Spermine Protects Mice Against Lethal Sepsis Partly by Attenuating Surrogate Inflammatory Markers

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The pathogenesis of sepsis is partly attributable to dysregulated inflammatory response mediated by pathogen-associated molecular patterns (PAMPs) (for example, endotoxin) and damage-associated molecular patterns (DAMPs) (for example, high-mobility group box 1 (HMGB1)). An endogenous ubiquitous polyamine, spermine, inhibits endotoxin-induced cytokine release *in vitro*, but its capacities to attenuate sepsis- and HMGB1-induced inflammatory responses was previously unknown. We thus tested the hypothesis that spermine protects mice against lethal sepsis by attenuating sepsis-induced local and systemic inflammatory responses. Intraperitoneal (i.p.) administration of spermine (10 mg/kg, twice daily, for 3 d) conferred a significant protection against lethal sepsis. The protective effects were associated with a significant reduction in peritoneal and serum levels of several surrogate markers of sepsis (for example, Interleukin-6 (IL-6), keratinocyte-derived chemokine (KC), monocytes chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-2 (MIP-2), tissue inhibitor of metalloproteinase-1 (TIMP-1), soluble tumor necrosis factor-α receptor I (sTNFRI), and soluble tumor necrosis factor-α receptor II (sTNFRII)) during a late stage of sepsis. *In vitro*, spermine effectively inhibited HMGB1-induced release of the above surrogate markers in peritoneal macrophages. Thus, spermine confers protection against lethal sepsis partly by attenuating sepsis- and HMGB1-induced inflammatory responses.

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INTRODUCTION

Sepsis refers to a systemic inflammatory response syndrome resulting from a microbial infection. Its pathogenesis is rather complex, but partly attributable to dysregulated inflammatory response. Innate immune cells (such as macrophages) are equipped with pattern recognition receptors (such as Toll-like receptors [TLRs] 2, 4, and 9) (1–3), which specifically recognize pathogen- or damage-associated molecular patterns (PAMPs or DAMPs) (3,4). Upon recognition of various PAMPs (such as bacterial peptidoglycan, endotoxin, and/or CpG-deoxyribonucleic acid [DNA]) (3–6) or DAMPs (such as HMGB1) (7–9), macrophages release various cytokines (such as tumor necrosis factor [TNF], IL-1, IL-6, and IL-12) and chemokines (such as IL-8, MIP-1s, MIP-2, and MCP-1) (10–12). Although an appropriate inflammatory response is required to defend against infection, an uncontrolled systemic inflammation may contribute to the pathogenesis of sepsis.

In animal models of lethal systemic inflammation, TNF (13), IL-1 (14), interferon (IFN)-γ (15), macrophage migration inhibitory factor (MIF) (16,17), and HMGB1 (5,18), individually or in combination, contribute to the pathogenesis of diseases. For instance, in murine models of endotoxemia and sepsis, anti-HMGB1 antibodies or inhibitors (for example, tanshinones, ethyl pyruvate, nicotine, or stearoyl lysophosphatidylcholine) significantly protect mice from endotoxemic and septic lethality (5,18–23). Recently, HMGB1 has been proposed as an alarmin signal to recruit, alert, and activate innate immune cells (24,25). For instance, HMGB1 can activate innate immune cells (for example, macrophage, monocytes, and dendritic cells) to produce various cytokines (such as TNF, IL-1α, IL-1β, IL-6) and chemokines (IL-8, MIP-1α, and MIP-1β) (7–9), thereby sustaining a potentially injurious inflammatory response in sepsis (24,25).

To counterregulate potentially injurious inflammatory response, mammals have evolved multiple negative regulatory mechanisms. For instance, spermine, a ubiquitous biogenic molecule

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that can be released passively by damaged cells, functions as a local feedback antiinflammatory mechanism at infection or injury sites (26). Indeed, spermine dose-dependently inhibited endotoxin-induced release of TNF, IL-1, IL-6, MIP-1α, and MIP-1β in human monocytes (26–29), and attenuated carrageenan-induced local inflammation (paw edema) (27). It was previously unknown, however, whether spermine affects sepsis- and/or HMGB1-induced inflammatory responses, and influences the outcome of lethal sepsis.

In this study, we found that administration of spermine conferred significant protection against lethal sepsis, and dramatically attenuated sepsis-induced local (peritoneal) and systemic (serum) accumulation of several surrogate markers of sepsis (such as IL-6, KC, MCP-1, MIP-2, TIMP-1, and sTNFRI). At the concentrations of HMGB1 (1.0 μg/mL) and LPS (100 ng/mL) that induced similar release of many cytokines (such as MCP-1, MIP-2, RANTES, sTNFRI, and sTNFRII), HMGB1 induced dramatically less IL-6, KC, MIP-1α, TIMP-1, and TNF than did LPS (100 ng/mL), confirming that HMGB1 and LPS induce distinct cytokine release. Furthermore, spermine effectively inhibited HMGB1-induced release of IL-6, KC, MIP-2, sTNFRI, and sTNFRII in primary peritoneal macrophages. Taken together, these experimental data suggest that spermine exerts protective effects against lethal sepsis partly by attenuating sepsis- and HMGB1-mediated inflammatory responses.

MATERIALS AND METHODS

Cell Culture

Murine macrophage-like RAW 264.7 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Primary peritoneal macrophages were isolated from Balb/C mice (male, 7–8 weeks, 20–25 grams) at 3 d after intraperitoneal injection of 2 mL thioglycollate broth (4%) as described previously (21,23,30). Murine macrophages were precultured in DMEM medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 2 mmol/L glutamine, and 1% penicillin.

Animal Models of 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, USA). Sepsis was induced in male Balb/C mice (7–8 wks, 20–25 g) by cecal ligation and puncture (CLP) as described previously (21,23). Briefly, mice were anesthetized with ketamine (75 mg/kg, intramuscular [i.m.]) and xylazine (20 mg/kg, i.m.), and a 15 mm midline incision was made to expose the cecum. After ligation of the cecum (with a 6–0 prolene suture at 5.0 mm from the cecal tip), the ligated cecal stump was then punctured once with a 22-gauge needle to extrude stool (1 mm in length). Immediately, the cecum was replaced back to its normal intra-abdominal position, and the abdomen wound was closed with a two-layer (peritoneum and fascia) running suture to prevent leakage of fluid. All animals were resuscitated with a normal saline solution (subcutaneously [s.c.], 20 mL/kg), and given a single dose of imipenem (0.5 mg/mouse, s.c.) at 30 min after the surgery. Spermine (Sigma, S4264) was administered i.p. into mice at indicated doses and time points, and animal survival rates were monitored for up to 2 wks.

In separate experiments, mice were euthanized at 24 h or 52 h post CLP, and the peritoneal cavity was lavaged immediately with 2.5 mL of sterile saline to collect peritoneal fluid. The peritoneal lavage fluid was centrifuged for 10 min at 1000*g* to collect cell-free supernatant for cytokine analysis. Immediately following peritoneal lavage, blood samples were harvested from mice by cardiac puncture, and allowed to clot (for 2 h at room temperature) to collect serum for cytokine analysis.

Preparation of Recombinant HMGB1

The cDNA encoding for rat HMGB1 was cloned onto a pCAL-n vector, and recombinant HMGB1 was expressed in *E. coli* BL21 (DE3) pLysS cells as described previously (5). Contaminating endotoxin was removed from the HMGB1 preparation by Triton X–114 extraction (31). Briefly, HMGB1 solution was mixed with Triton X–114 (5% v/v, 4° C), incubated at room temperature for 20 min. Subsequently, the Triton X–114 phase (containing the endotoxin) was precipitated by centrifugation (8 min, 16,000*g* [13,200 rpm], room temperature). The water-soluble fraction (containing HMGB1) was collected and assayed for LPS content by the chromogenic *Limulus* amebocyte lysate assay (Limulus Amebocyte Lysate [LAL] QCL-1000, Cambrex Bio Science Walkersville Inc, Walkersville, MD, USA). The endotoxin content was < 0.1 pg per microgram of HMGB1 protein.

LPS and HMGB1 Stimulation

Adherent macrophages were gently washed with, and cultured in, serum-free OPTI-MEM I medium 2 h before stimulation with endotoxin (lipopolysaccharide, LPS, *E. coli 0111:B4*, Sigma-Aldrich, MO, USA) or purified HMGB1 protein. At 16 h after stimulation, levels of various cytokines in the culture medium were determined by enzyme-linked immunosorbent assay (ELISA), and cytokine antibody array as described previously (21,30).

Cytokine Antibody Array

Murine cytokine antibody arrays (M0308003, RayBiotech Inc., Norcross, GA, USA) were used to determine levels of 62 cytokines in the culture medium, peritoneal lavage fluid, or serum as described previously (21). Briefly, the membranes were sequentially incubated with equal volume of cell-conditioned culture medium (150 μL), peritoneal lavage fluid (150 μL), or murine serum (15 μL, after 1:10 dilution), primary biotin-conjugated antibodies, and horseradish peroxidase–conjugated streptavidin. After exposing to X-ray film, the relative signal intensities were determined using a Gel &

Graph Digitizing Software (UN-SAN-IT gel version 6.1, Silk Scientific Inc., Orem, UT, USA). Each cytokine antibody is arrayed in duplicate to provide optimal reliability. The biotin-conjugated antibodies on every membrane serve as positive controls to identify the membrane orientation, normalize the results within the membrane, and compare results between different membranes.

ELISA

To validate the cytokine antibody array assay, levels of TNF and IL-6 in the culture medium, peritoneal lavage fluid, or serum also were determined using quantitative ELISA kits (MTA00, DY406, R & D Systems, Minneapolis, MN, USA) with reference to standard curves of purified recombinant TNF or IL-6 at various dilutions as described previously (21,23).

HMGB1 Western Blotting Analysis

The relative levels of HMGB1 in the peritoneal lavage fluid or serum were determined by Western blotting analysis as described previously (5,21,23,30). 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 as described previously (21).

Statistical Analysis

Data are expressed as mean ± SD of at least three independent experiments (n = 3–5). One-way analysis of variance (ANOVA) was used for comparison among all different groups. When the ANOVA was significant, *post hoc* 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 P value < 0.05 was considered statistically significant.

RESULTS

Spermine Protected Mice against Lethal Sepsis

To evaluate the role of spermine in lethal systemic inflammatory diseases, we examined its effects on survival in an animal model of sepsis. Given the late and prolonged kinetics of systemic HMGB1 accumulation in experimental sepsis (18), we first determined whether it is possible to rescue mice from lethal sepsis by giving spermine in a delayed fashion. Delayed administration of spermine (10 mg/kg), beginning 24 h *after* the onset of sepsis and followed by additional doses (twice a d, for 3 d), postponed animal lethality by 24–48 h, and slightly increased long-term animal survival rates from 48% to 60% (Figure 1A). At a higher dose (100 mg/kg), however, spermine decreased animal survival rate from 58% (for control group receiving saline, n = 12 mice/group) to 38% (for experimental group receiving spermine, n = 13 mice/group) at 48 h post CLP, and further decreasing it to 0% at 72 h post CLP.

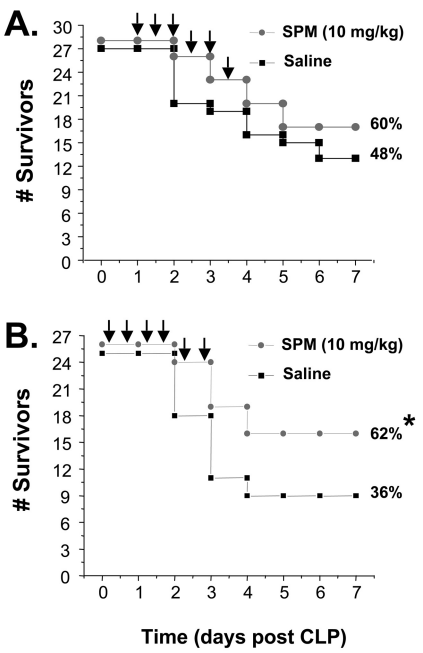

Figure 1. Spermine protected mice against lethal sepsis. Balb/C mice were subjected to lethal sepsis (induced by CLP), and spermine was administered intraperitoneally (i.p., 10 mg/kg, twice a day, for 3 d) beginning at +24 h (A) or +0.5 h (B) post the onset of sepsis. The Kaplan–Meier method was used to compare mortality rates between groups of two independent experiments (with 13–14 mice per group) with similar results. Arrows indicate the time points of spermine administration. *P < 0.05 versus saline control group.

To maximize its protective effects, the first dose of spermine was given immediately (30 min) after CLP, and additional doses were administered twice daily for 3 d. If given early, repetitive administration of spermine (10 mg/kg) conferred a reproducible and significant protection against lethal sepsis, increasing long-term survival rate from 36% to 62% (P < 0.05, Figure 1B). At a lower dose (1.0 mg/kg), spermine did not increase animal survival rate (36% for control group receiving saline, n = 25 mice/group; versus 38% for experimental group receiving spermine, n = 13 mice/group). Taken together, these observations suggest that spermine promotes a dose-dependent protection against experimental sepsis when given repetitively at moderate doses (1.0 to 10.0 mg/kg).

Spermine-Attenuated, Sepsis-Induced Local and Systemic Inflammatory Responses

To elucidate the mechanisms underlying spermine-mediated protection, we sought to cross-sectionally determine relative levels of multiple cytokines and chemokines in peritoneal fluid and serum using cytokine antibody array and ELISA. At 24 h post CLP, most (if not all) mice remained alive, but had already developed clear signs of sepsis (including lethargy, diarrhea, and piloerection). At this time point, HMGB1 was readily detected in peritoneal lavage fluid of most septic mice, but its levels were not reduced by spermine treatment (10 mg/kg, twice, data not shown). Paradoxically, peritoneal levels of IL-6 were slightly, but significantly, elevated by spermine treatment (7238 ± 706 pg/mL for CLP + saline group; versus 10598 ± 2702 pg/mL for CLP + spermine group; n = 3, P < 0.05). Similarly, serum levels of IL-6 were also significantly elevated in spermine-treated mice (5066 ± 529 pg/mL for CLP + saline group; versus 7045 ± 1029 pg/mL for CLP + Spermine group; n = 3, P < 0.05), suggesting that administration of

spermine may enhance local and systemic cytokine response during an early stage of sepsis.

At 52 h post CLP, some septic mice had not survived, but by then spermine already exhibited significant protection against lethal sepsis (see Figure 1). Peritoneal levels of G-CSF, IL-6, KC, MCP-1, MIP-1α, MIP-2, TIMP-1, sTNFRI, and sTNFRII were significantly reduced by spermine treatment (Figure 2A,2B). Similarly, serum levels of most of the above cytokines (except MIP-1α and MIP-2) also were reduced significantly in the spermine-treated group (Figure 2D). To validate the cytokine antibody array assay, levels of IL-6 in peritoneal lavage fluid or serum also were determined using quantitative ELISA. As shown in Figure 2C and 2E, actual IL-6 levels (in pg/mL) obtained from the more quantitative monoplex ELISA assays correlated well with its relative levels on multiplex cytokine antibody arrays (Figure 2B,2D). In addition, levels of HMGB1 in the peritoneal lavage fluid, but not in the serum, were reduced significantly by spermine treatment (Figure 3A,3B). Taken together, these experimental data suggest that repetitive administration of spermine attenuates both local (peritoneal) and systemic (serum) cytokine response during a late stage of sepsis.

HMGB1 and LPS Induced Distinct Cytokine Release in Macrophage Cultures

To elucidate mechanisms underlying sepsis-induced cytokine response, we confirmed whether HMGB1 stimulates macrophages to release all cytokines/chemokines that were seen in septic mice. Previously, we employed a polymyxin B purification method to remove contaminating endotoxin, and produced recombinant HMGB1 containing low concentration of endotoxin (< 600 pg endotoxin per microgram protein) (7). These low-endotoxin HMGB1 preparations effectively stimulated human peripheral blood mononuclear cells to produce TNF, IL-1α, IL-1β, IL-6, IL-8, MIP-1α, and MIP-1β even at

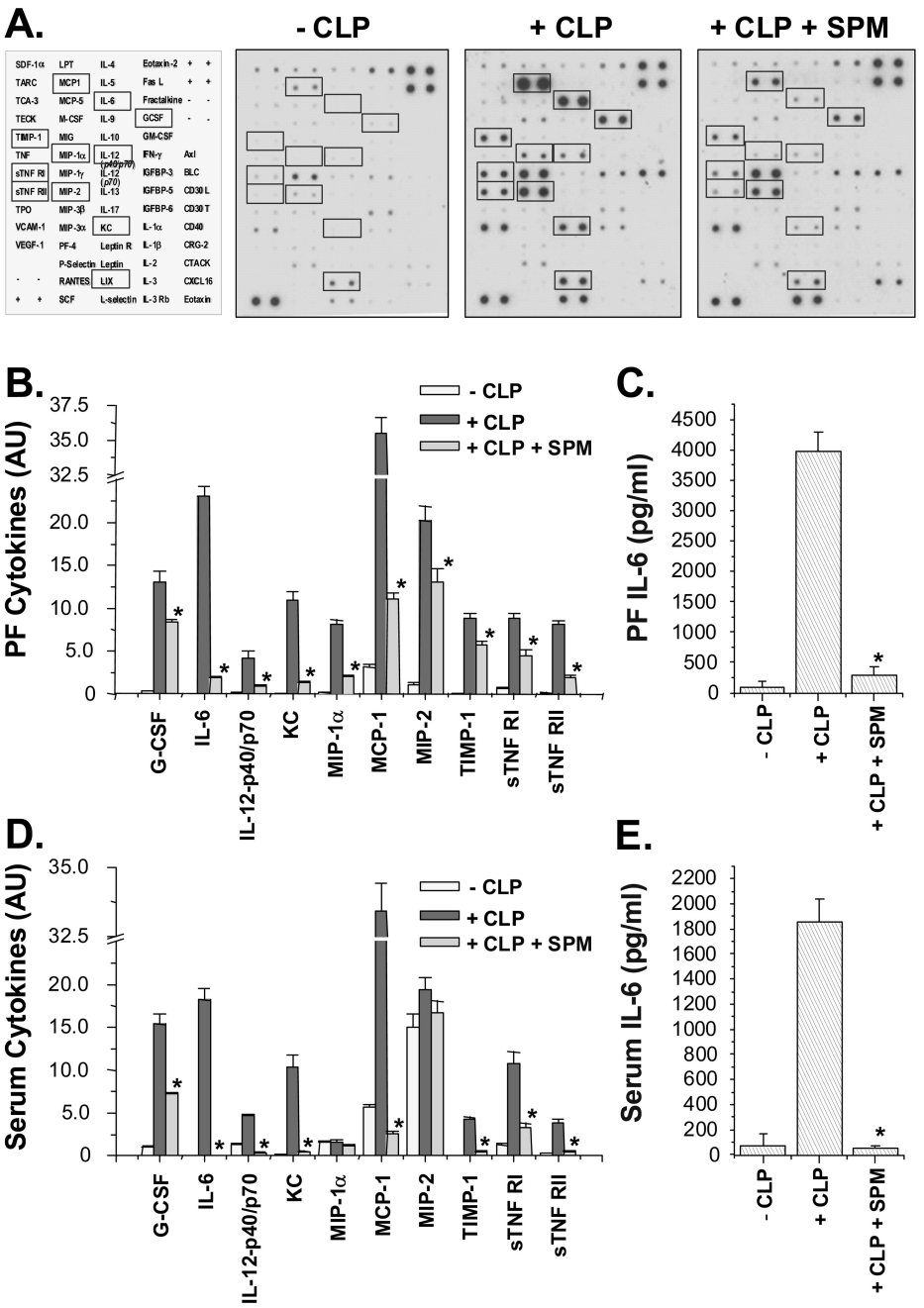

Figure 2. Spermine-attenuated, sepsis-induced local and systemic inflammatory responses. Balb/C mice were subjected to sepsis by CLP, and administered with control saline (0.2 mL/mouse) or spermine (10.0 mg/kg, twice a d) beginning at +0.5 h post CLP. At 52 h post CLP, levels of various cytokines in the peritoneal fluid (PF) or serum were determined by cytokine antibody array (A,B,D) and ELISA (C,E), respectively. (A) Representative cytokine antibody arrays. Peritoneal lavage fluid was pooled from three normal mice (–CLP), three septic mice receiving saline (+CLP), and three septic mice receiving spermine (+CLP +SPM), and assayed for cytokine levels by antibody arrays. (B) Relative peritoneal cytokine levels. The optical intensities (AU) for each pair of cytokine spots were determined using a Gel & Graph Digitizing Software, and expressed as mean ± SD of three independent experiments (n = 3). *$P < 0.05$ versus saline (+CLP). (C,E) ELISA analysis of IL-6 levels. (D) Relative serum cytokine levels. *$P < 0.05$ versus saline group (+CLP).

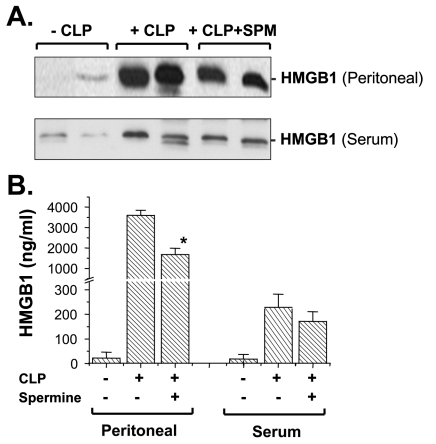

Figure 3. Spermine-attenuated peritoneal HMGB1 levels. Balb/C mice were subjected to sepsis by CLP, and administered with control saline (0.2 mL/mouse) or spermine (10 mg/kg, twice a d) beginning at + 0.5 h post CLP. At 52 h post CLP, levels of HMGB1 in the peritoneal fluid (PF) or serum were determined by Western blotting analysis. (A) Representative Western blots. –CLP, two normal mice; +CLP, two septic mice receiving saline; +CLP +SPM, two septic mice receiving spermine. (B) Relative HMGB1 levels. The relative band intensities were quantified using the NIH image 1.59 software to determine HMGB1 levels with reference to standard curves generated with purified HMGB1. *P< 0.05 versus saline group (+CLP).

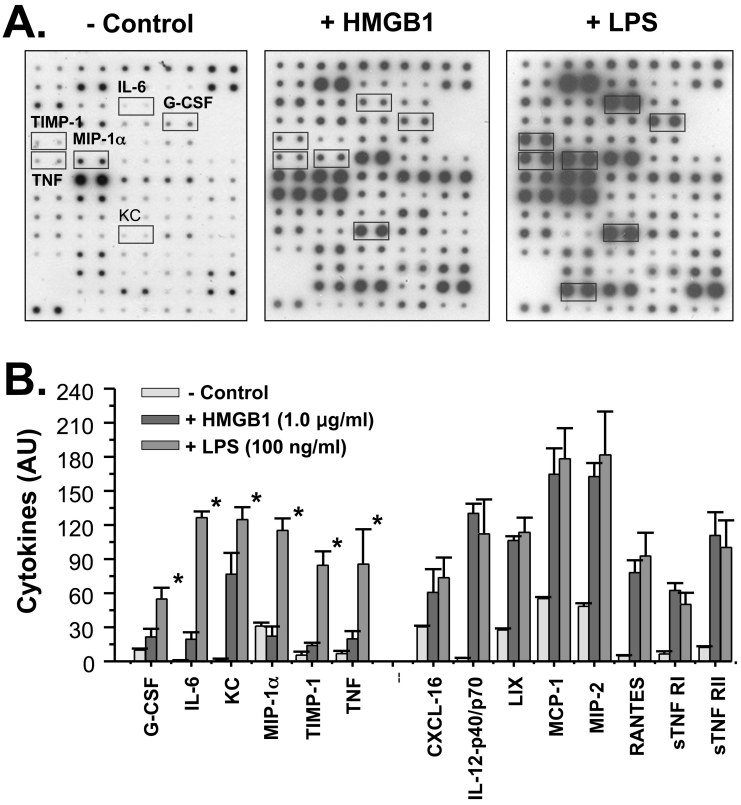

Figure 4. HMGB1 and LPS induce distinct cytokine release profiles in macrophage cultures. Primary peritoneal macrophages (MuMAcs) were stimulated with HMGB1 (+HMGB1, 1.0 μg/mL) or LPS (+LPS, 100 ng/mL) for 16 h, and levels of various cytokines in the culture medium were determined by cytokine antibody arrays. (A) Representative cytokine antibody arrays. (B) Relative cytokine levels. The optical intensities (AU) for each pair of cytokine spots were determined using a Gel & Graph Digitizing Software, and expressed as mean ± SD of three independent experiments (n = 3). *P < 0.05 versus HMGB1 group (+HMGB1).

a concentration as low as 1.0 μg/mL (7). To better characterize its cytokine-stimulating properties, we employed Triton X–114 extraction method to further remove contaminating endotoxin, and obtained highly purified HMGB1 containing < 0.1 pg endotoxin per microgram protein.

At the concentrations of HMGB1 (1.0 μg/mL) and LPS (100 ng/mL) that previously induced similar production of cytokines (for example, TNF, IL-6, IL-8, MIP-1α, and MIP-1β) in human monocytes (7), the highly purified HMGB1 and LPS induced similar release of IL-12, LIX, MCP-1, MIP-2, RANTES, sTNFRI, and sTNFRII in primary murine macrophages (Figure 4A,4B), confirming that highly purified HMGB1 is still capable of inducing cytokines (for example, IL-12) and chemokines (LIX, MCP-1, MIP-2, and RANTES) *in vitro*.

Interestingly, at the concentrations that induced similar release of the above cytokines and chemokines, HMGB1 induced significantly less amounts of G-CSF, IL-6, KC (IL-8 homologue), MIP-1α, TIMP-1, and TNF than those induced by LPS (see Figure 4A,4B). Similarly, at the concentrations that induced similar release of MCP-1, MIP-1α, MIP-2, and RANTES, highly purified HMGB1 induced dramatically less G-CSF, IL-6, and TNF in murine macrophage-like RAW 264.7 cells (data not shown). Taken together, these observations suggest HMGB1 and LPS induce distinct cytokine release profiles, supporting the notion that they use distinct mechanisms to activate innate immune cells (7,32,33).

Spermine Inhibited HMGB1-Induced Cytokine Release

To elucidate the mechanisms by which spermine attenuates sepsis-induced local and systemic inflammatory response, we assessed the effects of spermine on LPS- or HMGB1-induced cytokine release using cytokine antibody arrays. Consistent with our previous report (27), spermine dose-dependently inhibited LPS-induced release of TNF, IL-6, and MIP-1α in murine peritoneal macrophages (Figure 5A). At relative higher concentrations (500 μM), however, spermine effectively inhibited LPS-induced release of other

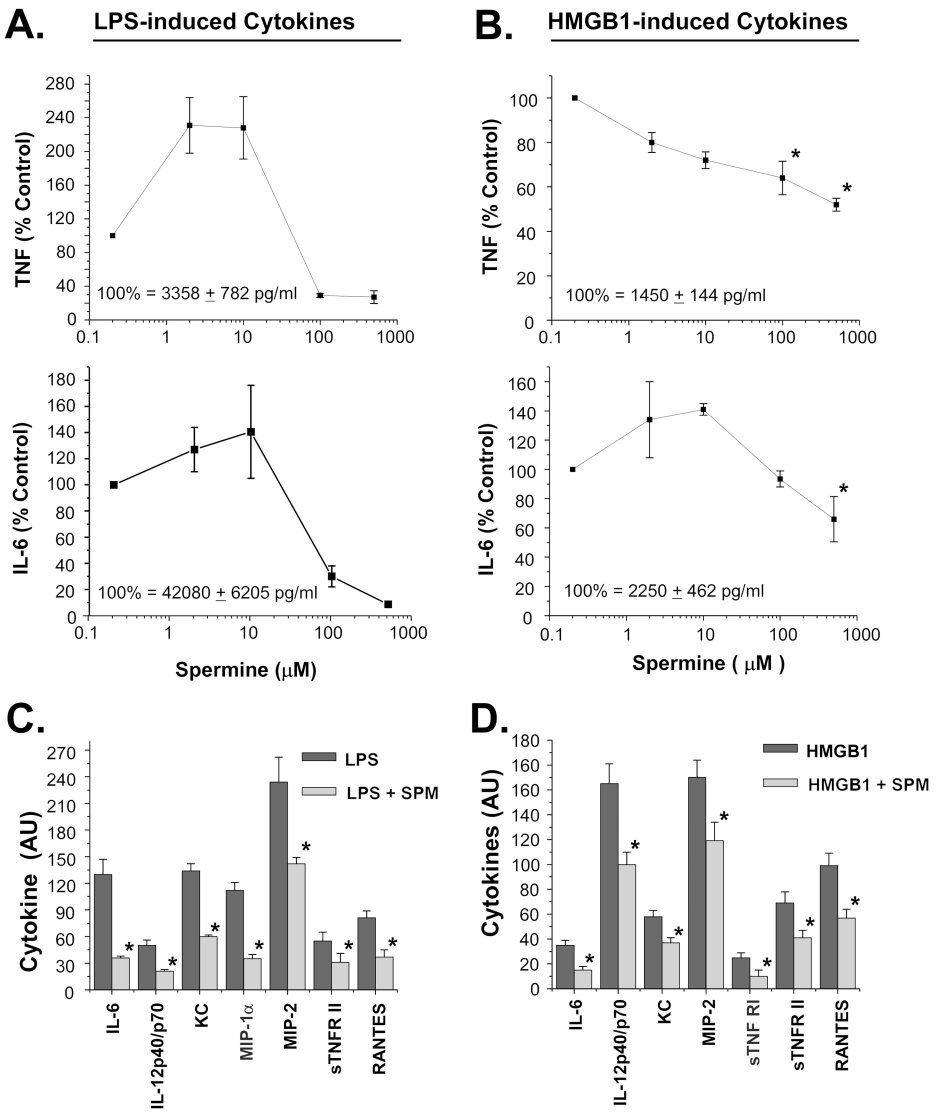

Figure 5. Spermine-attenuated, HMGB1-induced cytokine release. Primary peritoneal macrophages were stimulated with LPS (100 ng/mL), HMGB1 (1.0 μg/mL), either alone or in the presence of spermine at indicated doses for 16 h, and levels of various cytokines in the culture medium were assayed by ELISA (A,B) or cytokine antibody arrays (C,D). (A,B) ELISA assay of TNF and IL-6 levels. (C,D) Cytokine antibody arrays of various cytokines. The optical intensities (in arbitrary units, AU) for each pair of cytokine spots were expressed as mean ± SD of three independent experiments (n = 3). *$P < 0.05$ versus LPS or HMGB1 group, respectively.

cytokines such as IL-12, KC, MIP-2, sTNFRII, and RANTES in primary macrophage cultures (Figure 5C). Similarly, spermine significantly inhibited HMGB1-induced release of TNF, IL-6 (Figure 5B), as well as other cytokines such as IL-12, KC, MIP-2, RANTES, sTNFRI, and sTNFRII (Figure 5D). Paradoxically, spermine slightly enhanced LPS- or HMGB1-induced release of TNF or IL-6, particularly when given at relatively low concentrations (for example, 2-10 μM) (Figure 5A,5B).

DISCUSSION

In a clinically relevant model of sepsis (34), repetitive administration of spermine at moderate doses (1.0 –10 mg/kg, twice daily, for three days) confers a dose-dependent protection against lethal sepsis. However, when given at higher doses (for example, 100 mg/kg), spermine adversely reduced animal survival rates, indicating dual effects in experimental sepsis. On one hand, spermine might be protective by attenuating excessive inflammatory response when given at a moderate dose (for example, 10 kg/mL). On the other hand, it might be converted enzymatically by polyamine oxidases into cytotoxic metabolites (for example, 3-aminopropanal), and mediate cytotoxicity through direct chemical modification of cell membrane proteins (35,36). Although the observed dual (both beneficial and injurious) effects of spermine may pose limitations to its clinical use as a potential therapeutic agent, it provides us with a unique tool to elucidate the complex mechanisms underlying the pathogenesis of sepsis.

The pathogenesis of experimental sepsis is partly attributable to dysregulated inflammatory response that is mediated by peritoneal infection (due to spillage of bacteria and by-products) and cecal ischemia (due to ligation of cecum tissue). As a possible consequence of both active release (by peritoneal macrophages in response to bacterial products) and passive leakage (from ischemic cecum tissue), HMGB1 was readily detected in peritoneal lavage fluid of septic mice. However, repetitive administration of spermine led to a significant reduction of peritoneal levels of HMGB1, a critical late mediator of lethal endotoxemia (5) and sepsis (18,22). It is possible that spermine confers protection against lethal sepsis partly by attenuating peritoneal HMGB1 accumulation, and partly by inhibiting HMGB1-mediated inflammatory response.

To test the above hypothesis, we cross-sectionally determined the effects of spermine on local (peritoneal) and systemic (serum) levels of multiple cytokines and chemokines using cytokine antibody arrays. At 24 hours post CLP, peritoneal and serum levels of many cytokines and chemokines were not reduced by spermine treatment. Paradoxically, IL-6 levels

were slightly, but significantly, elevated by spermine treatment during an early stage of sepsis. At low concentrations (2–20 μM), spermine enhanced HMGB1-induced IL-6 release in primary peritoneal macrophage cultures. Although it is difficult to correlate the concentration-effect relationships of spermine *in vitro* and *in vivo*, a single injection of spermine (molecular weight = 202.3 Da) at 10 mg/kg theoretically could produce a maximal blood level of spermine about 500 μM (assuming an average blood volume of 10%), and a minimal tissue level of spermine about 50 μM (assuming an even distribution in all tissues including bone, muscle, blood, and others). It is possible that the spermine-mediated enhancement of IL-6 release *in vitro* (Figure 5A,5B) accounts for the observed increase of peritoneal and serum IL-6 levels *in vivo*.

At 52 hours post CLP, the spermine-mediated protective effects were associated with a dramatic decrease in peritoneal and serum levels of IL-6, KC, MCP-1, sTNFRI, sTNFRII, and TIMP-1. In addition, spermine also reduced peritoneal (but not serum) levels of HMGB1 and a few more chemokines (for example, MIP-1α and MIP-2) during a late stage of sepsis. In light of our previous observation that spermine effectively attenuated carrageenan-induced paw edema only when given locally (27), we propose that spermine confers protection against lethal sepsis by modulating a local (peritoneal) inflammatory response, which is partly sustained by HMGB1, either alone or in combination with other inflammatory stimuli (such as endotoxin, CpG-DNA, or IL-1) (6,37).

To test the above possibility, we reevaluated the proinflammatory properties of highly purified HMGB1 using peritoneal macrophage cultures. Consistent with previous reports (7,8), we found highly purified HMGB1 effectively stimulated macrophages to release various cytokines (for example, IL-12), chemokines (for example, KC, LIX, MCP-1, MIP-2, RANTES), and soluble TNF receptors. At the concentrations that induced similar release of the above cytokines and chemokines, highly purified HMGB1 induced dramatically lower amounts of TNF, IL-6, MIP-1α, and TIMP-1 than those induced by LPS. These experimental results were consistent with recent findings that highly purified HMGB1 induces much less TNF and IL-6 than those induced by LPS in macrophage/monocytes (38) and dendritic cells (9). It thus appears that HMGB1 and LPS induce distinct cytokine release in macrophage cultures, further supporting the notion that they may use distinct signaling pathways to activate innate immune cells (7,32,33).

To further elucidate mechanisms by which spermine attenuates local and systemic inflammatory response, we determined the effect of spermine on HMGB1-induced cytokine release. Spermine significantly inhibited HMGB1-induced release of IL-6, IL-12, KC, MIP-2, RANTES, and sTNFRs in primary peritoneal macrophages. The mechanisms by which spermine attenuates HMGB1-induced cytokine release remains a subject of future research. Because spermine only inhibits release of some cytokines, it is not likely that spermine merely binds HMGB1 (via ionic interaction) to neutralize its proinflammatory activities. Nevertheless, it is presently unknown whether spermine is taken up by macrophages, and interacts with intracellular signaling cascades activated by HMGB1.

Interestingly, most of the cytokines/chemokines that were inhibited by spermine both *in vivo* and *in vitro*, have been considered as surrogate markers of lethal experimental sepsis (39,40). For instance, serum levels of IL-6, MCP-1, and G-CSF have been suggested as potential predictors of mortality in patients with sepsis (41). Notably, there is ongoing controversy regarding the beneficial or detrimental roles for IL-6 in experimental sepsis (42–45). For example, administration of anti-IL-6 antibodies conferred protection against lethal sepsis only when given at middle (but not lower or higher) doses (45), suggesting too little or too much IL-6 is equally injurious to septic animals. We found that spermine-mediated protection was associated with a significant elevation of IL-6 levels during early sepsis, and an attenuation of IL-6 levels during a late stage of sepsis.

In addition to HMGB1, sepsis induces excessive accumulation of multiple other cytokines and chemokines. The activity of each cytokine/chemokine is dependent not only on its abundance, but also on the synergistic or antagonistic interactions with other cytokines/chemokines (cytokine network). Given the complexity of cytokine networks in sepsis, comprehensive surveys of multiple surrogate markers, rather than monoplex measurement of individual markers, may provide better prognostic evaluation of the inflammatory status in critically ill patients. Although multiplexed measurements might be technically challenging for less abundant cytokines/chemokines, we found that most surrogate markers (such as G-CSF, IL-6, MCP-1, MIP-2) were readily detected on multiplex cytokine antibody arrays *in vivo* and *in vitro*. Furthermore, the relative levels of these surrogate markers on the Antibody Arrays correlated well with the actual levels (in pg/mL) obtained from the more quantitative ELISA assays.

CONCLUSION

Administration of spermine conferred significant protection in a clinically relevant model of sepsis (induced by CLP). Its protective effects were attributable partly to attenuation of sepsis-induced local (peritoneal) and systemic (serum) levels of several surrogate markers such as IL-6, KC, MCP-1, MIP-2, TIMP-1, sTNFRI, and sTNFRII. At concentrations comparable to the doses used for animal studies, spermine effectively inhibited HMGB1-induced release of the above surrogate markers in peritoneal macrophages. Taken together, these experimental data suggest that spermine confers protection against lethal sepsis partly by attenuating sepsis- and HMGB1-induced inflammatory responses. In addition, we found that the relative levels of several surrogate markers (such as G-CSF, IL-6, MCP-1, MIP-2) on multiplex cytokine antibody arrays correlated well with the actual levels (in pg/mL) obtained from the more quantitative

monoplex ELISA assays. It will thus be important to use human cytokine antibody arrays to simultaneously measure multiple biomarkers to better profile the inflammatory status of the patient with sepsis and other systemic inflammatory diseases.

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DISCLOSURE

We declare that the authors have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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