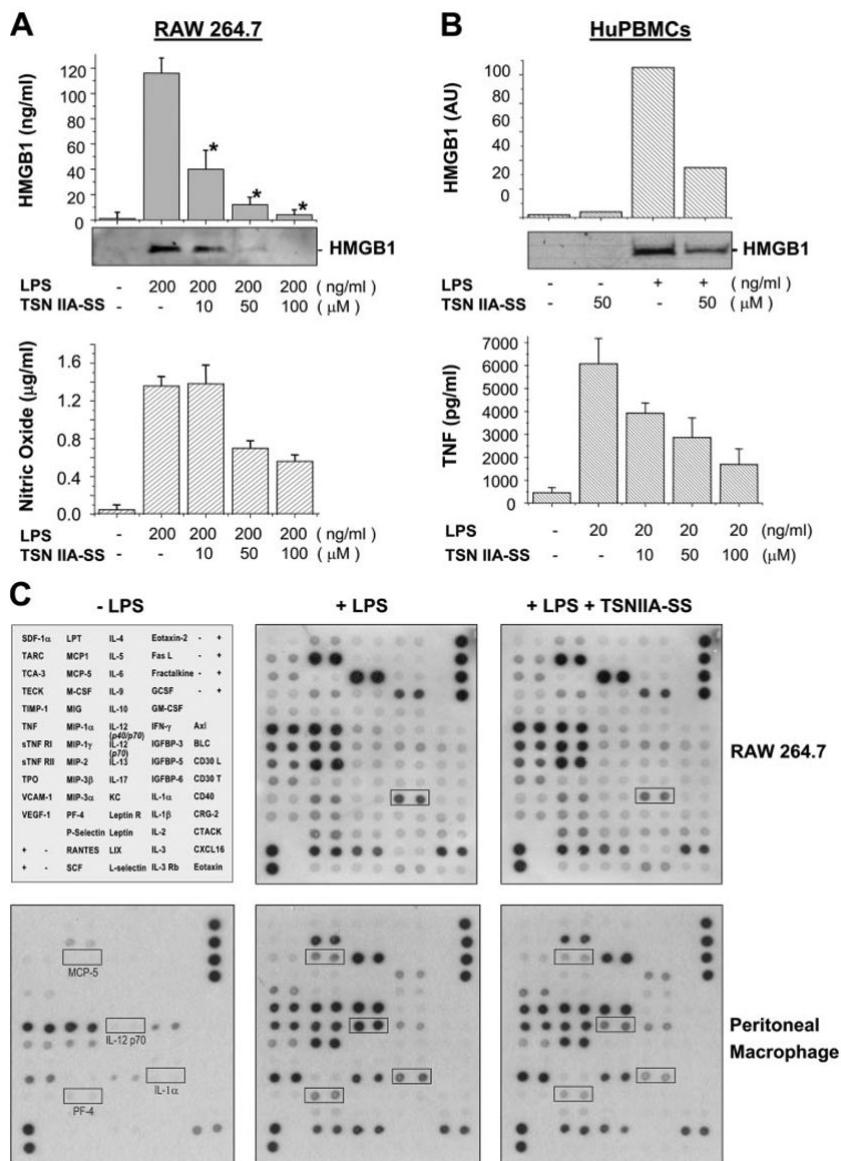
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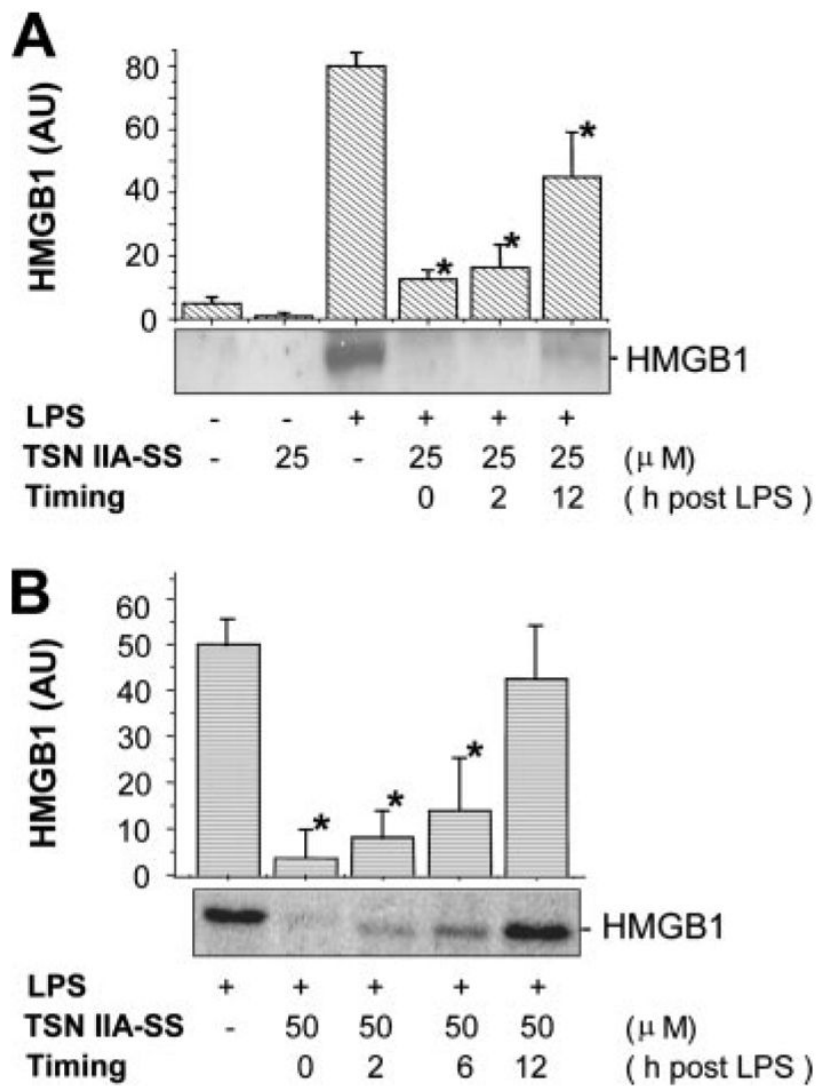
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**FIGURE 1.**

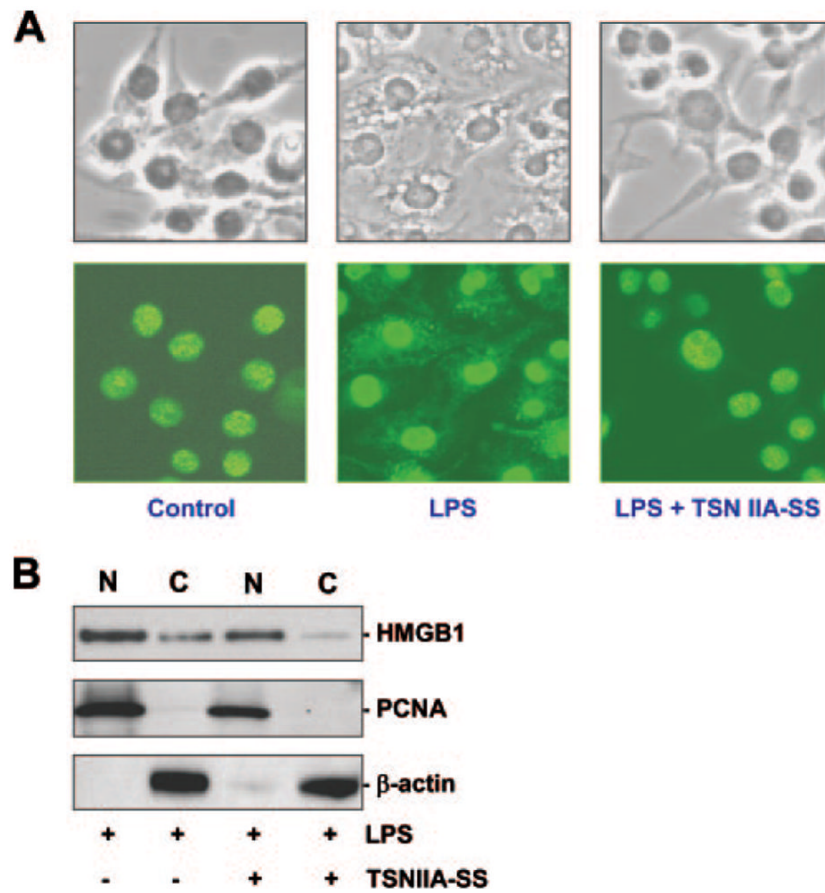
Danshen extract (A) and components (tanshinone I, tanshinone IIA, and cryptotanshinone) (B and C) attenuated endotoxin-induced HMGB1 release. Murine macrophage-like RAW 264.7 cells were stimulated with LPS in the absence or presence of herbal extract (A), and tanshinone I (TSN I), tanshinone IIA (TSN IIA), or cryptotanshinone (C-TSN) (B and C) for 16 h, and levels of HMGB1 in the culture medium were determined by Western blot analysis. A representative Western blot of three independent experiments with similar results is shown (bottom panel). \*,  $p < 0.05$  vs controls (+LPS alone).



**FIGURE 2.** TSNIIA-SS specifically abrogated endotoxin-induced HMGB1 release. Murine macrophage-like RAW 264.7 cells, primary murine peritoneal macrophages, or human PBMC were stimulated with LPS in the absence or presence of TSNIIA-SS at indicated concentrations. At 16 h after LPS stimulation, levels of HMGB1 (A and B), NO (A), or TNF (B and C) in the culture medium were determined by Western blot analysis (A and B), Griess reaction (A), ELISA (B), or cytokine array (C). Note that at concentrations that completely abrogated LPS-induced HMGB1 release, TSNIIA-SS did not completely block LPS-induced release of NO (A), TNF (B), or IL-1α, platelet factor 4 (PF-4), IL-12p70, and MCP-5 (C). A representative cytokine array (C) of two independent experiments with similar results is shown.

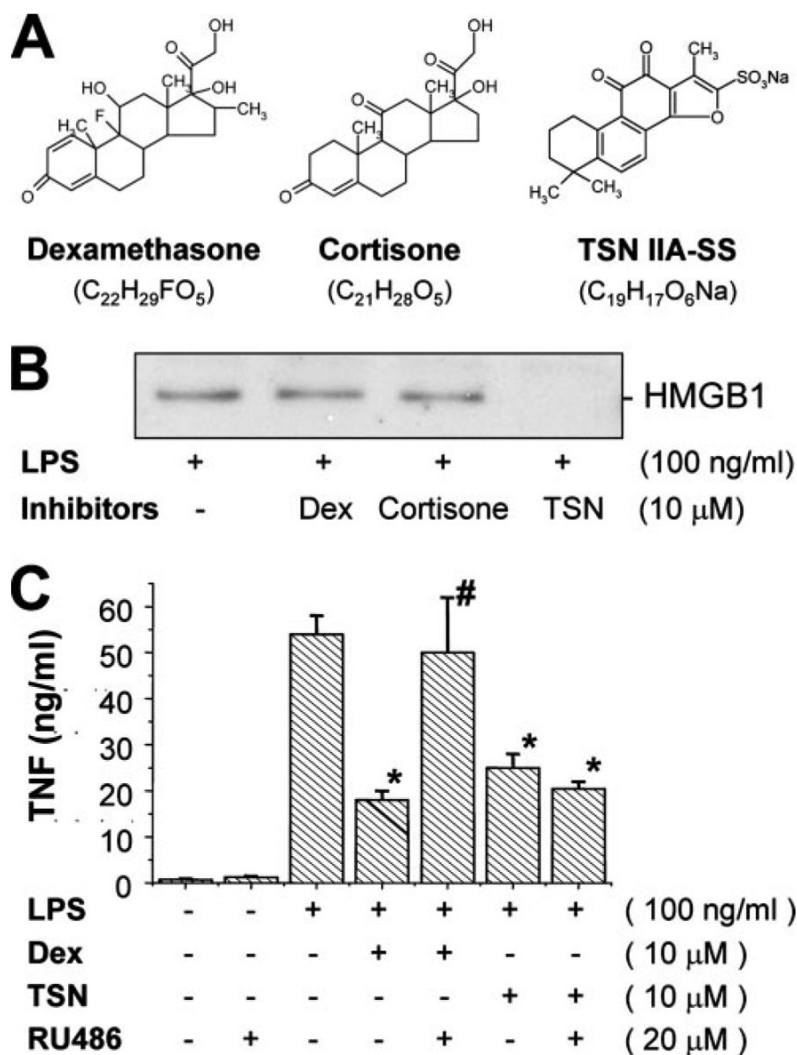
**FIGURE 3.**

Delayed administration of TSNIIA-SS still significantly attenuated endotoxin-induced HMGB1 release. Murine macrophage-like RAW 264.7 cells were stimulated with LPS, and TSNIIA-SS (25 μM (A) or 50 μM (B)) was added at 0, 2, 6, and 12 h after LPS stimulation. Levels of HMGB1 levels in the culture medium were determined at 16 h after LPS stimulation and expressed (in arbitrary unit, AU) as the mean ± SD of two independent experiments. A representative Western blot is shown at *bottom* of both experiments. \*,  $p < 0.05$  vs controls (+LPS alone).

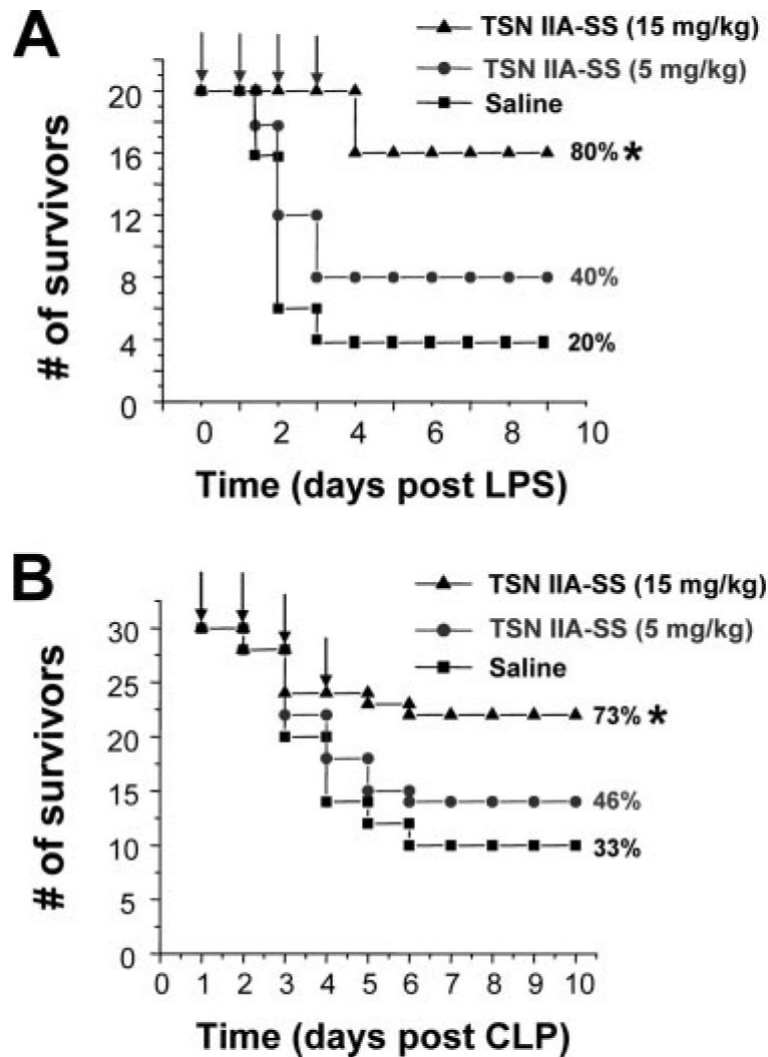
**FIGURE 4.**

TSNIIA-SS blocked endotoxin-induced cytoplasmic HMGB1 translocation. Macrophage cultures were stimulated with LPS in the absence or presence of TSNIIA-SS, and assayed for HMGB1 cytoplasmic translocation by immunohistochemistry (A) or cell fractionation (Western blot technique) (B) at 16 h after LPS stimulation. Note in A that HMGB1 was predominantly localized in the nuclear region of unstimulated macrophages (control) (*left column*), in both cytoplasmic and nuclear regions of LPS-stimulated macrophages (*middle column*). TSNIIA-SS (100  $\mu$ M) preserved HMGB1 in the nuclear regions (LPS + TSNIIA-SS) (*right column*). Following LPS stimulation, cytoplasmic (C) and nuclear (N) fractions were isolated and assayed for levels of HMGB1, a proliferating cell nuclear Ag protein, or cytoplasmic ( $\beta$ -actin) protein using Western blot analysis. Equal loading of samples was confirmed by Western blot analysis of fractions with cytoplasmic ( $\beta$ -actin) or proliferating cell nuclear Ag protein markers. Blots are representative of two independent experiments with similar results.

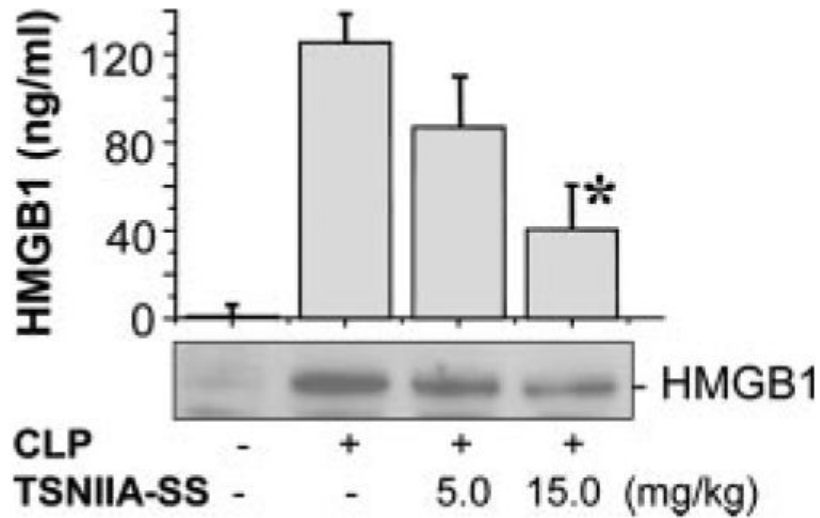


**FIGURE 5.**

TSNIIA-SS attenuates LPS-induced HMGB1 release in a glucocorticoid receptor-independent mechanism. Murine macrophage-like RAW 264.7 cells were stimulated with LPS in the absence or presence of dexamethasone, cortisone, or TSNIIA-SS alone (A), or in combination with a glucocorticoid receptor antagonist, RU486. At 16 h after stimulation, levels of HMGB1 (B) or TNF (C) in the culture medium were determined by Western blot analysis and ELISA, respectively. The representative Western blot of two independent experiments with similar results is shown. \*,  $p < 0.05$  vs “+ LPS alone” group; #,  $p < 0.05$  vs “+ LPS + Dex” group.

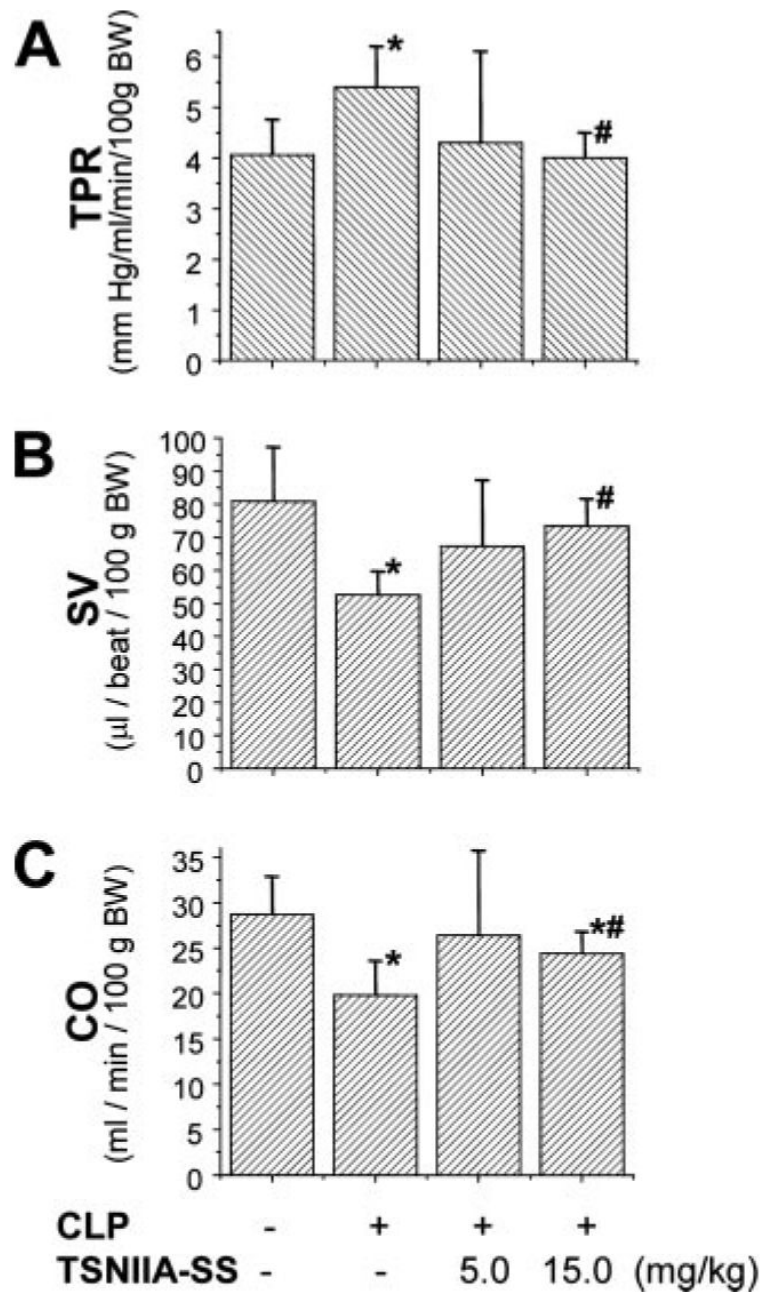


**FIGURE 6.** TSNIIA-SS dose dependently protects mice against lethal endotoxemia (A) and lethal sepsis (B). BALB/c mice were subjected to lethal endotoxemia (LPS, 15 mg/kg, i.p.), or sepsis (induced by CLP). At +0.5, +24, +48, and +72 h after the onset of endotoxemia or +24, +48, +72, and +96 h after the onset of sepsis, animals were i.p. administered with saline (0.2 ml/mouse) or TSNIIA-SS (0.2 ml/mouse at indicated doses), and animal survival was monitored for up to 2 wk. The Kaplan-Meier method was used to compare the differences in mortality rates between groups. \*,  $p < 0.05$  vs saline.



**FIGURE 7.**

TSNIIA-SS attenuates sepsis-induced systemic HMGB1 accumulation. BALB/c mice were subjected to lethal sepsis by CLP, and i.p. administered with control saline (0.2 ml/mouse) or TSNIIA-SS (at indicated doses) at +24 and +48 h after CLP. At 52 h after the onset of sepsis, serum HMGB1 levels were determined and expressed as the mean  $\pm$  SD ( $n = 10$  mice). \*,  $p < 0.05$  (by ANOVA or Tukey test).



**FIGURE 8.**

TSNIIA-SS prevents sepsis-induced cardiovascular dysfunction. Male Sprague-Dawley rats (290–310 g) were subjected to lethal sepsis by CLP, and TSNIIA-SS was administered via the femoral venous catheter using a Harvard Pump (Harvard Apparatus) at 5 h after CLP. At 20 h after CLP or sham operation, total peripheral resistance (TPR) (A), stroke volume (SV) (B), and cardiac output (CO) (C) were determined using radioactive microspheres as previously described (30). Data are expressed as the mean  $\pm$  SD ( $n = 6$  mice/group) and compared by one-way ANOVA and the Tukey test. \*,  $p < 0.05$  vs sham-operated animals (“-”); #,  $p < 0.05$  vs CLP animals treated with vehicle (+CLP).