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HMGB1 Enhances the Proinflammatory Activity of Lipopolysaccharide by Promoting the Phosphorylation of MAPK p38 through Receptor for Advanced Glycation End Products[1]

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High mobility group box-1 (HMGB1) protein was originally characterized as a nuclear DNA-binding protein, and was described to have an extracellular role when involved in cellular activation and proinflammatory responses. In the present study, we have found that the proinflammatory activity of recombinant HMGB1 proteins is determined by the containing endotoxin level, and HMGB1 that contains few endotoxins fails to stimulate macrophages to secrete proinflammatory cytokines. HMGB1 acts as a ligand of receptor for advanced glycation end products (RAGE) and works in synergy with LPS in activating the macrophages in vitro. In vivo, intra-articular injections of HMGB1 act in synergy with LPS to induce experimental arthritis in mice. HMGB1 promotes the phosphorylation of MAPK p38 and the activation of NF-κB through RAGE, and then enhances the expression of proinflammatory cytokines. These results demonstrate that HMGB1 enhances the proinflammatory activity of LPS by promoting the phosphorylation of MAPK p38 and by the activation of NF-κB through RAGE. ***The Journal of Immunology,*** **2009, 183: 6244–6250.**

The high mobility group box-1 (HMGB1)[4] that was first discovered as a nuclear protein with rapid electrophoretic migration was a 30 kDa, nonhistone, DNA-binding molecule that was highly conserved. It had a 99% sequence identity across mammalian species. HMGB1 seems to have different functions according to different cellular locations: intracellular, it plays a role in a number of fundamental cellular processes such as transcription, replication, DNA repair, and recombination; extracellular, it acts as a crucial cytokine that mediates the response to infection, injury, and inflammation (1). Extracellular HMGB1 may interact with TLR and/or receptor for advanced glycation end products (RAGE). In particular, an interaction between HMGB1 and TLR2 or TLR4 postulates that it may mediate the proinflammatory actions of HMGB1. Because of the dual functions of HMGB1, it plays a crucial role in many pathophysiological processes, such as rheumatoid arthritis, liver injury, and tumors (2–6).

Recent data have brought into question the proinflammatory properties of HMGB1. The HMGB1 isolated and purified from calf thymus or recombinant in mammal-expression system does not appear to be capable of inducing the activation of macrophages or dendritic cells unless bound to DNA-containing immune complexes or to damage-associated pattern molecules such as IL-1β and LPS (7–9).

In our study, we have found that the proinflammatory activity of recombinant HMGB1 protein is correlated with the endotoxin level it contained, and that HMGB1 significantly enhances the activity of LPS to induce proinflammatory cytokines both in vitro and in vivo by promoting the phosphorylation of MAPK p38 and the activation of NF-κB through RAGE.

Materials and Methods

Mice

Female DBA/1 mice, 6–8 wk old, were purchased from the Shanghai Laboratory Animal Center of Chinese Academy of Science. TLR2$^{-/-}$, TLR4$^{-/-}$, and wild-type C57BL/6 mice were purchased from the Model Animal Research Center of Nanjing University. The mice were bred and housed in the animal facility at Changhai Hospital. They were kept under standard conditions of temperature and light, and were fed laboratory chow and water ad libitum. The study was approved by the Ethics Committee of the Second Military Medical University.

Reagents

For in vitro experiments, recombinant HMGB1 proteins were purchased from Sigma-Aldrich and R&D Systems. Abs that block the TLR2 and TLR4 were purchased from eBioscience. RAGE-Fc was purchased from R&D Systems. PE-antiTLR2 and allophycocyanin-antiTLR4 Abs were purchased from eBioscience, and anti-RAGE Abs were purchased from Millipore. LPS from *Escherichia coli* serotype O55:B5 and glycated albumin were purchased from Sigma-Aldrich. Pam_3Cys was purchased from Alexis. Inhibitors of ERK (PD98059) and MAPK p38 (SB203580) were purchased from Calbiochem, whereas the JNK inhibitor (SP600125) and NF-κB inhibitor (Bay11–7085) were from Sigma-Aldrich.

Isolation and treatment of mouse peritoneal macrophages

C57BL/6J mice were injected i.p. with 2 ml of 9% thioglycollate broth to elicit peritoneal macrophages. Mice were euthanized by CO_2 asphyxiation

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[4] Abbreviations used in this paper: HMGB1, high mobility group box 1; RAGE, receptor for advanced glycation end products.

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60–72 h later, and cells were collected by lavaging the peritoneal cavity three times with 5 ml of ice-cold RPMI 1640 medium. Cells were washed with PBS and resuspended in an RPMI 1640 medium supplemented with 10% heat-inactivated FBS (Invitrogen Life Technologies), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were seeded at 5×10^5/ml in triplicate in 24- or 96-well plates (Corning Costar) and were allowed to adhere for 2 h at 37°C under 5% CO_2. Cells were then washed twice with PBS to remove nonadherent cells and supplied with fresh medium, as described above, and allowed to rest for 18–24 h. In all experiments, LPS and HMGB1 (Lot 018K4157) were premixed for at least 30 min under 37°C before they were added into the macrophages. Cells were cultured with 0.1 ng/ml LPS, with or without 0.1 μg/ml HMGB1 for 3.5 or 24 h to detect the expression mRNA and cytokines, respectively. In some experiments, cells were cultured in the presence of Abs (5 μg/ml, 30 min) or inhibitors (5 μM, 1 h) before LPS and HMGB1 were added.

Quantitative PCR

Total RNA was purified with TRIzol reagent (Invitrogen Life Technologies) and cDNA was synthesized using PrimeScript reverse transcription reagents (TaKaRa). Real-time PCR was performed on a LightCycler (Roche) using SYBR Premix EX *Taq* reagent (TaKaRa). The mouse IL-1β transcript was amplified using the following primers: forward, 5′-TCC AGG ATG AGG ACA TGA GCA C-3′ and reverse, 5′-GAA CGT CAC ACA CCA GCA GGT TA-3′. The mouse IL-6 transcript was amplified using the following primers: forward, 5′-TCC AGT TGC CTT CTT GGG AC-3′ and reverse, 5′-GTG TAA TTA AGC CTC CGA CT TG-3′. The mouse TNF-α transcript was amplified using the following primers: forward, 5′-AAG CCT GTA GCC CAC GTC GTA-3′ and reverse, 5′-GGC ACC ACT AGT TGG TTG TCT TTG-3′. The mouse CXCL2 transcript was amplified using the following primers: forward, 5′-GCG CTG TCA ATG CCT GAA GA-3′ and reverse, 5′-TTT GAC CGC CCT TGA GAG TG-3′. Mouse β-actin transcript was used as an internal control: forward primer, 5′-CAT CCG TAA AGA CCT CTA TGC CAA-3′ and reverse, 5′-ATG GAG CCA CCG ATC CAC-3′. The housekeeping gene, β-actin, was used to normalize all tested genes, and data quantification was performed using the $\Delta\Delta$CT method.

Cytokine assay

The levels of TNF-α or IL-6 released from mice peritoneal macrophages into the culture medium were determined using commercially available ELISA kits (eBioscience) following the manufacturer's instructions.

Induction of experimental arthritis

Injection procedures were conducted according to reference (10). In brief, following the inhalation (Isoflurane) of anesthesia, DBA/1 mice were injected intra-articularly into the knee joint with a total volume of 15 μl of solution containing 5 μg HMGB1 with or without 2 ng LPS. The contralateral knee joints of mice or knee joints of controlled mice received an equivalent volume of PBS with or without a matching concentration of LPS. All mice were sacrificed after 7 days for histological evaluation of joint appearance.

Histologic examination

Seven days after the intra-articular injections, the mice were sacrificed and knee joints were removed, fixed in 4% formaldehyde, decalcified, embedded in paraffin, sectioned, and stained with H&E. All the slides were assessed in a blind manner by two researchers (Li Gao, Yanghua Qin) with regard to synovial hypertrophy (membrane thickness $>$ 2 layers), pannus formation (synovial tissue overlaying joint cartilage), and cartilage and bone destruction. The extent of synovitis was judged on a scale of grade 0 to grade 3 (0 = no signs of inflammation; 1 = mild synovial hypertrophy consisting of up to five cell layers; 2 = moderate inflammation characterized by hyperplasia of synovial membrane up to 10 cell layers and influx of inflammatory cells throughout the synovial tissue; and 3 = marked synovial hypertrophy consisting of $>$ 10 cell layers, and the synovial tissue infiltrated by inflammatory cells).

Flow cytometric analysis

Mouse peritoneal macrophages (5×10^5) were incubated at room temperature for 30 min with fluorochrome-conjugated mAbs against RAGE (Millipore), TLR2, and TLR4 (eBioscience) in PBS containing 2% FBS. Labeled cells were washed twice and analyzed using a FACSCalibur (BD Biosciences). Data were analyzed using the Cell Quest software (BD Biosciences).

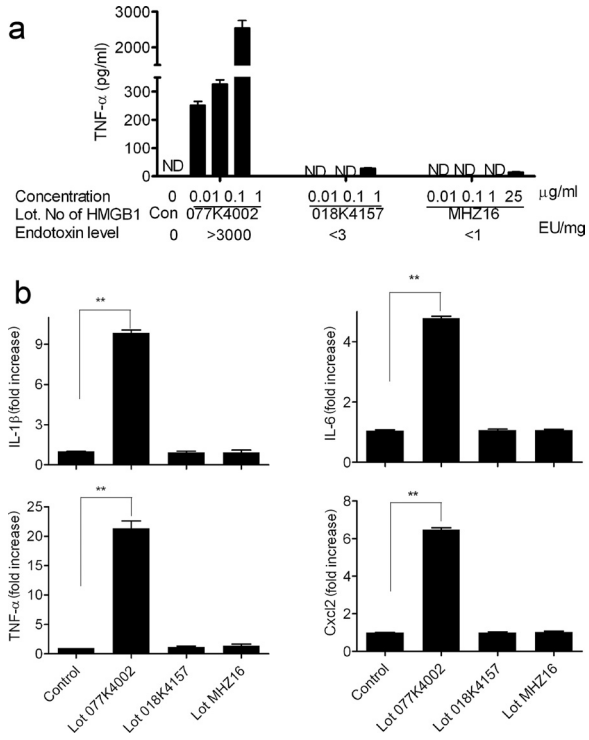

FIGURE 1. Secretion of TNF-α and expression of proinflammatory cytokines of mouse peritoneal macrophages exposed to different HMGB1 proteins. Mouse peritoneal macrophages were cultured in triplicate with different recombinant HMGB1 for 24 or 3.5 h; TNF-α concentrations in the culture supernatants, as well as intracellular IL-1β, IL-6, TNF-α, and Cxcl2 mRNA levels, were then determined by ELISA and real-time PCR, respectively. Protein levels for TNF-α (*a*) and mRNA for IL-1β, IL-6, and TNF-α or Cxcl2 by mouse peritoneal macrophages (*b*) are shown. *, $p < 0.05$ and **, $p < 0.01$.

Immunoblot analysis

Peritoneal macrophages were treated for 10 min or 30 min with LPS (0.1 ng/ml) and/or HMGB1 (0. 1 μg/ml). Cells were then lysed in a lysis buffer containing 1.0% (vol/vol) Nonidet-P40, 150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, and a protease inhibitor mixture (Roche). Cell lysates were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were blotted with the appropriate antibodies (Cell Signaling Technologies) and were visualized with an ECL Western Blotting System (Pierce Protein Research Products).

Statistics

Each experiment was repeated at least three times, and one representative result was shown. A one-way ANOVA test was used for the analysis with GraphPad Prism version 4.0 (GraphPad Software).

Results

Proinflammatory activity of recombinant HMGB1 from different resources

In previous studies, both HMGB1 isolated from *Escherichia coli*, and eukaryotic HMGB1 obtained from pig or calf thymus induced the secretion of proinflammatory cytokines from macrophages in vitro. However, recent studies have shown that HMGB1 or eukaryotic HMGB1 had no or only marginal activity when exposed to macrophages. To determine whether HMGB1 has proinflammatory properties, we examined the cytokine expression by mouse peritoneal macrophages exposed to HMGB1 purchased from Sigma-Aldrich (Lot 077K4002, Lot 018K4157) and R&D Systems (Lot MHZ16). As shown in Fig. 1, there was a significant amount of secreted protein for TNF-α or an increased mRNA level for

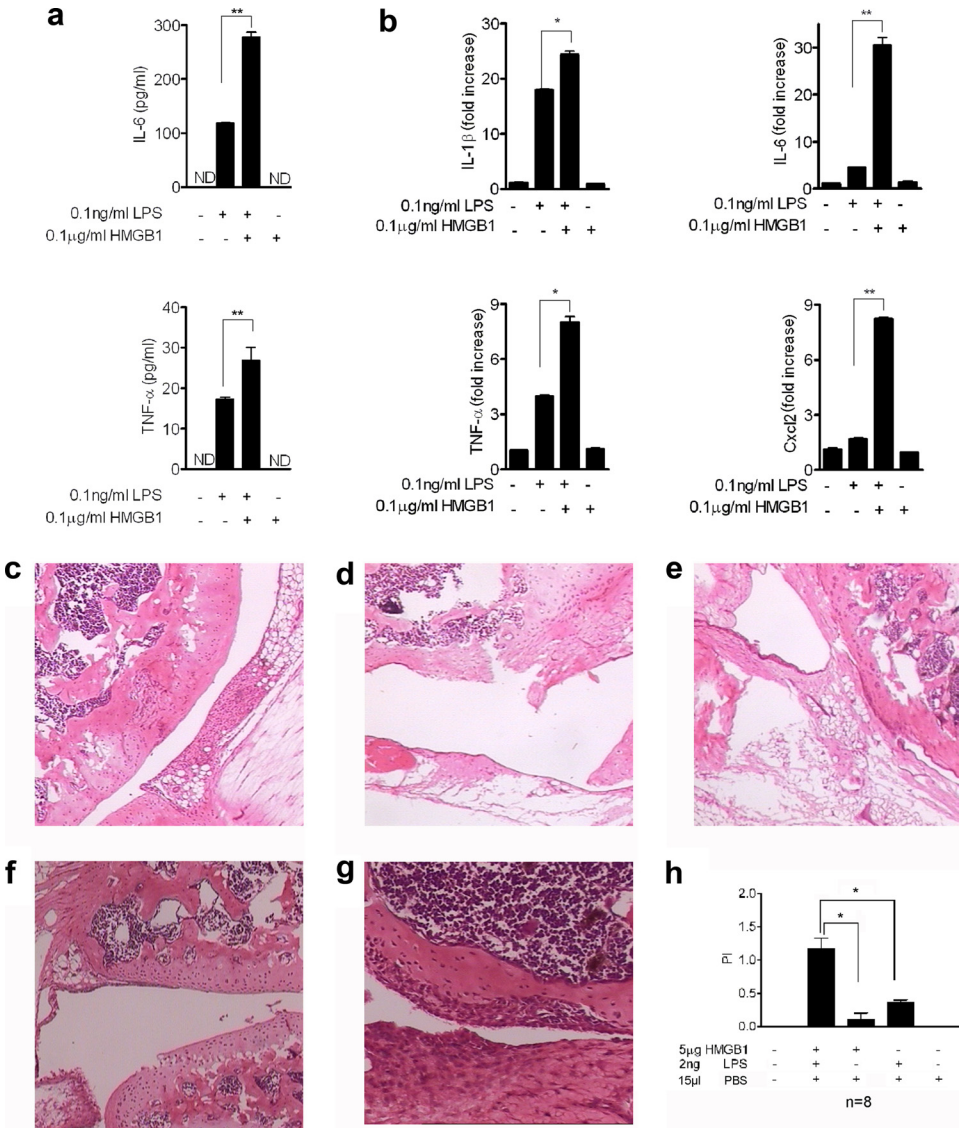

FIGURE 2. Secretion and expression of proinflammatory cytokines of mouse peritoneal macrophages exposed to LPS and/or HMGB1 and the HMGB1-induced experimental arthritis. Mouse peritoneal macrophages were cultured in triplicate with 0.1 ng/ml LPS and/or 0.1 μg/ml HMGB1 for 24 or 3.5 h; TNF-α and IL-6 concentrations in the culture supernatants (*a*), as well as intracellular IL-1β, IL-6, TNF-α, and Cxcl2 mRNA levels (*b*), were then determined, respectively. DBA/1 mice were intra-articular injected with 5 μg HMGB1 with or without 2 ng LPS for 7 days, and then sacrificed. The knee joints were stained for histological evaluation of joint appearance. Photomicrographs showed a normal DBA/1 mouse knee joint from an untreated mouse (*c*), a PBS-treated mouse (*d*), a HMGB1-treated mouse (*e*), a LPS-treated mouse (*f*), and a LPS plus HMGB1-treated mouse (*g*). The pathological index of each group ($n = 8$) was shown (*h*). Histologic staining was performed using H&E. $*$, $p < 0.05$ and $**$, $p < 0.01$.

proinflammatory cytokines by macrophages stimulated with HMGB1 that had been purchased from Sigma-Aldrich (Lot 077K4002). In contrast, other HMGB1 (Lot 018K4157, Lot MHZ16) marginally induced the secretion of TNF-α or increased mRNA levels for proinflammatory cytokines in macrophages. We then detected the endotoxin contamination with *Limulus* amebocyte lysate (ZhanJiang A&C Biological). The endotoxin concentration in Lot 077K4002 was >3,000 EU/mg, and was <3 in Lot 018K4157 and Lot MHZ16.

HMGB1 acts in synergy with LPS to induce proinflammatory cytokines

Although Lot 077K4002 contained significantly more amounts of endotoxin than others, the minimal amount of endotoxin does not fully explain the different activities. Subsequently, we hypothesized that HMGB1 could enhance LPS-induced proinflammatory cytokine secretion. To confirm this hypothesis, we cultured macrophages with LPS, HMGB1 (Lot 018K4157), and the combination of both. As a result, HMGB1 significantly enhanced the immunostimulatory properties of LPS (Fig. 2). No significant secretions of IL-6 or TNF-α were detected by stimulation with HMGB1 alone. The secretion of IL-6 or TNF-α from macrophages cultured with LPS-HMGB1 was significantly higher than those cultured with equivalent amounts of LPS alone (Fig. 2*a*). HMGB1 significantly enhanced the proinflammatory activity of LPS at a higher concentration (supplemental Fig. 1).[5] Real-time PCR showed similar results, that is, HMGB1 acted in synergy with LPS in inducing the mRNA expression of proinflammatory cytokines (Fig. 2*b*).

To further confirm that HMGB1 acted in synergy with LPS in vivo, we used an intra-articular injection of HMGB1-induced experimental arthritis model. It was reported that, after injections with 5 μg HMGB1 for 4–7 days, ~80% of mice developed arthritis (10, 11). After intra-articular injections with 5 μg HMGB1 alone for 7 days, one of eight DBA/1 mice developed mild synovitis, and seven of eight mice developed arthritis in the group that was injected with 5 μg HMGB1 plus 2 ng LPS. Given LPS alone, three of eight mice developed mild arthritis. The control group injected with PBS or that which remained untreated showed no signs of arthritis (Fig. 2, *c–h*).

HMGB1 acted in synergy with LPS to up-regulate the expression of RAGE, TLR2, and TLR4

HMGB1 was reported to bind to and signal through the multivalent Ig receptor RAGE, as well as TLR2 and TLR4. To further assess

[5] The online version of this article contains supplementary material.

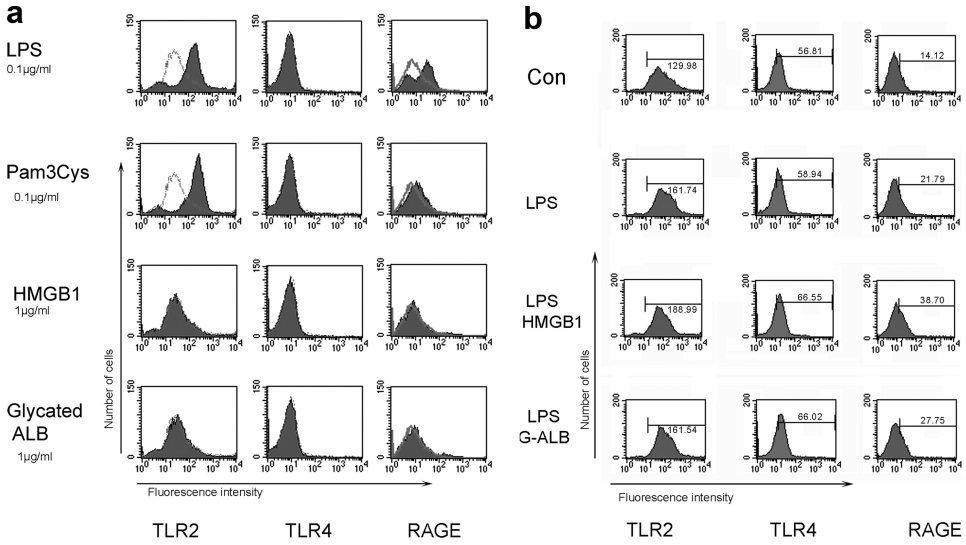

FIGURE 3. Recombinant HMGB1 proteins act as a RAGE ligand in vitro. *a*, Mouse peritoneal macrophages were cultured in triplicate with 0.1 μg/ml LPS (a TLR4 agonist), 0.1 μg/ml Pam_3Cys (a TLR2 agonist), 1 μg/ml HMGB1, or 1 μg/ml glycated ALB (a RAGE agonist). After incubation for 24 h, expression of RAGE, TLR2, and TLR4 molecules was assessed by flow cytometry. *b*, Mouse peritoneal macrophages were cultured with 0.1 ng/ml LPS with or without 0.1 μg/ml HMGB1/glycated albumin. After incubation for 24 h, expression of RAGE, TLR2, and TLR4 molecules was assessed by flow cytometry. Numbers below the marker indicate the mean fluorescence intensity of gated, and the number above the marker indicate the proportion of gated.

the extracellular function of HMGB1 (with no detectable endotoxins), we cultured the mouse peritoneal macrophages with 0.1 μg/ml LPS (a TLR4 agonist), 0.1 μg/ml Pam_3Cys (a TLR2 agonist), 1 μg/ml HMGB1, or 1 μg/ml glycated albumin (a RAGE agonist) for 24 h. The cells were then collected and stained for a flow cytometric analysis. The results shown in Fig. 3 demonstrated that HMGB1 alone, like the glycated albumin, did not up-regulate nor down-regulate the receptors (TLR2, TLR4, and RAGE) on the macrophage surface (Fig. 3*a*). We next cultured the macrophages with 0.1 ng/ml LPS with or without 0.1 μg/ml HMGB1/glycated albumin. We found that HMGB1 acted in synergy with LPS to up-regulate the RAGE, TLR2, and TLR4 on the macrophage surface and that there was little synergistic effect between LPS and glycated albumin (Fig. 3*b*).

Synergistic effect between HMGB1 and LPS is RAGE-dependent

To further assess which receptor was involved in the synergistic response, we added blocking Abs (anti-TLR2, anti-TLR4, and RAGE-Fc) 30 min before LPS and/or HMGB1 were added to the medium and measured the secretion and expression of proinflammatory cytokines. The results shown in Fig. 4 revealed that the treatment of mouse peritoneal macrophages with anti-TLR4 Abs almost completely inhibited the response (below the examination limit) to LPS or LPS plus HMGB1, whereas cytokine production by macrophages treated with anti-TLR2 remained unchanged. In the treatment of macrophages with RAGE-Fc, the synergistic effect in inducing the secretion of proinflammatory cytokines between LPS and HMGB1 was absent. The concentration of IL-6 or TNF-α in the medium of LPS and HMGB1 treated with RAGE-Fc was similar to that treated with LPS alone. Real-time PCR showed similar results, that is, Anti-TLR4 Abs inhibited the mRNA expression levels of proinflammatory cytokines to the control levels, while RAGE-Fc reduced the expression levels of proinflammatory cytokines (Fig. 4*b*).

It seemed that both TLR4 and RAGE might have contributed to the enhanced activity of LPS through binding HMGB1. Recently, HMGB1 has been reported to bind to IL-1β and then acquired the proinflammatory activity. An addition, anti-IL-1β Abs or the IL-1 receptor antagonist to cell cultures blocked the proinflammatory activity of HMGB1, indicating that such activity was dependent on

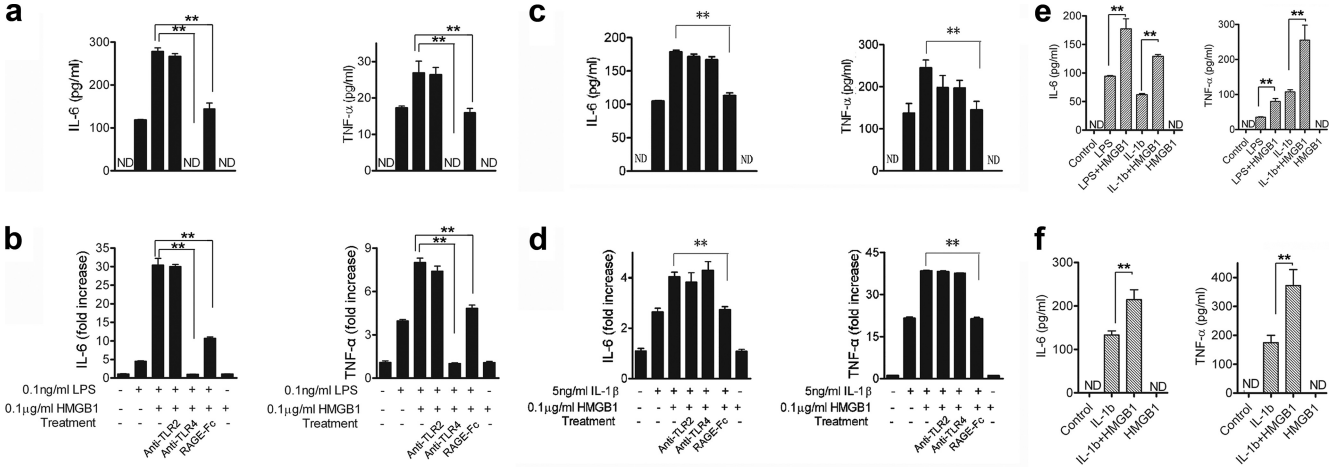

FIGURE 4. RAGE may be involved in the synergistic effect between HMGB1 and LPS or IL-1β. *a–d*, Mouse peritoneal macrophages were pretreated with blockade Abs (anti-TLR2, anti-TLR4, and RAGE-Fc) 30 min before stimulated with 0.1 ng/ml LPS plus 0.1 μg/ml HMGB1 or 5 ng/ml IL-1β plus 0.1 μg/ml HMGB1 for 24 or 3.5 h; TNF-α and IL-6 concentrations in the culture supernatants, as well as intracellular IL-6, and TNF-α mRNA levels were then determined, respectively. *e* and *f*, Mouse peritoneal macrophages isolated from $TLR2^{-/-}$ and $TLR4^{-/-}$ were cultured with 0.1 ng/ml LPS/0.1 μg/ml HMGB1 and 5 ng/ml IL-1β/0.1 μg/ml HMGB1 for 24 h. Protein levels for TNF-α and IL-6 (*a*, *c*, *e*, and *f*) and mRNA levels for IL-1β, IL-6, and TNF-α or Cxcl2 by mouse peritoneal macrophages (*b* and *d*) are shown. $*$, $p < 0.05$ and $**$, $p < 0.01$.

the interaction with the IL-1 receptor (7). Further, IL-1 receptors and TLR4 have similar intracellular Toll-IL-1 receptor domains, and initiate innate immunity via NF-κB activation leading to the production of proinflammatory cytokines. To further determine the involvement of RAGE or TLR4 in the HMGB1-induced enhancement activity of LPS, we added blocking Abs (anti-TLR2, anti-TLR4, and RAGE-Fc) 30 min before IL-1β and/or HMGB1 were added to the medium, and measured the secretion and expression of proinflammatory cytokines. Results shown in Fig. 4, *c* and *d*, indicate that IL-1β premixed with HMGB1 resulted in an enhanced activity in the mediation of mouse peritoneal macrophages to secrete proinflammatory cytokines. Treatment of mouse peritoneal macrophages with RAGE-Fc reduced the enhanced activity of IL-1β and HMGB1, whereas cytokine production by macrophages treated with anti-TLR2 or anti-TLR4 Abs remained unchanged. Real-time PCR showed similar results, that is, RAGE-Fc reduced the mRNA expression levels of the IL-1β and HMGB1 groups, anti-TLR2, and anti-TLR4 Abs, which had no significant influence on the mRNA expression levels (Fig. 4*d*).

To further rule out the TLR2 and TLR4 mediating the effect of HMGB1, we cultured the mouse peritoneal macrophages isolated from TLR2$^{-/-}$ and TLR4$^{-/-}$ mice with LPS/HMGB1 or IL-1β/HMGB1 for 24 h. HMGB1 acted in synergy with LPS and IL-1β in activating TLR2$^{-/-}$ macrophages (Fig. 4*e*), and HMGB1 acted in synergy with IL-1β in activating TLR4$^{-/-}$ macrophages (Fig. 4*f*).

Enhanced activation of p38 MAPK and NF-κB contributes to the enhancement of activity

The Toll-IL-1 receptor superfamily initiates innate immunity via NF-κB activation, leading to the production of proinflammatory cytokines. TLRs share common intracellular MyD88-dependent signal pathways to recruit the TRAF6, which leads to activation of four protein kinase cascades and culminates in the activation of NF-κB and the MAPKs p38, JNK, and ERK1/2. These molecules, in turn, promote the production of many proinflammatory proteins and enhance immune reactivity. It is also reported that, after being activated, RAGE can lead to the activation of MAPK p38 and NF-κB (12–14). To further figure out which molecules play a crucial role in mediating the production of proinflammatory cytokines, we used certain inhibitors to block these proteins from being activated. The results in Fig. 5*a* show that blockading the activation of NF-κB abolished the production of TNF-α or IL-6, and blockading the activation of MAPK p38 partly inhibited the production of TNF-α or IL-6, whereas blockading the activation of ERK or JNK had no significant effect on the production of proinflammatory cytokines (Fig. 5*a*).

To further confirm the roles of MAPK p38 and NF-κB, we cultured peritoneal macrophages with the indicated reagents for 30 min, and cells were collected for immunoblot analysis. The results in Fig. 5*b* show that the phosphorylation level of MAPK p38 in cells cultured with LPS plus HMGB1 was significantly higher than those cultured with LPS or HMGB1 alone. The activation of NF-κB inhibitor I-κB and NF-κB p65 were similar with that of MAPK p38. Pretreatment with RAGE-Fc significantly down-regulated the phosphorylation level of MAPK p38, I-κB, and NF-κB p65 induced by HMGB1-LPS. Image analysis and statistical calculations of the blots are shown in supplemental Fig. 2.

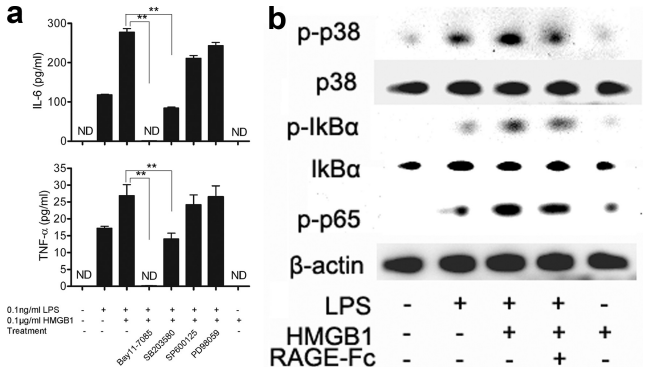

FIGURE 5. Recombinant HMGB1 enhances the activation of p38 MAPK and NF-κB contributed to the enhanced activity of LPS. *a*, Mouse peritoneal macrophages were pretreated with inhibitors (Bay11–7085, SB203580, SP600125, and PD98059) 1 h before culture with 0.1 ng/ml LPS plus 0.1 μg/ml HMGB1 for 24 h. TNF-α and IL-6 concentration in the culture supernatants was determined with an ELISA kit. *b*, Mouse peritoneal macrophages were cultured with 0.1 ng/ml LPS and/or 0.1 μg/ml HMGB1 for 30 min. The cells were lysed and the proteins were resolved by SDS-PAGE. The activation of MAPK p38 and NF-κB was studied by Western blotting. *, $p < 0.05$ and **, $p < 0.01$.

Discussion

HMGB1 has been recognized for many years as an abundant nuclear chromatin protein that binds to and distorts DNA. In doing so, it regulates many transcriptional events. HMGB1 was cloned initially in 1991 by Merenmies et al. (15), which eventually promoted the research of HMGB1. After being found as a late mediator in endotoxemia (16), the extracellular functions of HMGB1 as a cytokine have become a research highlight. In the past decade, many discoveries about HMGB1 had been reported. We are now aware that HMGB1 is a highly mobile nuclear protein that has influenced transcription and other nuclear transactions (17, 18), and that it has an important extracellular role as a signal for tissue damage (19). HMGB1 is either secreted actively by inflammatory cells or released passively as a soluble molecule from necrotic cells to signal tissue injury and initiate the inflammatory response and/or repair. In contrast to necrotic cells, apoptotic cells retain HMGB1 in their nuclei, and thus do not activate inflammation (20). Some studies have reported that apoptotic cells can also release HMGB1 and the function of HMGB1 has been determined by the redox state (21). Although HMGB1 has been proposed to potently induce acute inflammatory processes, the mechanisms through which it activates cells and increases the expression of proinflammatory mediators have not been completely characterized. The HMGB1 used in many experiments are recombined in *E. coli*, and even after strict purification procedures, we still cannot completely abolish the influence of endotoxin. We have found that HMGB1 proteins from different companies have different proinflammatory activities, which are concordant with the endotoxin contamination. Recombinant HMGB1 proteins with little endotoxins show marginal proinflammatory activity. Except endotoxins, other components of *E. coli*-like DNA may also contribute to the activity of HMGB1 (8).

Although HMGB1 (Lot 077K4002) contained more endotoxins than others, the contaminated endotoxin level could not induce macrophages to secrete high concentrations of cytokines. We showed evidence that HMGB1 actually enhanced the proinflammatory activity of the LPS. HMGB1 enhanced the LPS-induced production of proinflammatory cytokines (IL-6 and TNF-α) and the expression levels of TLR2 and RAGE in vitro, and HMGB1 acted in synergy with LPS in vivo in mice experimental arthritis model. HMGB1 has little proinflammatory activity itself, but HMGB1 can act with LPS, IL-1β (7), or DNA (8, 22) in activating the immune system and sustaining the activated state. Youn et al. (9) recently reported that HMGB1 can bind to LPS and may facilitate the transfer of LPS to CD14 and enhance LPS-induced TNF-α production in human monocytes; however, the mechanisms

through which receptors and signal pathway HMGB1 activates cells and increases the expression of proinflammatory cytokines have not been completely characterized. Many of the biological effects of HMGB1 are proposed to be mediated by TLR2, TLR4, and RAGE, thus we cultured the macrophages with agonists of RAGE, TLR2, and TLR4, and found that LPS and Pam3Cys up-regulated the expression of RAGE and TLR2, but HMGB1 and glycated albumin did not change the expression of RAGE or TLR2. Results indicated that HMGB1 alone did not have the ability to agitate TLR2 or TLR4. Next, we used the blocking Abs to impede the receptors from figuring out which receptor participated in the synergistic effect. We found that the application of anti-TLR4 or RAGE-Fc abolished the synergistic effect between HMGB1 and LPS, and it indicated that TLR4 and RAGE were involved in the synergistic effect. Because both HMGB1 and LPS were reported to signal through TLR4, we could not determine whether TLR4 mediated the effect of HMGB1. Recently, a study has found that HMGB1 proteins and IL-1β have similar synergistic responses, and IL-1 receptor and TLRs have similar intracellular signal pathways to activate NF-κB (7). We added anti-TLR4 and RAGE-Fc 30 min before IL-1 β and/or HMGB1 were added into the medium, and RAGE-Fc, which eliminated the synergistic response while blockading TLR4, did not change the production of cytokines. We next confirmed this finding in TLR2 and TLR4-deficient mice and showed evidence that the binding of HMGB1 and RAGE was crucial for the synergistic effect between HMGB1 and LPS.

The activation of RAGE and TLR4 can lead to the phosphorylation of MAPK p38 and the activation of NF-κB, while the activation of NF-κB is crucial to the production of proinflammatory cytokines such as TNF-α and IL-6 in macrophages. The mechanism of HMGB1 enhances the LPS-induced production of proinflammatory cytokines. In the case where the binding to RAGE and the signal pathway after the activation of RAGE were not well interpreted, we used signal protein inhibitors as a tool to figure out which protein was crucial to the synergistic effect between HMGB1 and LPS. After the inhibitors were added, we found that MAPK p38 was crucial to the synergistic effect, while NF-κB was crucial to the production of proinflammatory cytokines. We further confirmed the notion with immunoblotting that the phosphorylation of MAPK p38 and the activation of NF-κB were significantly up-regulated when macrophages were cultured with LPS plus HMGB1. The cross-talk between TLRs and other signal pathways was ubiquitous in macrophage activation, and it had been reported that HMGB1 can signal through both RAGE and TLR4 to activate the NF-κB (23–25). We thought it was from the LPS that HMGB1 contained signals through the TLR4 to activate the macrophages, and that the cross-talk between TLR4 and RAGE led to the enhanced phosphorylation of MAPK p38 and NF-κB. We also found that LPS acted in synergy with HMGB1 to up-regulate the expression level of receptors (TLR2, TLR4, and RAGE) in macrophages, which indicated the cross-talk between TLR4 and RAGE.

HMGB1 has two DNA binding domains, and has been shown to be capable of binding to other molecules except DNA. Using affinity chromatography, Rouhiainen et al. (26) found that some bacterial components (including DNA and LPS) bind tightly to HMGB1, and when released from HMGB1, can elicit a proinflammatory response. Youn et al. (9) showed evidences that HMGB1 actually binds tightly to LPS by ELISA, Western blot, and surface plasmon resonance. Most recently, Hreggvidsdottir et al. (27) used biotinylated LPS and streptavidin Sepharose high-performance beads to pull-down HMGB1 proteins and showed direct evidence of the formation of HMGB1-LPS complex. We used direct ELISA, naive PAGE, and Western blot to investigate the affinity between HMGB1 and LPS, and found out that HMGB1 binds tightly to LPS and forms a complex (supplemental Fig. 3). We discovered that the binding of HMGB1 and LPS was crucial to the synergistic effect, because the simultaneous addition of HMGB1 and LPS failed to significantly increase the secretion of proinflammatory cytokines in macrophages compared with the addition of LPS alone (supplemental Fig. 4). The HMGB1-LPS complex can activate their receptors instantaneously and spatial adjacents allow the cross-talk between two signal pathways to happen. We also found that the synergistic effect between glycated albumin and LPS in up-regulating the expression level of receptors was not as evident as the synergistic effect between HMGB1 and LPS, although glycated albumin can signal through RAGE. The cause may be that glycated albumin cannot bind tightly to LPS.

In conclusion, our data indicate that the proinflammatory activity of HMGB1 recombined from *E. coli* is in accordance with the endotoxin levels it contains. HMGB1 acts in synergy with LPS in activating macrophages to secrete proinflammatory cytokines, signals through RAGE, and enhances the phosphorylation of MAPK p38 and the activation of NF-κB. The cross-talk between TLR4 and RAGE leads to the enhancement of inflammatory responses in macrophages.

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Disclosures

The authors have no financial conflict of interest.

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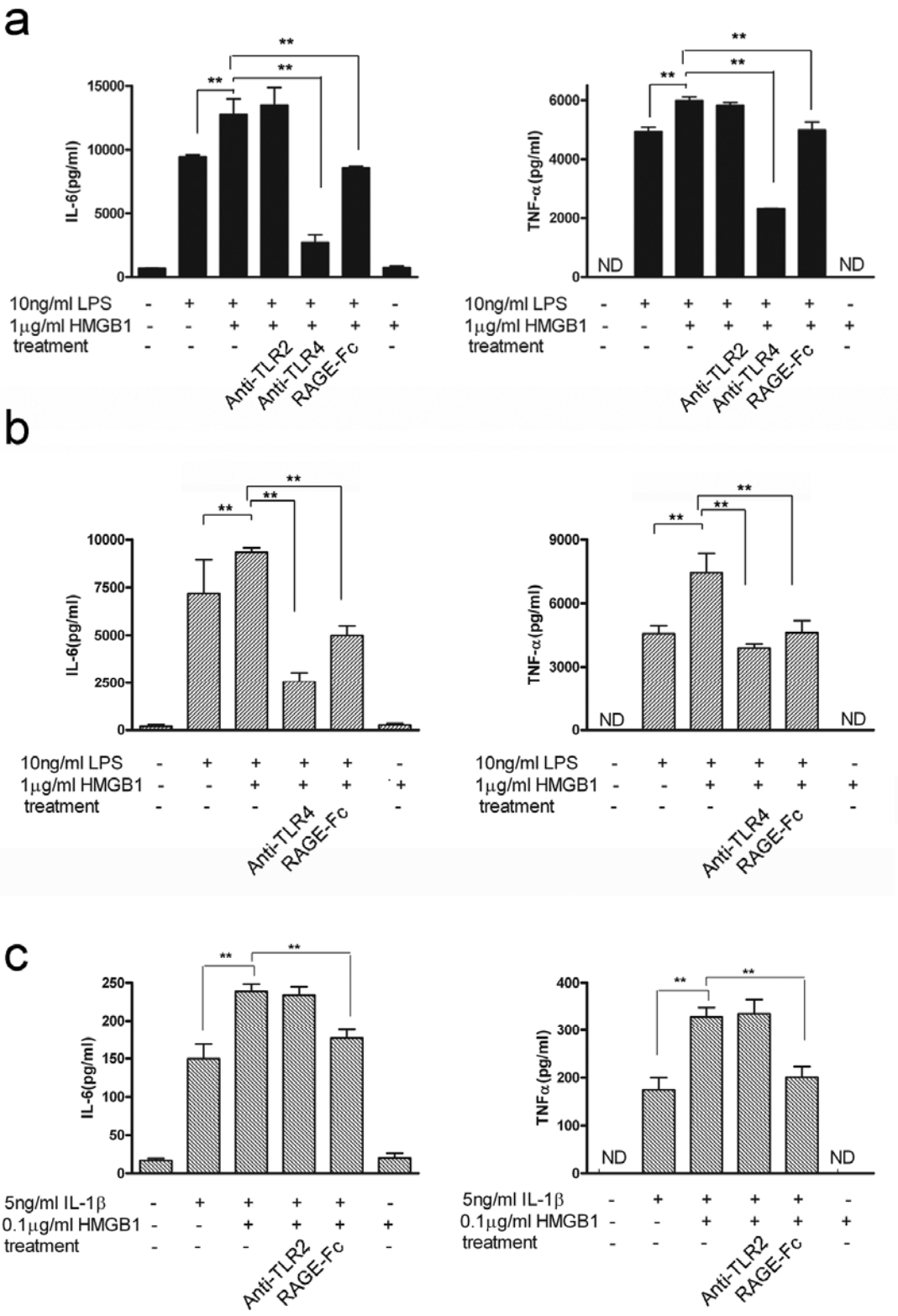
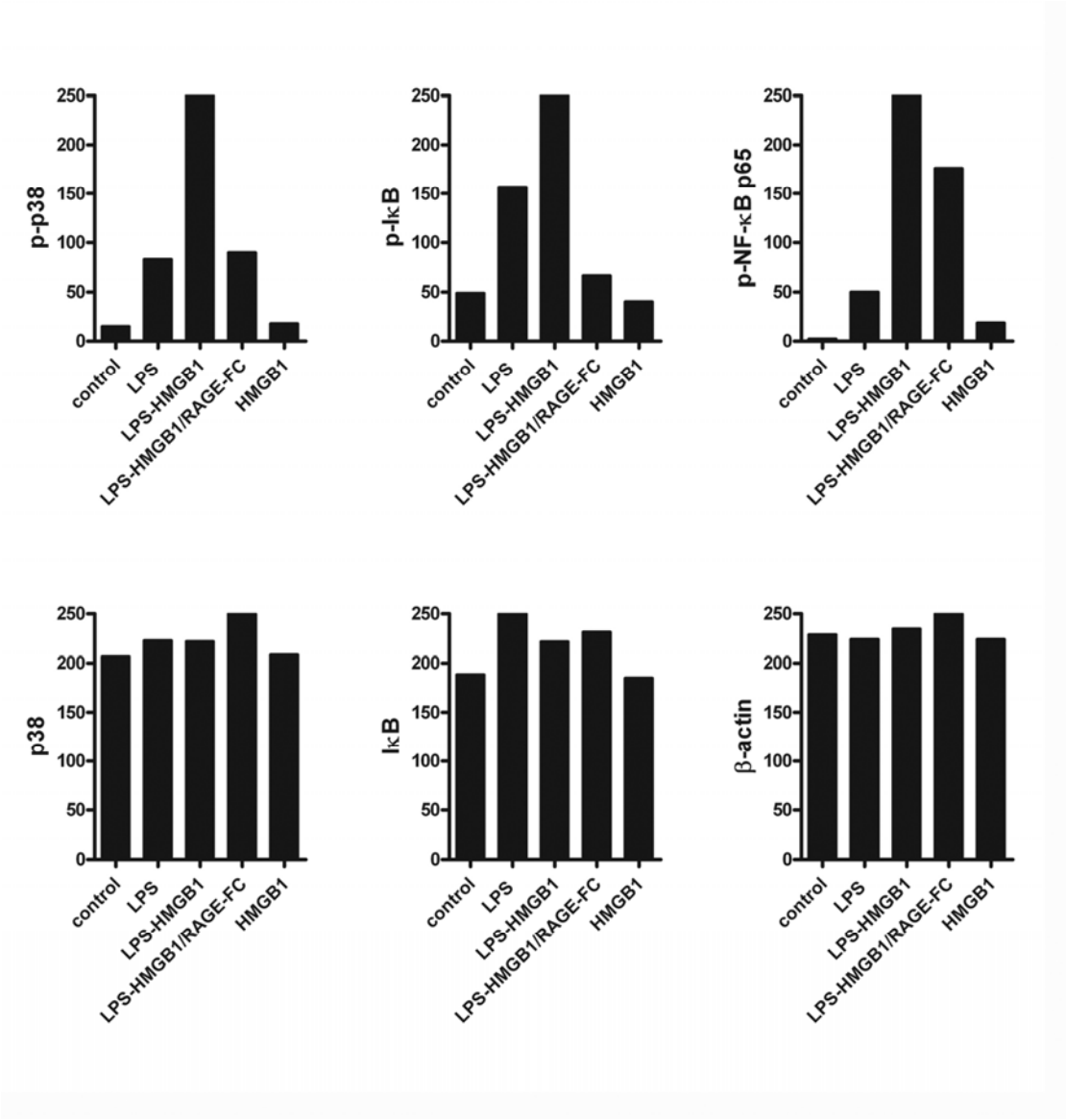
Supplemental figures



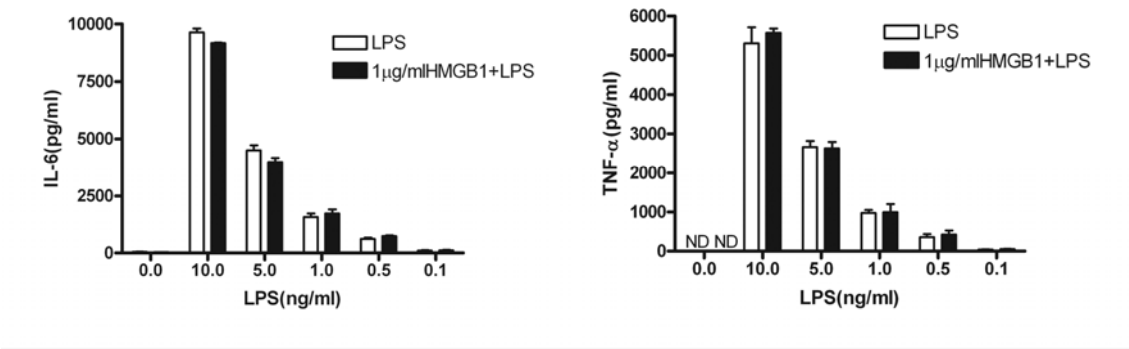
Supplemental figure1. Secretion of proinflammatory cytokines of mouse peritoneal macrophages exposed to high dose LPS and/or HMGB1

Wild type and TLR2$^{-/-}$ macrophages were cultured in triplicate with 10ng/ml LPS and/or 1μg/ml HMGB1 for 24h, TNF-α and IL-6 concentrations in the culture supernatants were then determined (a, b). TLR4$^{-/-}$ macrophages were pretreated with blockade antibodies (anti-TLR2 and RAGE-Fc) 30 minutes before stimulated with 5ng/ml IL-1β plus 0.1μg/ml HMGB1 for 24h, TNF-α and IL-6 concentrations in the culture supernatants were then determined(c) (*, $p< 0.05$, and **, $p <0.01$).



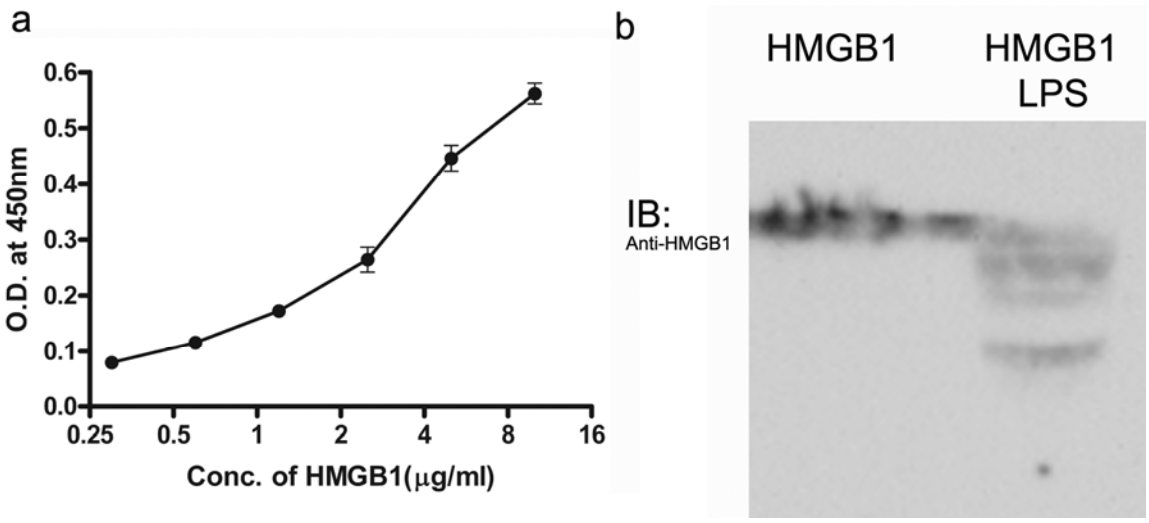
Supplemental figure.2. Image analysis and statistical calculations of the blots

All blots were quantitive analyzed with AlphaEase FC software (Alpha Innotech Corporation).



Supplemental figure 3. Secretion of proinflammatory cytokines of mouse peritoneal macrophages simultaneous exposed to LPS and HMGB1

Mouse peritoneal macrophages cultured with simultaneous addition of indicated concentration LPS and 1μg/ml HMGB1 or indicated concentration LPS alone for 24h, TNF-α and IL-6 concentrations in the culture supernatants were then determined. (Data were analyzed by A two-way ANOVA test with GraphPad Prism version 4.0 (GraphPad Software). *, $p < 0.05$, and **, $p < 0.01$).



Supplemental figure 4. HMGB1 binds tightly to LPS

To observe the binding of HMGB1 to LPS, microtiter plates (Corning) were coated with 10μg/ml LPS suspended in PBS (0.137 M NaCl, 0.005 M KCl, 0.009 M Na_2HPO_4, and 0.001 M KH_2PO_4 (pH 7.4)) containing 0.05% Tween 20 (v/v, PBST) over night at 4℃. The plates were then washed with PBST and blocked with 3% BSA in PBST. Recombinant HMGB1 proteins were added to the wells that had been coated with LPS and incubated for 2 h at room temperature. After washing, rabbit anti-HMGB1 antibodies (Saier Biotechnology) was added and incubated for 1h at room temperature. After a second wash, HRP-labeled anti-rabbit IgG (Cell Signal) was added. TMB solution (eBioScience) was used for color development and ODs were measured at 450 nm. (a). To demonstrate the formation of HMGB1-LPS complex, native gel eletrophoresis was performed. One μg LPS was incubated with 0.5μg HMGB1 for 30 min at 37°C and was electrophoresed on polyacrylamide gradient gel without detergents. Then the gel was transferred to Polyvinylidene Fluoride membranes. HMGB1 was detected with anti-HMGB1 antibodies (Saier Biotechnology) to show the formation of HMGB1-LPS complex (b).