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High Mobility Group Box 1 Protein Binding to Lipopolysaccharide Facilitates Transfer of Lipopolysaccharide to CD14 and Enhances Lipopolysaccharide-Mediated TNF-α Production in Human Monocytes[1]

Ju Ho Youn,* Young Joo Oh,* Eun Sook Kim,* Ji Eun Choi,[§] and Jeon-Soo Shin[2]*[†‡]

LPS-binding protein (LBP) is a central mediator that transfers LPS to CD14 to initiate TLR4-mediated proinflammatory response. However, a possibility of another LPS transfer molecule has been suggested because LBP-deficient mice showed almost normal inflammatory response after LPS injection. In this study, we describe the novel finding that high mobility group box 1 protein (HMGB1) recently identified as a mediator of sepsis has a function of LPS transfer for a proinflammatory response. We used ELISA and surface plasmon resonance to show that HMGB1 binds LPS in a concentration-dependent manner and that the binding is stronger to lipid A moiety than to the polysaccharide moiety of LPS. This binding was inhibited by LBP and polymyxin B. Using native PAGE and fluorescence-based LPS transfer analyses, we show that HMGB1 can catalytically disaggregate and transfer LPS to both soluble CD14 protein and to human PBMCs in a dose-dependent manner. However, this effect was dramatically reduced to the baseline level when HMGB1 was heat inactivated. Furthermore, a mixture of HMGB1 and LPS treatment results in a higher increase in TNF-α production in human PBMCs and peripheral blood monocytes than LPS or HMGB1 treatment alone or their summation. Thus, we propose that HMGB1 plays an important role in Gram-negative sepsis by catalyzing movement of LPS monomers from LPS aggregates to CD14 to initiate a TLR4-mediated proinflammatory response. ***The Journal of Immunology,*** **2008, 180: 5067–5074.**

Sepsis is a systemic inflammatory response that can induce severe organ damage and high mortality (1). When Gram-negative bacteria are introduced into blood, LPS is released and forms micellar structures due to the acyl residues of lipid A. LPS consists of a lipid A moiety and a sugar moiety of a core polysaccharide and an *O*-polysaccharide of variable length, with lipid A playing an essential role in evoking the inflammatory response (2). In the earliest cell-mediated events after LPS release, LPS-binding protein (LBP)[3] in plasma binds to the LPS molecules and catalyzes the movement of LPS monomers from LPS aggregates (3–5). LBP transfers LPS to CD14 (6), a GPI-anchored protein, which concentrates LPS and increases the sensitivity of LPS signaling through the TLR4-MD2 receptor (7, 8). LPS-mediated stimulation of $CD14^+$ cells is enhanced when LBP is added to serum-free systems (9). Therefore, the transfer LPS to CD14 by LBP is important in the initiation of LPS-induced shock.

However, an in vivo study using LBP-deficient mice have yielded conflicting results (10, 11). TNF-α production was almost normal after LPS injection into the mice although ex vivo TNF-α production in peritoneal macrophages by LPS treatment was ~1,000-fold less responsive than in $LBP^{+/-}$ mice, suggesting that LBP is not the only mediator of LPS-induced cell activation but a possible existence of another LPS transfer molecule involved in the inflammatory response (11). And another study using rabbit whole blood cells that TNF-α was released by high concentrations of LPS in LBP-depleted conditions, also suggested a possibility of LBP-independent cell stimulation (5), but remains unidentified.

High mobility group box 1 protein (HMGB1) is a nuclear protein involved in nucleosome stabilization, gene transcription, and neurite outgrowth (12). Recent evidence indicates that HMGB1 triggers inflammation (13) and also functions as a late mediator of endotoxemia and sepsis in both animal models and human patients (14–17). Specific inhibition of endogenous HMGB1 with HMGB1 antagonists of soluble receptor for advanced glycation end products (RAGE) or anti-HMGB1 Ab could reverse the lethality of established sepsis (18). After treatment with LPS or various cytokines such as TNF-α, IL-1β, or IFN-γ, HMGB1 is released from activated macrophages or monocytes within 4 h and reaches a plateau in expression levels around 18–24 h. HMGB1 is released into the extracellular space through acetylation (19) or phosphorylation (20). HMGB1 binds to several transmembrane receptors of the RAGE, TLRs 2 and 4, and syndecan-1 (CD138) and activates NF-κB and ERK1/2 (21–23).

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[3] Abbreviations used in this paper: LBP, LPS-binding protein; HMGB1, high mobility group box 1 protein; RAGE, receptor for advanced glycation end product; PBMo, peripheral blood monocyte; PMB, polymyxin B; SPR, surface plasmon resonance; CETP, cholesteryl ester transfer protein; PLTP, phospholipid transfer protein; BPI, bactericidal/permeability-increasing protein; DC, dendritic cell; ΔC-HMGB1, acidic tail-deleted HMGB1; GPI, glycosylphosphatidylinositol.

Although HMGB1 has been found to act as a mediator of sepsis, HMGB1 induces a weak TNF-α production in vitro treatment (24) or shows no significant difference of neutrophilic inflammatory response in mice which were i.p. challenged with necrotic *Hmgb1*$^{+/+}$ and *Hmgb1*$^{-/-}$ cells (25), suggesting that HMGB1 may augment the inflammatory response by binding to bacterial substances (24). HMGB1 is an amphipathic heparin-binding protein (26) that binds to some lipid molecules such as phophatidylserine and sulfatide lipids (27, 28).

In the present report, we sought to determine whether HMGB1 could interact with LPS and transfer LPS to CD14 to enhance LPS-mediated inflammation. We show that HMGB1-mediated LPS transfer increases TNF-α production in human PBMCs and peripheral blood monocytes (PBMo), indicating that there is another mechanism by which HMGB1 transfers LPS to CD14 through TLR4-mediated signaling.

Materials and Methods

LPS and recombinant proteins

For our analysis, wild-type LPS extracted from *Escherichia coli* 0111:B4, *Salmonella enterica* serotype minnesota (*S. minnesota*) and *Salmonella enterica* serotype typhimurium (*S. typhimurium*) were purchased from Sigma-Aldrich. We also used Re mutant LPS composed of lipid A and 3-deoxy-D-manno-octulosonic acid (2-keto-3-deoxyoctonate, KDO) as the sole constituent of the core from *S. minnesota* Re595 and delipidated LPS (Sigma-Aldrich), which had been partially delipidated by alkaline hydrolysis from *E. coli* 0111:B4 and resulted in endotoxin levels ~10,000 times lower than those of the parent LPS (29).

Recombinant human LBP (R&D Systems) and soluble CD14 proteins (sCD14, aa 1–352; R&D Systems) and polymyxin B (PMB; USB) were purchased. Six His-tagged recombinant human HMGB1, HMGB1 boxes A (aa 1–79) and B (aa 88–162) were produced in *E. coli* BL21 (DE3) pLysE and purified using Ni^{2+}-NTA, Sephadex G75 and ion-exchange columns (20). A six-His-tagged acidic tail-deleted HMGB1 (ΔC-HMGB1, aa 1–185) was subcloned and produced in *E. coli*. We determined the LPS level using a *Limulus* amebocyte lysate assay (Sigma-Aldrich), and the level was under 1.0 EU/μg protein. We used these bacterially produced recombinant HMGB1 proteins in this study. In a BODIPY FL-LPS transfer assay, we additionally used eukaryotic recombinant HMGB1 (Euk. HMGB1, see below).

ELISA and competition ELISA

LPS ELISAs were performed with minor modifications as previously described (30). To observe the binding of HMGB1 to LPS, microtiter plates (Corning) were coated with 10 μg/ml LPS from *S. minnesota* 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). 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, mouse anti-His Ab was added and incubated for 1.5 h. After a second wash, HRP-labeled anti-mouse Ig (Sigma-Aldrich) was added. TMB solution was used for color development and ODs were measured at 450 nm. GST protein was used as a negative control and anti-GST was used for the primary Ab. The reciprocal experiment was also performed. Microtiter plates were coated with 5 μg/ml HMGB1 and biotin-labeled LPS (InvivoGen) was added to the wells. The binding was probed with HRP-conjugated streptavidin (R&D Systems).

A competitive ELISA was performed to confirm whether HMGB1 and LBP share the competitive binding motif on LPS. On the microtiter plates coated with 1 μg/ml LPS, a constant amount of 5 μg/ml HMGB1 was added to the wells in the presence of various concentrations of LBP. GST and PMB were used as controls. After incubation for 4 h at room temperature with a gentle agitation, the plates were incubated with rabbit anti-HMGB1 Ab (Abcam) and HRP-conjugated anti-rabbit Ig as the primary and secondary Abs, respectively.

Surface plasmon resonance (BIAcore)

Analysis of LPS binding to HMGB1 was also conducted using a BIAcore 2000 instrument (BIAcore). In brief, HBS running buffer was used for sample dilution and analysis (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, and 0.005% Tween 20 (pH 7.4)). The research grade CM5 dextran sensor chip was activated with equal amounts of 0.2 M *N*-ethyl-*N*′-[3-diethylamino-propyl]-carbodiimide and 0.05 M *N*-hydroxysuccinimide. HMGB1, ΔC-HMGB1, HMGB1 boxes A and B proteins at a concentration of 100 μg/ml were immobilized in 10 mM sodium acetate buffer (pH 4.0) followed by 1 M ethanolamine-hydrochloride (pH 8.0) to deactivate excess *N*-hydroxysuccinimide-esters. This coupling resulted in 4,400 response units of immobilized proteins per flow-cell that corresponded to ~10 ng protein/mm^2. To evaluate binding, each LPS was diluted in HBS buffer and analyzed at various concentrations and passed over the sensor chip at a flow rate of 10 μl/min. LBP and GST proteins were immobilized on the adjacent vacant flow-cell under the same conditions as positive and negative control proteins, respectively. An activated and blocked flow-cell without immobilized ligand was used to evaluate nonspecific binding. For all samples, response curves were also recorded on control surfaces. Results were calculated after subtraction of the control values using the BIAevaluation 3.0 software (BIAcore).

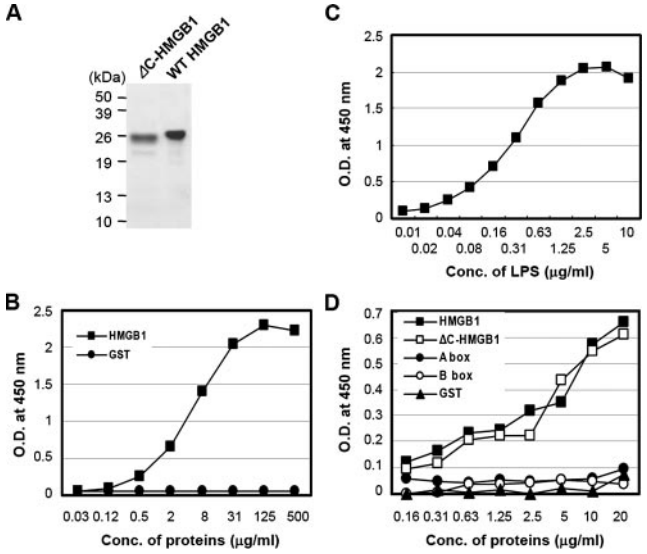

FIGURE 1. Binding of HMGB1 to LPS. *A*, Six His-tagged wild type HMGB1 and ΔC-HMGB1 proteins were expressed in *E. coli* and purified. Each protein was observed at the expected size by Coomassie blue staining after SDS-PAGE. *B* and *D*, Microtiter plates were immobilized with 10 μg/ml *S. minnesota* LPS and the binding of each His-tagged HMGB1, ΔC-HMGB1 protein, and HMGB1 boxes A and B were tested by ELISA. GST protein was used as a negative control. *C*, Microtiter plate was immobilized with 5 μg/ml HMGB1 and variable concentrations of biotin-LPS were incubated to test the binding to HMGB1.

Native PAGE and Western blotting

To demonstrate the mobility shift of HMGB1-mediated transfer of LPS to sCD14, native gel eletrophoresis was performed. A mixture of constant quantities of sCD14 protein and 1 μg/ml *S. minnesota* LPS was incubated with differing amounts of purified HMGB1 for 1.5 h at 37°C and was electrophoresed on 4–20% gradient polyacrylamide gradient gels (Bio-Rad) without detergents. After running the gels at 100 V for ~2 h with a continuous buffer (24 mM Tris, 192 mM glycine (pH 8.3)), the gels were transferred to nitrocellulose membranes. CD14 was detected with anti-CD14 Ab (R&D Systems).

Measurement of fluorescence of BODIPY FL-LPS

Disaggregation of BODIPY FL-LPS and the transfer to sCD14 was performed as described previously (31, 32). In brief, 1 μg/ml (100 nM) of BODIPY FL-LPS (LPS from *S. minnesota*; Molecular Probes) and 5 μg/ml (100 nM) of sCD14 produced from CHO cells, were added to 100 μl of PBS lacking Ca^{2+} and Mg^{2+} in the presence or absence of 5 μg/ml (200 nM) of recombinant HMGB1 protein. Two μg/ml (40 nM) of human LBP was used as a positive control. After 10 h at 25°C, the fluorescence rise was digitally measured with a LS50B spectrofluorometer (PerkinElmer). The fluorescence emission of BODIPY FL-LPS at 525 nm was recorded with a 488-nm excitation. The addition of 2% SDS completely solubilizes LPS, so the increase in BODIPY FL fluorescence emission represents a fully disaggregated state of LPS. The relative fluorescence intensities (%) were

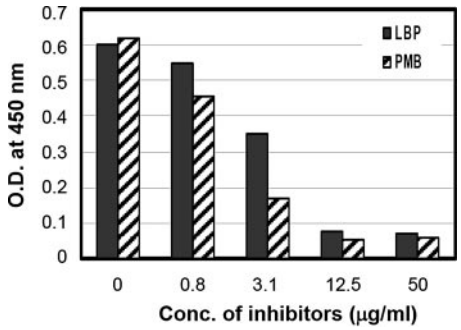

FIGURE 2. Competitive binding of HMGB1 and LBP to LPS. Microtiter plates were immobilized with 1 μg/ml *S. minnesota* LPS and a constant amount of each His-tagged HMGB1 protein was added to the wells in the presence of various concentrations of LBP. The binding of HMGB1 to LPS was measured by ELISA. PMB was used as a positive control.

calculated based on the fluorescence of disaggregated LPS alone. Eukaryotic recombinant human HMGB1 (R&D Systems), which was expressed in a mammalian cell of mouse myeloma cell line NS0, was used to confirm this assay. To observe the real-time change of fluorescence levels, the fluorescence was measured under the same conditions over 240 min at 5-min intervals after the addition of HMGB1 or LBP to a mixture of BODIPY FL-LPS and sCD14.

To investigate the LPS transfer at the cellular level, 5×10^5 human PBMCs were incubated with a mixture of BODIPY-LPS and HMGB1 or LBP for 60 min at 37°C. The mixture was preincubated for 60 min at 37°C, before addition to the cells. The cells were washed with cold PBS and the cell-associated fluorescence was measured by a spectrofluorometer.

Cytokine production

For the cytokine production experiments, human PBMCs and PBMo cells were used. Human PBMo cells, which purity was ~90% using anti-CD68 by flow cytometric analysis, were harvested from the adhesive cells on the culture flask, after yielding PBMCs from normal subjects by a Ficoll-Hypaque gradient centrifugation after obtaining the permission of IRB (YUHS Protocol 4–2007-0059). The cells were cultured in serum-free OPTI-MEM medium (Invitrogen Life Technologies) at 5×10^6 cells/ml for human PBMCs and at 5×10^5 cells/ml for human PBMo in 96-well plates. LPS in the presence or absence of HMGB1 or LBP was preincubated for 2 h and then added to the cultures. The cultures were incubated for 16 h at 37°C and the culture supernatants were collected after centrifugation. The concentration of TNF-α was determined using a sandwich ELISA (R&D Systems).

Statistics

A one-way ANOVA test was used for the analysis with GraphPad Prism version 4.0 (GraphPad Software).

Results

HMGB1 binds to LPS

To understand the role of HMGB1 in innate immunity, we produced six His-tagged wild HMGB1, HMGB1 boxes A and B proteins in *E. coli* (20). ΔC-HMGB1 was subcloned and produced with the predicted molecular size (Fig. 1*A*). To observe the interaction of HMGB1 with LPS, microtiter wells were coated with 10 μg/ml *S. minnesota* LPS and HMGB1 protein was added to the wells for an ELISA. HMGB1 bound to solid-phase LPS in a concentration-dependent manner and reached saturated binding curve

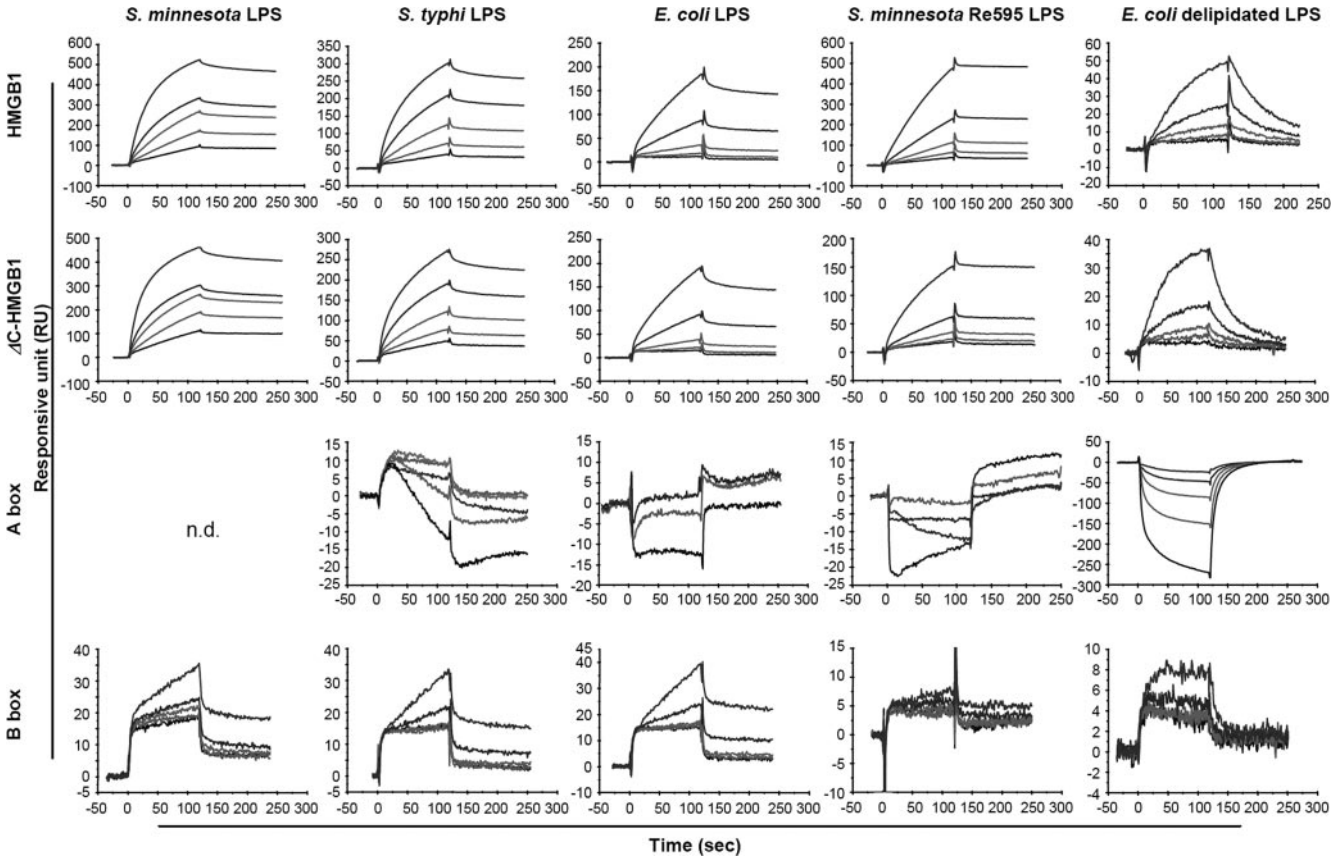

FIGURE 3. Surface plasmon resonance analyses of LPS binding to HMGB1. Three types of LPS isolated from *S. minnesota, S. typhi*, *E. coli* 0111:B4, and two types of mutant LPS of Re mutant LPS from *S. minnesota* Re595 and of delipidated LPS from *E. coli* 0111:B4, were flowed over HMGB1-, ΔC-HMGB1-, and HMGB1 boxes A and B-immobilized CM5 dextran sensor chips. Three types of wild-type LPS were applied at 0.63, 1.25, 2.5, 5, and 10 μM, and Re mutant LPS and delipidated LPS were applied at 0.06, 0.13, 0.25, 0.5, and 1 μg/ml. An activated and blocked flow-cell without immobilized ligand was used to evaluate nonspecific binding. n.d., Not done.

(Fig. 1*B*). And the reciprocal experiment of LPS binding to solid-phase HMGB1 showed the similar result (Fig. 1*C*). HMGB1 is composed of two similar HMG boxes A and B and an acidic tail (12). Both HMG boxes bind to the chromatin minor grooves and the acidic tail has important biological functions such as modulating the interaction with nucleosomes and chromatin remodeling (33). When we used ΔC-HMGB1 to observe the effect of the acidic tail on the binding of LPS, the ELISA data showed a negligible difference when compared with wild type HMGB1. These results show that HMGB1 binds well to LPS, but the influence of the acidic tail on LPS binding is little or minimal (Fig. 1*D*). HMGB1 boxes A and B, however, showed almost no binding to LPS in ELISA.

LBP competes with HMGB1 for LPS binding

A competition ELISA was performed to investigate whether the LPS-transferring molecule of LBP could compete with HMGB1 for the binding to LPS. Five μg/ml HMGB1 was added to LPS-immobilized microtiter wells in the presence of various concentrations of LBP. LBP inhibited HMGB1 binding to LPS in a concentration-dependent manner similar to PMB (Fig. 2), a well-known LPS-neutralizing peptide. These results imply that HMGB1 recognizes the lipid A moiety, which is the region that binds to LBP (4, 34).

Surface plasmon resonance (SPR) analyses

To further evaluate the binding of HMGB1 to LPS in detail, SPR analyses were performed. Because of the heterogenicity of LPS, three different types of LPS isolated from *S. minnesota*, *S. typhimurium,* and *E. coli* were applied to a CM5 dextran sensor chip, which was immobilized with HMGB1, ΔC-HMGB1, HMGB1 boxes A and B proteins to generalize the binding characteristics of LPS. The sensograms demonstrate that each LPS has a high affinity binding for both HMGB1 and ΔC-HMGB1 proteins in a concentration-dependent manner and the binding was not easily dissociated by washing (Fig. 3). LPS binding to ΔC-HMGB1 protein was similar to the binding of wild type HMGB1, confirming that the acidic tail of HMGB1 showed a negligible influence on the binding to LPS (Fig. 1*C*). Next, Re mutant LPS and delipidated LPS from *E. coli* 0111:B4 were applied to the chip to investigate which moiety of LPS is involved in the binding. Re mutant LPS showed a similar binding pattern to wild type LPS from *S. minnesota* and was hardly dissociated by washing. However, the delipidated LPS had a weak association to HMGB1 and was quickly dissociated by washing (Fig. 3), suggesting that the lipid A moiety is required for binding to HMGB1. When LPS bindings to HMGB1 boxes A and B were tested, no characteristic binding pattern was observed in HMGB1 box A and a severely reduced binding, which was not discernable in ELISA, was observed in HMGB1 box B protein.

HMGB1 facilitates the gel shift of LPS-CD14 complexes

In LPS-mediated inflammation, LPS transfer to CD14 is important for the detection of minute amounts of LPS (6). CD14-deficient mice were found to be highly resistant to shock induced by either live Gram-negative bacteria or LPS (35). LBP is a lipid transfer protein that catalyzes the transfer of LPS monomers from micelles to a binding site on CD14 (36, 37), resulting in the initiation of the TLR4-mediated signaling of LPS (7). To test whether HMGB1 can bind and catalyze LPS micelles, HMGB1 was incubated with LPS from *E. coli* and *S. minnesota* for 1.5 h at 37°C and the electrophoretic mobility shift using native PAGE was observed. HMGB1 could complex with both LPSs and the migration of HMGB1-LPS complexes was shifted (Fig. 4*A*). We next tested whether HMGB1

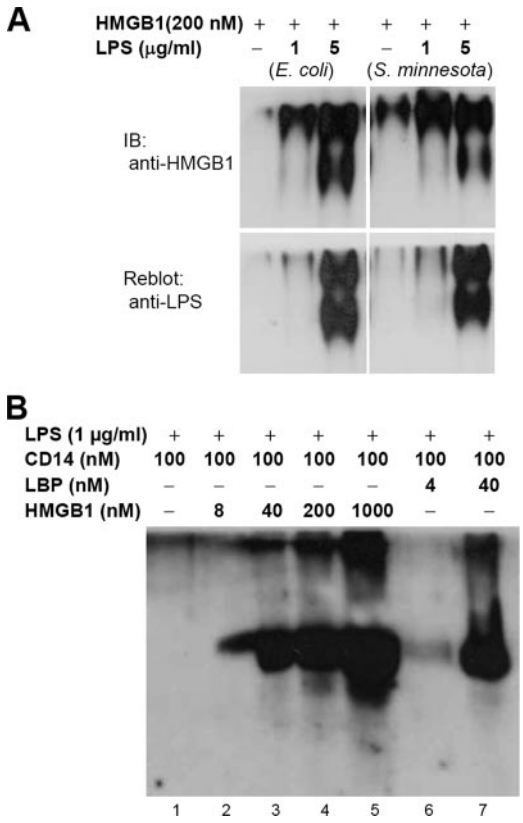

FIGURE 4. Native gel mobility shift assay of LPS transfer to sCD14 by HMGB1. *A*, *E. coli* LPS or *S. minnesota* LPS was incubated with HMGB1 for 1.5 h at 37°C and subjected to 4–20% native PAGE for membrane transfer. The membrane was blotted with anti-HMGB1 and reblotted with anti-LPS. *B*, One μg/ml *S. minnesota* LPS was incubated with the indicated amount of HMGB1 or LBP in the presence of sCD14 and subjected to native PAGE. The membrane was blotted with anti-CD14.

can transfer LPS to CD14-like LBP using the electrophoretic mobility shift of sCD14 complexed with LPS. In the presence of LBP as a positive control, LPS-sCD14 complexes were shifted in a LBP dose-dependent manner, which had been observed in a previous report (36). In the presence of HMGB1, an increasing amount of the LPS-sCD14 complex was shifted by HMGB1 in a concentration-dependent manner (Fig. 4*B*).

HMGB1 catalyzes the movement of BODIPY FL-LPS from micelles to both sCD14 protein and human PBMCs

Next, the change of fluorescence upon transition from an aggregated LPS state was measured using BODIPY FL-LPS. The fluorescence of BODIFY FL is increased upon transition from an aggregated to a disaggregated LPS state. First, to assess the ability of HMGB1 to transfer LPS, BODIPY FL-LPS was incubated with sCD14 in the presence or absence of HMGB1 for 10 h at 25°C and the fluorescence level was measured. When BODIPY FL-LPS was incubated with sCD14, LBP or HMGB1 alone, the fluorescence levels were 0.481, 0.429, and 0.541, respectively, which represent an increase of 145, 129, and 163% from BODIPY FL-LPS alone (0.332, 100%) (Fig. 5*A*). When HMGB1 was added to a mixture of BODIPY FL-LPS and sCD14, the fluorescence level was increased to 1.187 (358%, $p < 0.001$ vs the treatment of BODIPY-LPS with sCD14). This was similar to the level of positive control, a mixture containing BODIPY FL-LPS, sCD14, and LBP (387%). This increment is similar to the level of BODIPY FL-LPS that was forcibly solubilized with 2% SDS (392%) and to the previously published data (38). The LPS transfer activity by HMGB1 was

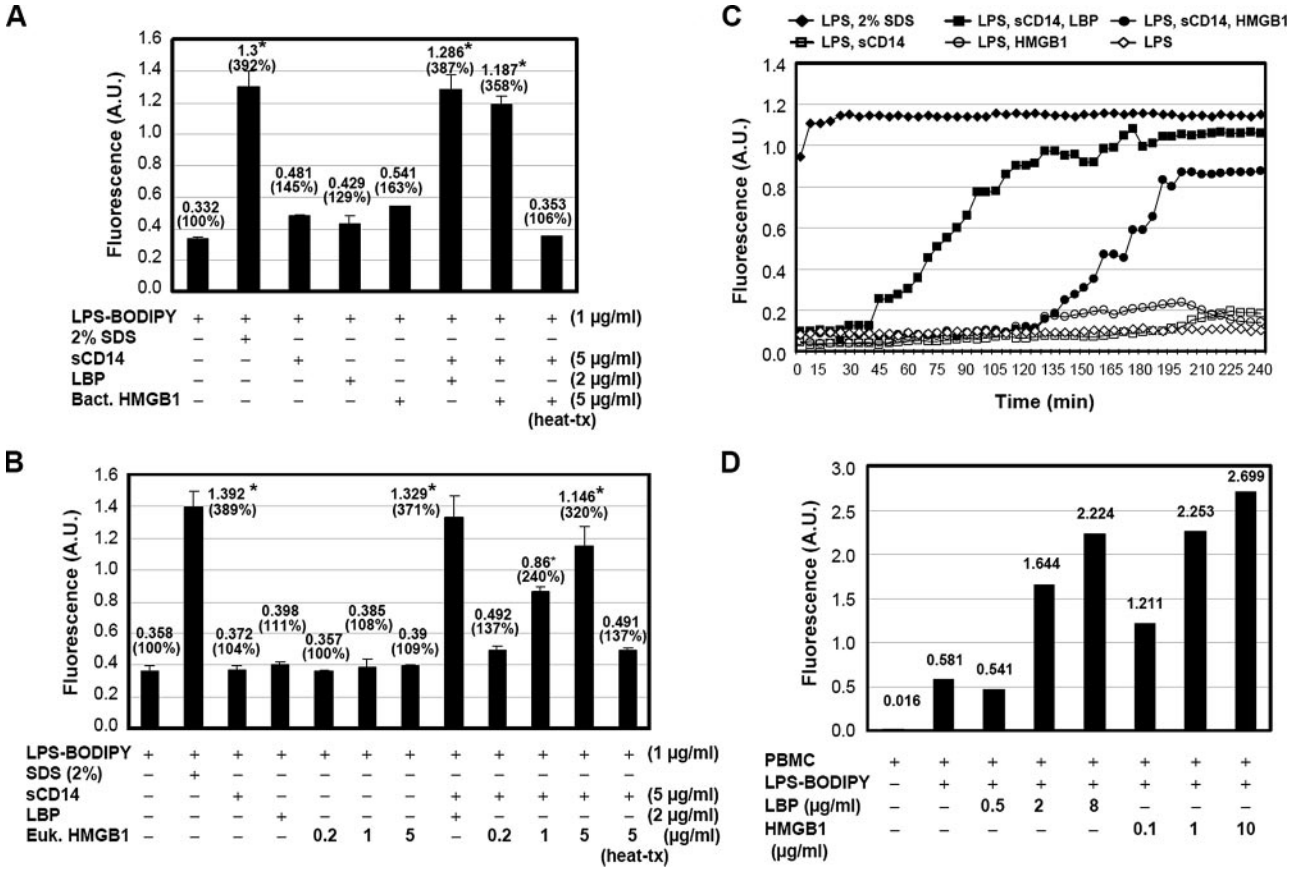

FIGURE 5. HMGB1-mediated transfer of BODIPY FL-LPS to sCD14 protein and human PBMCs. *A* and *B*, A mixture of BODIPY FL-LPS and sCD14 was incubated in the presence of bacterially produced HMGB1 (Bact. HMGB1; *A*) or eukaryotic recombinant HMGB1 (Euk. HMGB1; *B*), and fluorescence levels were measured after 10 h at 25°C. LBP was used as a positive control and 2% SDS was used to completely solubilize the disaggregated state of LPS for maximum fluorescence. Heat-treated (tx) HMGB1 was added to confirm the effect of HMGB1. Each fluorescence level is shown as the mean ± SD of three experiments. *, $p < 0.001$, vs the treatment of BODIPY-LPS with sCD14. *C*, The real-time change in BODIPY FL-LPS fluorescence levels was recorded for 240 min at 5-min intervals after incubation. A mixture of BODIPY FL-LPS (1 μg/ml) and sCD14 (5 μg/ml) was incubated with bacterially produced recombinant HMGB1 (5 μg/ml) or LBP (2 μg/ml) or none as described in *Materials and Methods*. *D*, Human PBMCs of 5×10^5 cells were incubated with a mixture of BODIPY-LPS and HMGB1 or LBP for 60 min at 37°C. The mixture was preincubated for 60 min at 37°C before its addition to the cells. The cell-associated fluorescence levels were measured after washing. The figure is a representative of two independent experiments with similar results.

dramatically reduced to almost baseline level when HMGB1 was used after heat-inactivation by boiling over 20 min (Fig. 5*A*). We also compared eukaryotic recombinant HMGB1 with bacterially produced recombinant HMGB1 because eukaryotic recombinant HMGB1 is a less potent TNF-α secretion inducer than bacterially produced recombinant HMGB1 (24). With eukaryotic recombinant HMGB1, LPS transfer increased in a HMGB1 concentration-dependent manner and was very similar to the level of transfer seen with bacterially produced HMGB1 (Fig. 5*B*).

To further evaluate the real-time change of LPS transfer, the fluorescence level was monitored over 240 min at 5-min intervals. Real-time fluorescence levels were increased in a mixture of sCD14 and BODIPY FL-LPS with the addition of HMGB1, although the initiation times between LPS transfers and the fluorescence levels between LBP and HMGB1 were different, possibly due to the lower catalytic activity of HMGB1 (Fig. 5*C*).

We then investigated whether the LPS transfer by HMGB1 could be observed at the cellular level. Human PBMCs were incubated at 37°C for 60 min with a mixture of BODIPY FL-LPS and various concentrations of HMGB1 or LBP. The sCD14 protein was removed from the reaction mixture because the monocytes in PBMCs express membranous CD14. As expected, the fluorescence level of human PBMCs was sharply increased by the addition of HMGB1 in a concentration-dependent manner (Fig. 5*D*). These data demonstrate that HMGB1 can catalyze the transfer of LPS to CD14 in both in vitro and ex vivo.

HMGB1 facilitates LPS-induced TNF-α production in human PBMCs and PBMo

As part of this study, we measured LPS-induced TNF-α levels in the culture supernatants of human PBMCs and PBMo, because some lipid transfer/LBP family members of cholesteryl ester transfer protein (CETP), phospholipid transfer protein (PLTP), and bactericidal/permeability-increasing protein (BPI) are able to interact with LPS and either mediate the diffusion of LPS monomers from the aggregates or neutralize LPS, rendering LPS noninflammatory (38, 39). Exposure of human PBMCs in serum-free medium to HMGB1 alone increased TNF-α in a concentration-dependent manner, with the TNF-α level increasing to 104 pg/ml with 1,000 ng/ml HMGB1 treatment from 24 pg/ml with 16 ng/ml HMGB1 treatment (Fig. 6*A*). Treatment with HMGB1 alone or in combination with PMB showed little difference in TNF-α production. When PBMCs were treated with various concentrations of HMGB1 in addition to 1 ng/ml LPS, TNF-α levels were dose-dependently increased from 89 pg/ml with LPS alone to 303 pg/ml with the addition of 1,000 ng/ml HMGB1. TNF-α production was

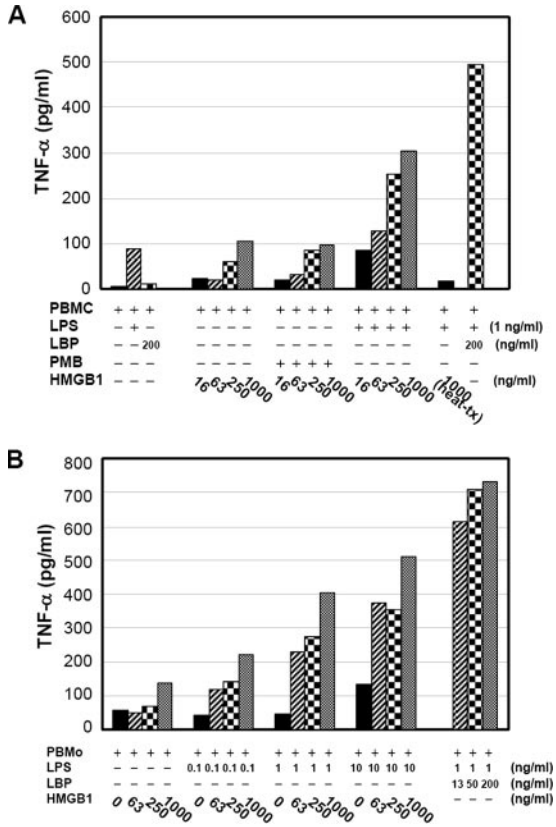

FIGURE 6. TNF-α production in human PBMCs and PBMo after HMGB1-mediated LPS transfer. *A* and *B*, Human PBMCs (5×10^6 cells/ml; *A*) and PBMo (5×10^5 cells/ml; *B*) were stimulated with the indicated concentrations of LPS in the presence of HMGB1 or LBP for 16 h at 37°C. The figures are representatives of three and two independent experiments with similar results, respectively. Heat-treated (tx) HMGB1 was added to confirm the effect of HMGB1. PMB was added at the concentration of 10 μg/ml.

reduced to almost baseline level when heat-inactivated HMGB1 was used, suggesting a role for HMGB1 in LPS transfer-mediated TNF-α production (Fig. 6*A*).

We next measured TNF-α production in human PBMo cells that were purified and then treated with various concentrations of LPS in the presence or absence of the increasing concentrations of HMGB1. TNF-α production significantly increased at each concentration of LPS with the addition of the increasing concentrations of HMGB1 (Fig. 6*B*). HMGB1 treatment resulted in a higher level of LPS-induced TNF-α production over either LPS or HMGB1 treatment alone or their summation. These results suggest that HMGB1 can transfer LPS to CD14 to mediate the inflammatory response.

Discussion

Previous investigations have shown that HMGB1 mediates proinflammatory functions by inducing TNF-α, IL-1, IL-6, IL-8, MIP-1α, and −1β (40). Further, HMGB1 activates endothelial cells, induces the release of chemokines and cytokines, up-regulates adhesion molecules to increase the adhesion of neutrophils and monocytes (41), and mediates the migration of monocytes (42) and maturation of dendritic cells (DCs) (43).

In this study, we have demonstrated the novel finding that HMGB1 could bind and catalyze the movement of LPS monomers from LPS aggregates and render LPS to CD14 and eventually to TLR4-MD2 receptors for TNF-α production. Since LBP has been found as the first LPS transferring molecule to CD14, a possibility of another LPS transfer molecule to enhance inflammation has been suggested because LBP-deficient mice produced TNF-α almost similar to $LBP^{+/-}$ mice after LPS injection (10, 11). Of the lipid transfer/LBP family members (BPI, PLTP, CETP, and LBP) (38, 39), LPS-binding histone proteins (H1, H2A, H2B, H3, H4) (44), and palate lung nasal epithelial clone family members (45), which were recently suggested to function in innate immune response in human upper airway, no LPS-transferring molecule except LBP is known to transfer LPS to CD14 and enhance the inflammatory response. We propose in this study that HMGB1 is another LPS transfer molecule to enhance LPS-mediated inflammation and able to activate monocytes at LPS concentrations lower than required with LPS alone.

HMGB1 is a highly inducible protein that is present at an undetectable plasma level of ~50 pg/ml in healthy people to an average of 25.2 ng/ml and 83.7 ng/ml in survivors and nonsurvivors, respectively, in septic patients with bacteremia and sepsis-induced organ dysfunction (14). Considering that normal plasma LBP levels are 200–500 ng/ml (6), LBP probably play a dominant role for LPS transfer before HMGB1 is secreted. HMGB1, however, may be the main molecule of LPS transfer to CD14 under conditions where LBP is absent such as in LBP-deficient mice (11) or where and when the level of HMGB1 is highly increased such as with Gram-negative bacterial infections (14). HMGB1 is secreted from not only immune competent cells of activated macrophages and monocytes, NK cells, DCs, and endothelial cells but also from nonimmune cells such as neurons, smooth muscle cells, osteoclasts, and intestinal epithelial cells (12), so HMGB1-mediated LPS transfer can take place in systemic circulation and microenvironmental circumstances.

Based on the crystal structure of human BPI, which is the only lipid transfer/LBP family protein with a known three-dimensional structure, BPI is organized into two related N- and C-terminal sequences (~200 aa) with a boomerang shape that are connected by a proline-rich linker and a single disulfide bond in the N-terminal domain. Alignment of the amino acid sequences of other lipid transfer/LBP family proteins of PLTP, CETP, and LBP shows that they all share the two-domain structure of BPI, although they have different physiological functions for their two domains and variable sequence homologies, and the two cysteine residues that form the single disulfide bond in N-terminal repeat (34). Interestingly, HMGB1 shows striking parallels of structural similarity to BPI although HMGB1 has less amino acid (215 aa) than BPI (456 aa). HMGB1 has two internal repeats of boxes A and B (~70 aa) with a proline-rich linker (six prolines in residues 80 to 99) and a disulfide bond between Cys23 and Cys45 in box A domain (12, 46). In the BPI model, the carbon tails of lipid A have been proposed to bind to the apolar lipid binding pocket in a manner similar to that observed for phosphatidylcholine (34). The binding region of LBP is residues 91–108 in N-terminal repeat (47). In our SPR data, LPS shows high affinity binding to wild type and ΔC-HMGB1 proteins but low affinity binding to box B protein, suggesting that LPS-binding region exists in or around the linker region between A and B boxes or between B box and acidic tail. Thus, we are now studying the LPS-binding region of HMGB1 for a better understanding of molecular interactions between HMGB1 and LPS.

There are many intracellular proteins that have been known to exhibit LPS-binding activity: histones H1, H2A, H2B, H3, H4 (44), and Nod1 (48). Histone H2A, a well studied protein against LPS, is not exclusively localized in the nucleus but can accumulate in the cytoplasm and on the membrane of various cell types such as neuronal cells (49), monocytes (50), and human placenta (51)

and in the extracellular milieu by secretion, suggesting a new class of intra and extracellular LPS sensor. Intracellular protein of Nod1 binds to LPS and confers cell responsiveness to LPS via NF-κB activation (48). HMGB1 is a nuclear protein that enters the nucleus via nuclear karyopherin-α1 (20) and export to the cytoplasm through CRM1 (19) and is able to localize to the cell membrane (26). It is possible that HMGB1 can act as an intra and extracellular LPS sensor. Histone H2A has a different function from HMGB1, inhibiting LPS-induced production of TNF-α and functioning as an endotoxin-neutralizing protein (44, 52).

In our study, the role of HMGB1 in LPS transfer in vivo situation is not shown because HMGB1-deficient mice are not available. HMGB1-deficient mice cannot survive after birth by hypoglycemia (53). In the ex vivo study of using HMGB1-deficient DCs, the cytokine response to LPS was not defective (54), probably because they used the culture medium containing 10% FBS, which has a significant level of LBP (6). We think that this cytokine response to LPS in HMGB1-deficient DCs was mediated by LBP-CD14-TLR4 pathway. It is not clear whether HMGB1 is the molecule that functions as an alternative LPS transfer in LBP-deficient mice. However, the enhanced productions of TNF-α by HMGB1-mediated LPS transfer in human PBMC and PBMo cells help to understand the in vivo phenomenon of HMGB1 as a mediator of sepsis, although HMGB1 alone is a minor proinflammatory trigger. Further studies regarding the in vivo role of HMGB1 in LPS transfer will provide a better understanding for LPS-mediated inflammation.

In summary, HMGB1 can bind and transfer LPS to CD14, thus enhancing LPS-mediated signaling and this pathway of HMGB1-mediated LPS transfer should be considered when managing Gram-negative sepsis and endotoxic shock.

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Disclosures

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