

Signaling of High Mobility Group Box 1 (HMGB1) through Toll-like Receptor 4 in Macrophages Requires CD14

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High mobility group box 1 (HMGB1) is a DNA-binding protein that possesses cytokinelike, proinflammatory properties when released extracellularly in the C23–C45 disulfide form. HMGB1 also plays a key role as a mediator of acute and chronic inflammation in models of sterile injury. Although HMGB1 interacts with multiple pattern recognition receptors (PRRs), many of its effects in injury models occur through an interaction with toll-like receptor 4 (TLR4). HMGB1 interacts directly with the TLR4/myeloid differentiation protein 2 (MD2) complex, although the nature of this interaction remains unclear. We demonstrate that optimal HMGB1-dependent TLR4 activation *in vitro* requires the coreceptor CD14. TLR4 and MD2 are recruited into CD14-containing lipid rafts of RAW264.7 macrophages after stimulation with HMGB1, and TLR4 interacts closely with the lipid raft protein GM1. Furthermore, we show that HMGB1 stimulates tumor necrosis factor (TNF)- α release in WT but not in TLR4^{-/-}, CD14^{-/-}, TIR domain-containing adapter-inducing interferon- β (TRIF)^{-/-} or myeloid differentiation primary response protein 88 (MyD88)^{-/-} macrophages. HMGB1 induces the release of monocyte chemoattractant protein 1 (MCP-1), interferon gamma-induced protein 10 (IP-10) and macrophage inflammatory protein 1 α (MIP-1 α) in a TLR4- and CD14-dependent manner. Thus, efficient recognition of HMGB1 by the TLR4/MD2 complex requires CD14.

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INTRODUCTION

The toll-like receptors (TLRs) are a major family of pattern recognition receptors (PRRs) that reside in cell membranes, both at the cell surface and in endosomes, that recognize and respond to a variety of bacterial products, called pathogen-associated molecular patterns (PAMPs) (1). Some members of the TLR family, notably TLR2, TLR4 and TLR9, also recognize multiple endogenous damage-associated molecular patterns (DAMPs) such as high mobility group box 1 (HMGB1), heat shock proteins (HSPs), heparan sulfate and mammalian DNA, which are released after cellular

stress or injury and can drive sterile inflammatory responses (2–5). Whereas the molecular bases for TLR recognition of many microbial molecules are well characterized, the mechanisms by which TLRs detect DAMPs are less clear.

HMGB1 is an archetypal DAMP that was originally identified as a nuclear protein involved in binding DNA and stabilizing DNA interactions with transcription factors to regulate gene transcription (6). Although the cytokinelike properties of HMGB1 were initially described in models of sepsis, HMGB1 has more recently been shown to be a mediator of inflammation in models of sterile

injury (7–9) and chronic inflammation (10). Whereas HMGB1 triggers signaling through a wide range of receptors, it is the capacity of HMGB1 to trigger TLR4 signaling that is thought to define its cytokinelike and cytokine-inducing activities (11,12). Recent studies show that only HMGB1 in which cysteine 106 is maintained in the thiol state, and also in which cysteines 23 and 45 form a disulfide bond, is capable of activating TLR4 signaling (13–15). It is unknown whether HMGB1 recognition by the TLR4/myeloid differentiation protein 2 (MD2) complex shares similarities with other prototypical activators of TLR4 signaling.

Optimal activation of TLR4 by bacterial lipopolysaccharide (LPS) involves the formation of a signaling complex that includes the coreceptor molecules MD2 and CD14, as well as intracellular signaling molecules including myeloid differentiation primary response protein 88 (MyD88) and TIR domain-containing adapter-inducing interferon- β (TRIF) (16,17). This interaction facilitates an intracellular signaling cascade that culminates in the translocation of the tran-

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scription factor nuclear factor (NF)- κ B to the nucleus (16). LPS responsiveness is enhanced by dimerization of TLR4 molecules and mobilization of the signaling complex to a portion of the plasma membrane known as a lipid raft (18). Lipid rafts are defined as glycosphingolipid-enriched domains within the cell membrane that form detergent-resistant membrane fractions (19). These fractions have light buoyancy density on sucrose gradients and are rich in both cholesterol and glycosphingolipids (20). Glycosylphosphatidyl inositol-anchored proteins such as CD14 were the first group of proteins reported to be enriched in lipid rafts (20). These lipid rafts, or membrane rafts, are believed to be small, dynamic domains that compartmentalize cellular processes and facilitate cellular signaling (19,20).

Although LPS can bind to CD14, this interaction alone is not sufficient to induce proinflammatory signaling (21,22). CD14 is thought to shuttle LPS to TLR4-coupled MD2 (16). This interaction may in turn serve to activate the TLR4 transmembrane signaling apparatus (18). Recruitment of signaling molecules to the lipid rafts may also lead to internalization of both TLR4 and LPS, a process that may be required for an adequate inflammatory response to LPS (23). The importance of this process is demonstrated by the attenuation of LPS-dependent TLR4 activation on disruption of the raft complex (18).

Whereas HMGB1 was demonstrated to display TLR4-dependent activity, it is unclear what role CD14 and lipid rafts play in this process. Here we report that CD14 is required for activation of TLR4-dependent signaling by HMGB1. Furthermore, this occurs in association with CD14, TLR4 and MD2 accumulation within lipid rafts. Thus, recognition of cytokinelike HMGB1 by macrophages requires CD14.

MATERIALS AND METHODS

Animal Care

All experimental protocols were approved by the Institutional Animal Use and Care Committee of the University of

Pittsburgh. Experimental procedures were carried out in accordance with all regulations regarding the care and use of experimental animals as published by the National Institutes of Health. Animals used in all experiments were maintained in laminar flow cages in a specific pathogen-free atmosphere at the University of Pittsburgh (Pittsburgh, PA, USA). Male C57BL/6 (WT) mice, 8–12 wks old, were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Animals were allowed access to rodent chow and water *ad libitum*. TLR4^{-/-} mice were generated in our laboratory on a C57BL/6 background and backcrossed at least six times (24). Briefly, mice harboring a floxed allele of TLR4 (TLR4^{lox[FRT-neo-FRT]/+}) were generated initially by Ozgene (Bentley, Bentley DC, Western Australia) and were mated with an EIIa-Cre transgenic mouse (B6.FVBTg [EIIa-cre]C5379Lmgd/J) to generate a null allele of TLR4 (TLR4^{del/+} or TLR4^{+/-}). The transgenic EIIa-Cre mouse expresses Cre recombinase in almost all tissues, including those of preimplantation embryos, and has been used previously to mediate recombination between loxP sites in germ cells (25). TLR4^{+/-} progeny were confirmed by polymerase chain reaction genotyping by using multiple primer pairs and interbred to generate TLR4^{-/-} mice, which were unresponsive to LPS (data not shown). TLR4^{-/-} mice showed no differences in overall health or viability compared with WT littermates. CD14^{-/-} mice (a gift from Mason Freeman, Massachusetts General Hospital, Boston, MA, USA) also on a C57BL/6 background were back-crossed at least six times and bred in our facility (26). MyD88-competent (MyD88^{+/+}) and MyD88^{-/-} mice on a C57BL/6 background were provided by Ruslan Medzhitov (Howard Hughes Medical Institute, Yale University, New Haven, CT, USA). TRIF-deficient (Trif^{LPS2/LPS2}, synonymous TRIF^{-/-}) mice on a C57BL/6 background were provided by Bruce Beutler (Scripps Institute, La Jolla, CA, USA). MyD88^{-/-} and TRIF^{-/-} strains were backcrossed at least 10 times into a C57BL/6 background and bred in our facility (27).

MyD88-deficient mice and their MyD88^{+/+} WT controls were provided water supplemented with trimethoprim (4 mg/mL) and sulfamethoxazole (40 mg/mL) until 8 wks of age. Antibiotics were stopped for at least 2 wks before mice were used in experiments.

Reagents

All tissue culture plates and flasks were purchased from BD Biosciences (San Diego, CA, USA). Dulbecco's modified Eagle medium (DMEM) and RPMI 1640 and fetal bovine serum (FBS) was from Lonza BioWhittaker (Walkersville, MD, USA). OPTI-MEM was purchased from Invitrogen/Life Technologies (Carlsbad, CA, USA). Ultrapure LPS (*Escherichia coli* 0111:B4) was purchased from List Biological Laboratories (Vandell Way, CA, USA). Polymixin B was from Sigma-Aldrich (St. Louis, MO, USA). Rabbit anti-mouse TLR4 was acquired from Santa Cruz Biotechnology (H-80; Santa Cruz, CA, USA). Mouse anti-mouse flotillin-1 and rat anti-mouse CD14 were purchased from BD Biosciences. Rabbit anti-mouse MD2 was obtained from Abcam (Cambridge, MA, USA). Pam3CSK4 (TLR2/1 ligand) and polyI:C (TLR3 ligand) were from InvivoGen (San Diego, CA, USA). Pam3CSK4 and poly I:C were tested for LPS contamination by Limulus amoebocyte lysate assay (Associates of Cape Cod, East Falmouth, MA, USA; or BioWhittaker), which determined that there was no detectable LPS (minimum sensitivity of 0.125 EU/mL). Concentrations of all ligands and inhibitors were used, as indicated in the corresponding figure legends.

Recombinant Human HMGB1

Recombinant rat HMGB1, expressed in *E. coli* and purified to homogeneity as previously described (7,28), was a gift from Kevin Tracey (The Feinstein Institute for Medical Research, Manhasset, NY, USA). HMGB1 was tested for LPS contamination by Limulus amoebocyte lysate assay, which determined that there was no detectable LPS (minimum sensitivity of 0.125 EU/mL).

Cell Culture and Transfection

RAW264.7 cells and HEK293 cells (American Type Culture Collection, Manassas, VA, USA) were maintained in DMEM containing 10% FBS, 2 mmol/L L-glutamine and 100 U/mL penicillin and streptomycin. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. HEK293 cells were stably transfected with the following: (a) empty expression vector; (b) TLR4, (c) TLR4 with MD2 or (d) TLR4, MD2 and CD14. The vectors for TLR4, MD2 and CD14 were a gift from Jeffrey L Platt (University of Michigan, Ann Arbor, MI, USA), as previously described (29). Subsequent cell lines were maintained in DMEM supplemented with 10% fetal bovine serum and the appropriate selection antibiotics. Control cell lines were transfected with empty expression vectors and maintained in selection conditions as described above. Adenovirus vectors for CD14 were constructed using plasmid vectors generously provided by Regine Landmann (University Hospital, Basel, Switzerland). Recombinant adenoviruses were generated by cotransfection of Ψ 5 and pAdlox-CD14 linearized with *Swa*I into the adenovirus packaging cell line CRE8, which expressed Cre recombinase. Recombinant adenoviruses were propagated in CRE8 cells, purified by a ViraBind Adenovirus Purification kit (Cell Biolabs, San Diego, CA, USA) according to manufacturer directions. Subsequent dialysis proceeded according to standard protocols, with the purified product being stored at -80°C. Adenoviral vectors were infected into RAW264.7 cells at a multiplicity of infection of 50. Control adenoviral vectors (Ad Ψ 5) were also infected into similar groups of cells obtained at the same time. Infection was allowed to occur for 4 h before cell treatment. Cells were collected for each assay as detailed above.

Isolation and Culture of Murine Peritoneal Macrophages

Peritoneal macrophages were isolated as previously described (30). Thioglycollate-elicited peritoneal macrophages were collected 4 d after intraperitoneal injection of

1.5 mL 4% Brewer thioglycollate medium (Sigma-Aldrich) by peritoneal washout with RPMI 1640. Peritoneal washout fluid was subsequently strained, pelleted and treated with ACK lysing buffer (Lonza Walkersville, Walkersville, MD, USA) to remove any red blood cells. Extracted cells were counted and resuspended in RPMI 1640 containing 10% FBS, 2 mmol/L L-glutamine and 100 U/mL penicillin and streptomycin. Macrophages were plated at 7×10^5 cells/well in a volume of 100 μ L/well into 96-well cell culture plates, and cells were allowed to recover overnight at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Cell media were changed the following day, and cells were treated with a total volume of 100 μ L/well of medium with indicated experimental treatment. At designed time points after treatment, supernatants were collected and stored at -80°C until analyzed for cytokines and chemokines. All cell culture experiments were performed in triplicate.

NF- κ B Luciferase Reporter Assays

Activation of NF- κ B was measured by using an NF- κ B dual luciferase reporter assay as previously described (29). Briefly, HEK293 cell lines stably expressing TLR4, TLR4 and MD2, or TLR4, MD2 and CD14, were seeded into 24-well tissue culture plates (3×10^4 cells/well) in 0.1 mL DMEM containing 10% fetal bovine serum with L-glutamine. Cells were allowed to adhere at 37°C overnight and were then transfected with 0.05 μ g/well of pTK Renilla-luciferase (Promega, Madison, WI, USA) and 0.025 μ g/well of NF- κ B firefly luciferase (a gift from Carlos Paya from Institute Pasteur, Paris, France) using LipofectamineTM-2000 (Invitrogen/Life Technologies). After transfection, cells were washed once with phosphate-buffered saline (PBS) and cultured for 24 h at 37°C overnight in 100 μ L DMEM containing 10% fetal bovine serum and L-glutamine. After various treatments, media were removed, and cells were washed once with PBS. The cells were then lysed by using 100 μ L passive lysis buffer (Promega) in a dual-luciferase re-

porter assay system from Promega with rocking at room temperature for 15 min. Renilla and firefly luciferase were then assayed simultaneously by using a Synergy Mx Monochromator-Based Multi-Mode Microplate Reader (Biotek, Winooski, VT, USA). Activation of NF- κ B is reported as a ratio of firefly luciferase to the constitutively expressed Renilla luciferase internal control and is the mean of triplicate wells.

Sucrose Gradient Fractionation

RAW264.7 cells were grown on 10-cm tissue culture plates and stimulated with LPS or HMGB1 for the indicated times. Cells were then washed twice with ice-cold PBS and lysed in ice-cold MBS buffer [25 mmol/L 2-(N-morpholino)ethanesulfonic acid solution (MES) (pH 6.5), 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L Na₃VO₄ and protease inhibitors] and incubated on ice for 30 min. Lysates were separated by density centrifugation in a discontinuous sucrose gradient (40%, 36% and 5% sucrose in MBS). Samples were spun at 250,000g in a Beckman SW41 swinging bucket rotor for 18 h at 4°C, by using slow acceleration and no brake. Fractions were removed and stored at -80°C until needed. Samples were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to identify proteins within each fraction. Lipid raft laden fractions were identified via immunoblot for flotillin-1 via Western blot analysis.

Western Blot

Samples were separated by 8–12% SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was blocked for 1 h in Tris-buffered saline (TBS)-Tween-20 (0.1%) with 5% milk, followed by immunostaining with optimized dilutions of primary antibody in 1% milk in TBS-Tween-20 (0.1%) overnight at 4°C. Membranes were washed three times for 10 min in TBS-Tween-20 (0.1%), and antibody binding was detected with horseradish peroxidase-conjugated secondary antibodies in a standard enhanced chemiluminescence reaction according to manu-

facturer's instructions (Pierce) and exposed to Kodak X-Omat film (Kodak, Rochester, NY, USA).

Cytokine/Chemokine Measurement

Tumor necrosis factor (TNF)- α , interferon gamma-induced protein 10 (IP-10), monocyte chemoattractant protein 1 (MCP-1) and a macrophage inflammatory protein 1 α (MIP-1 α) enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA) were used to assay collected supernatant in experiments. All cytokine measurements were performed according to the manufacturer's instructions.

Immunofluorescent Microscopy

RAW264.7 cells on coverslips were fixed with 4% paraformaldehyde (Canemco-MARivac) for 15 min, permeabilized with 0.1% Triton X-100 in PBS for 10 min, blocked with 2% bovine serum albumin for 1 h and incubated with anti-TLR4 (1:50) antibody. Alexa Fluor 488- or Cy3-conjugated Fab fragment secondary antibodies were from Jackson ImmunoResearch Laboratories. GM-1 ganglioside, a component of lipid raft, was detected by incubating live cells with rhodamine (TRITC)-conjugated cholera toxin B (1:200; List Biological Laboratories) before fixation.

Imaging Cytometry

Measurements of pERK (Abcam), pp38 and pJNK (Cell Signaling, Danvers, MA) were performed in WT, TLR4^{-/-} or CD14^{-/-} peritoneal exudate cells using Cellomics Arrayscan VTi (ThermoFisher Scientific, Pittsburgh, PA, USA) as previously described (31, 32). Cells were plated on 384-well plates and stimulated with either HMGB1 or LPS for time points up to 4 h; then cells were permeabilized, stained with appropriate antibodies and imaged.

Biolayer Interferometry

Affinity of target proteins to HMGB1 protein was measured by using a FortéBio Octet QK platform and default settings for the sample stage orbital rate (1,000 rpm) at 30°C. Amine reactive biosensors were

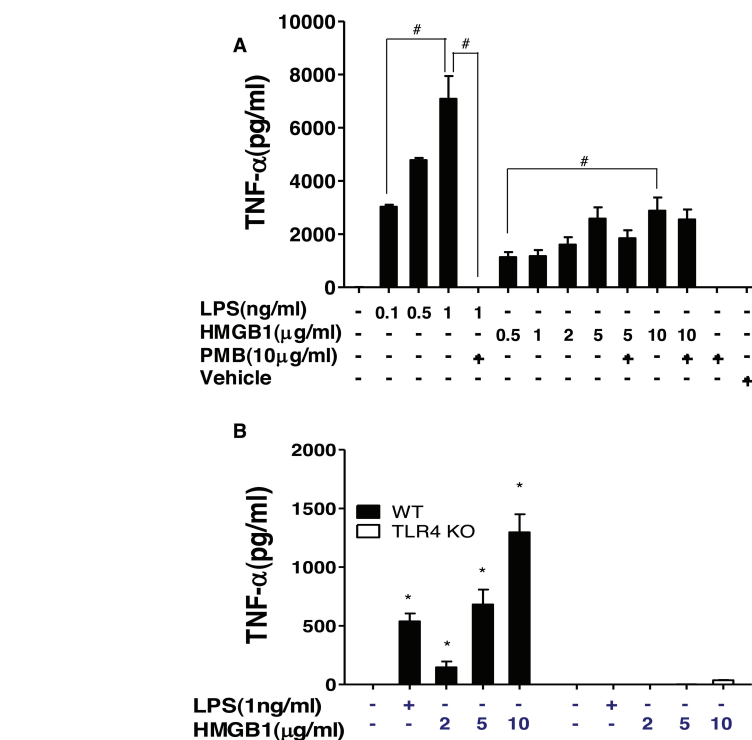


Figure 1. HMGB1-induced TNF- α release is TLR4 dependent. (A) Supernatants from RAW264.7 cells treated with vehicle, LPS (0.1, 0.5 or 1 ng/mL) or HMGB1 (0.5, 1, 2, 5, or 10 μ g/mL) with or without polymyxin B (PMB) (10 μ g/mL) for 18 h were assessed for TNF- α production by using ELISA. (B) Supernatants of peritoneal macrophages from WT or TLR4^{-/-} mice treated with LPS (1 ng/mL) and HMGB1 (2, 5 or 10 μ g/mL) for 18 h were assessed for TNF- α by using ELISA. Data are representative of three independent experiments in triplicate (mean \pm SEM, # P < 0.05 between designated, * P < 0.05 versus control).

equilibrated in a 100 mmol/L MES. Biosensors were activated with a 1:50 0.4 mol/L 1-ethyl-3-(3-dimethylamino) propyl carbodiimide, hydrochloride (EDC)/N-hydroxysuccinimide (NHS):MES solution. The amine biosensors were loaded with a solution of 25 μ g/mL HMGB1 and then introduced into solutions containing nanomolar concentrations of sTLR4, sCD14 or MD2. Recombinant antibodies to HMGB1 (RD, N-term, 5H and GLIOA5) were also assessed for HMGB1 affinity. Spastic paraplegia 4 was used as a positive binding control. The binding reaction was quenched by 1 mmol/L glycine, and PBS was used to equilibrate the protein. Dissociation was assessed by washing biosensors in PBS, and affinity to HMGB1 was calculated using FortéBio software (Menlo Park, CA, USA), controlling for the buffer only sample.

Statistical Analysis

Data are presented as mean \pm standard error of mean (SEM). Experimental results are analyzed for their significance by the Student t test by using SigmaStat (SPSS, Chicago, IL, USA). Significance was established at the 95% confidence level (P < 0.05).

All supplementary materials are available online at www.molmed.org.

RESULTS

HMGB1 Signaling through TLR4 Induces TNF- α Release and NF- κ B Activation

HMGB1 stimulates mouse macrophages to produce TNF- α through TLR4 (13). However, the role of CD14 (a known coreceptor for LPS recognition by TLR4) in

TLR4-mediated signaling of HMGB1 is unknown (13). We first confirmed that nonreduced, nonoxidized recombinant HMGB1 would stimulate TNF- α release in RAW264.7 murine macrophages in a TLR4-dependent manner. To do this, RAW264.7 cells were stimulated for 18 h with PBS (control—no stimulation) or 0.1, 0.5 or 1 ng/mL LPS; and TNF- α levels in supernatants were measured by ELISA. Even the lowest concentration of LPS used was able to stimulate RAW264.7 to produce TNF- α after 18 h (Figure 1A). TNF- α induced by 1 ng/mL LPS was completely abrogated by concurrent treatment with 10 μ g/mL polymyxin B (PMB). Similarly, HMGB1 at concentrations from 0.5 to 10 μ g/mL induced significantly increased levels of TNF- α in supernatants after 18 h in a concentration-dependent manner. The concentrations of TNF- α were not significantly decreased with concurrent treatment with PMB, confirming that LPS contamination did not account for the TNF- α release induced by the HMGB1 used for these studies (see Figure 1A). To confirm that the induction of TNF- α release was TLR4 dependent, we isolated peritoneal macrophages from WT (C57BL/6 mice) and TLR4^{-/-} mice and stimulated the cells *ex vivo* with either LPS (1 ng/mL) or HMGB1 (2, 5 or 10 μ g/mL) for 18 h. As expected, LPS induced TNF- α in peritoneal macrophages from WT mice but not in TLR4^{-/-} mice (Figure 1B). HMGB1 also stimulated TNF- α release in peritoneal macrophages from WT mice, even at the lowest concentration of 2 μ g/mL (see Figure 1B). Similarly to LPS, HMGB1 did not induce TNF- α release in TLR4^{-/-} peritoneal macrophages, confirming that HMGB1-induced TNF- α increase occurs via TLR4-dependent signaling (see Figure 1B).

HMGB1-Dependent TLR4 Activation Requires CD14 *In Vitro*

To assess the role of CD14 in TLR4-dependent recognition of HMGB1, we measured NF- κ B activation by reporter assay in HEK293 cells transfected with (a) TLR4, (b) TLR4 with MD2 or (c) TLR4, MD2 and CD14 and stimulated the cells

with LPS (100 ng/mL), HMGB1 (5 and 10 μ g/mL) or TNF- α (10 ng/mL as a positive control) for 18 h. As expected, all cells responded to the positive control, TNF- α , to exhibit significant NF- κ B activation. Only HEK293 cells transfected with the whole TLR4/MD2/CD14 complex significantly induced NF- κ B activation in response to LPS compared with controls (Figure 2A). Similarly, HMGB1 only significantly activated NF- κ B in HEK293 cells transfected with TLR4/MD2/CD14, at concentrations of 5 and 10 μ g/mL (see Figure 2A). In HEK293 cells transfected

with TLR4 alone, HMGB1 as well as LPS failed to induce significant NF- κ B activation, even in the presence of MD2. Moreover, to verify the importance of CD14 in signaling and to determine if TLR4 and CD14 alone were sufficient to allow downstream signaling, the HEK/TLR4 and HEK/TLR4/MD2 cell lines were exposed to HMGB1 with or without transfection with an adenoviral vector carrying a CD14 cDNA (AdCD14). The cell line expressing TLR4 and MD2 responded to HMGB1 if transfected with AdCD14 (Figure 2B). These findings demonstrate that

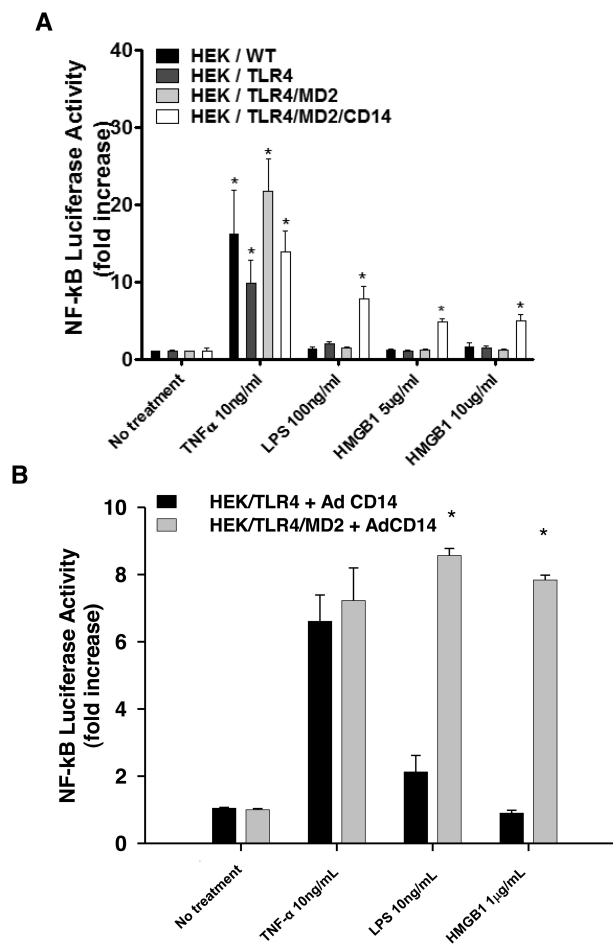


Figure 2. HMGB1-dependent TLR4 activation requires MD2 and CD14 *in vitro*. (A) HEK293 cells expressing components of the TLR4 signaling complex were stimulated with 5 or 10 μ g/mL HMGB1, 100 ng/mL LPS or 10 ng/mL TNF- α . NF- κ B activity is expressed as a fold-increase in the inducible form of luciferase. (B) HEK/TLR4 and HEK/TLR4/MD2 cell lines were transfected with AdCD14 and similarly stimulated with LPS (10 ng/mL) or HMGB1 (1 μ g/mL) treatment as above, and NF- κ B activation was measured (mean \pm SEM, * P < 0.05 versus HEK/WT in (A) and versus HEK/TLR4 + AdCD14 in (B)). Results shown are representative of at least three separate repeats in triplicate.

CD14 enhances HMGB1-dependent TLR4 activation in HEK cells.

HMGB1-Induced TNF- α and Chemokines Are TLR4/CD14 Signaling Dependent

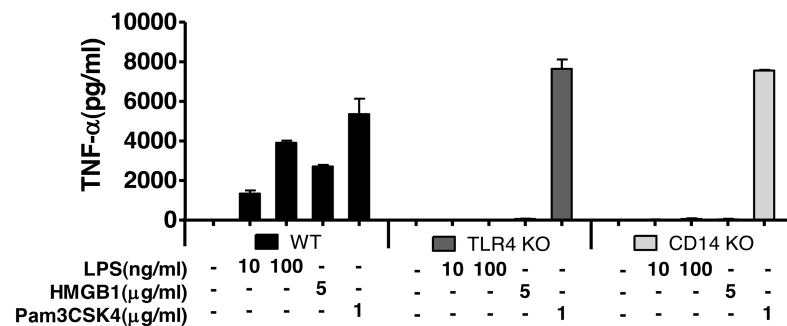
In a previous study, it was shown that HMGB1 can directly interact with CD14 (13). We confirmed this interaction by using biolayer interferometry measured using the FortéBio instrument. Recombinant HMGB1 was shown to associate with recombinant CD14 as well as with MD2 (Supplementary Figure S1). To further investigate the role of CD14 and TLR4 in HMGB1 signaling in peritoneal macrophages, we stimulated elicited peritoneal macrophages from WT, TLR4^{-/-} and CD14^{-/-} mice with HMGB1 (5 μ g/mL), LPS (10 ng/mL) or Pam3CSK4 (TLR2 agonist; 1 μ g/mL) and measured levels of TNF- α in cell supernatants after 18 h. As expected, LPS stimulated TNF- α in WT macrophages but not in TLR4^{-/-} or CD14^{-/-} macrophages. Pam3CSK4 stimulated TNF- α release from WT, TLR4^{-/-} and CD14^{-/-} macrophages (Figure 3A). HMGB1 (5 μ g/mL) also stimulated TNF- α release from WT macrophages but not from TLR4^{-/-} or CD14^{-/-} macrophages (see Figure 3A).

To determine if inflammatory mediators induced by HMGB1 in macrophages other than TNF- α were also TLR4 and CD14 dependent, we also measured macrophage production of three chemokines: MCP-1 (CCL2), IP-10 and MIP-1 α (CCL3). As shown in Figure 3B, both LPS and HMGB1 induced significant increases in the supernatant levels of these three chemokines at 18 h from WT macrophages. However, no increase was observed in either TLR4^{-/-} or CD14^{-/-} macrophages (see Figure 3B).

HMGB1 Stimulates Clustering of the TLR4/MD2/CD14 Complex in the Lipid Raft

Having demonstrated that CD14 is essential in HMGB1/TLR4 signaling, we next investigated the role of CD14-enriched lipid rafts in the recognition of

A



B

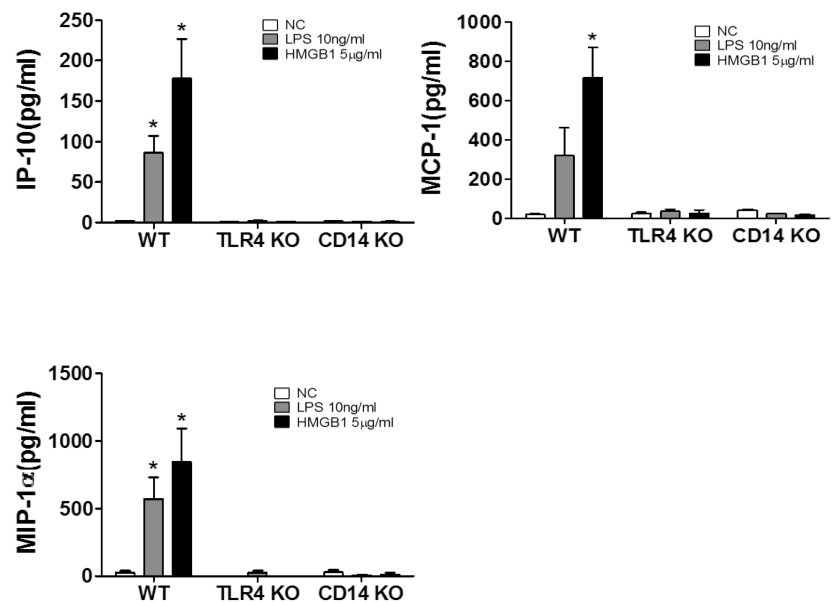


Figure 3. HMGB1-induced TNF- α and chemokine release are TLR4/CD14 dependent. (A) Peritoneal macrophages from WT, TLR4^{-/-} and CD14^{-/-} mice were treated with LPS (10, 100 or 1,000 ng/mL), HMGB1 (5 or 50 μ g/mL) or Pam3CSK4 (1 μ g/mL) in Opti-MEM for 18 h. TNF- α was then measured in supernatants by ELISA. (B) MCP-1, IP-10 and MIP-1 α in supernatants of WT, TLR4^{-/-} or CD14^{-/-} macrophages stimulated with LPS (10 ng/mL) or HMGB1 (5 μ g/mL) for 24 h. Data are representative of three independent experiments performed in triplicate (mean \pm SEM, * P < 0.05 versus negative control).

TLR4/MD2. To determine whether TLR4 was present in the lipid raft of RAW264.7 cells at baseline, untreated cell lysates were subjected to sucrose gradient fractionation. After fractionation, lysates were drawn off in 12 separate 1-mL aliquots and protein was subsequently

precipitated. An equal volume of each fraction was separated by SDS-PAGE, and Western blot was performed by using an antibody to flotillin-1 to identify raft-enriched fractions. The membrane was then probed by using an antibody to TLR4. In resting cells, no TLR4 was de-

tected in the raft fractions (Figure 4A). Similarly, RAW264.7 cells were then stimulated with 1 $\mu\text{g}/\text{mL}$ HMGB1 for 15–90 min, and the cell lysates were subsequently subjected to sucrose gradient fractionation. Maximal clustering of TLR4 in the lipid raft was seen after 60 min of stimulation with HMGB1, with clustering occurring as early as 15 min after treatment (data not shown). Colocalization of TLR4 and the raft protein GM1 was also assessed by confocal microscopy at multiple time points (Figure 4B). Minimal colocalization was observed at baseline; however, increased colocalization was observed at both 15 and 60 min, which diminished by 90 min of stimulation with HMGB1. These findings suggest that TLR4 migrates into the lipid raft transiently on stimulation with HMGB1, as shown by the close association with the raft protein GM1.

The adaptor protein MD2 closely associates with TLR4 and enhances TLR4-dependent signaling after stimulation with LPS or HMGB1 (13,16,22). To determine if HMGB1 similarly causes the migration of MD2 to the lipid raft, RAW264.7 cells were subjected to sucrose gradient fractionation both before and after stimulation with HMGB1. As expected, MD2 was limited to nonraft fractions at baseline (Figure 5). After stimulation with HMGB1, however, there was migration of MD2 to the raft complex (Figure 5). CD14 is a glycosylphosphatidylinositol-linked protein found in relative high abundance within the lipid raft (18). We confirmed this observation in unstimulated RAW264.7 cells. After stimulation with 1 $\mu\text{g}/\text{mL}$ HMGB1, there may be a slight increase in CD14 protein within the raft fraction (Figure 5).

HMGB1-Induced TNF- α Release Depends on MyD88 and TRIF

Others have shown that HMGB1-induced TNF- α production is MyD88-dependent (33). By using peritoneal macrophages from both TRIF^{-/-} and MyD88^{-/-} mice, we showed that TNF- α production by HMGB1-stimulated macrophages is both TRIF^{-/-} (Figure 6) and MyD88^{-/-} dependent (Fig-

A

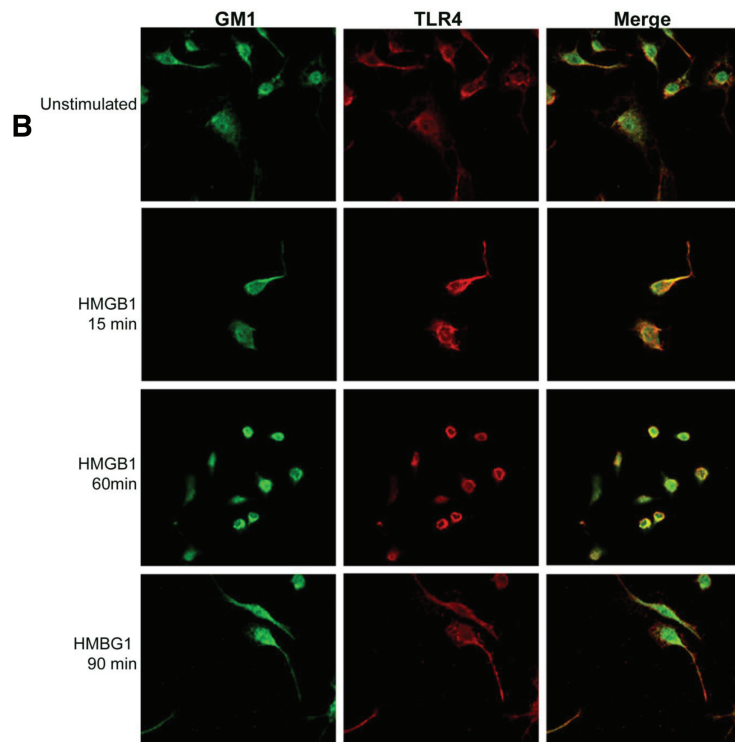
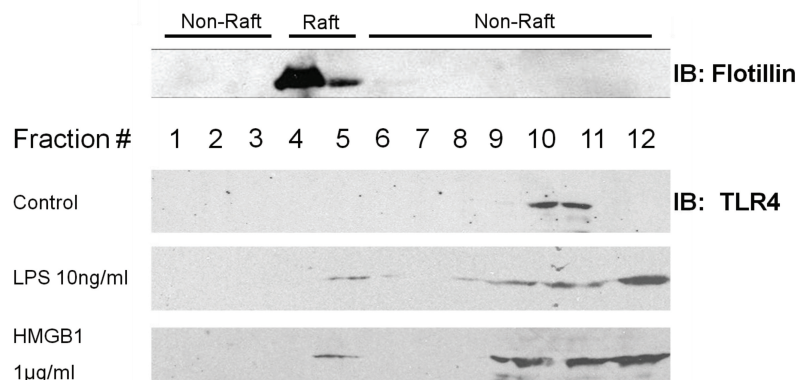


Figure 4. HMGB1 stimulates clustering of TLR4 in the lipid raft. RAW264.7 macrophages were treated with 10 ng/mL LPS or 1 $\mu\text{g}/\text{mL}$ HMGB1 for 60 min, and lysates were subjected to sucrose gradient fractionation. (A) Lysates were then drawn off in 12 separate fractions and run on SDS-PAGE before immunoblot (IB) with flotillin-1 to identify raft-enriched fractions. Fractions were then probed for TLR4. (B) RAW264.7 cells were treated with 1 $\mu\text{g}/\text{mL}$ HMGB1 for 0, 15, 60 or 90 min and fixed. Immune fluorescence microscopy was performed for TLR4 (red) and the lipid raft protein GM1 (green), and colocalization was assessed (yellow). Images are representative of at least three independent experiments.

ure 6) at the 18-h time point. As expected, TNF- α production in response to the TLR3 agonist (PolyI:C) and TLR2 agonist (Pam3CSK4) was TRIF and MyD88 dependent, respectively.

HMGB1-Induced Mitogen-Activated Protein Kinase Activation Depends on TLR4 and CD14

To determine if signaling pathways downstream of MyD88 and TRIF were ac-

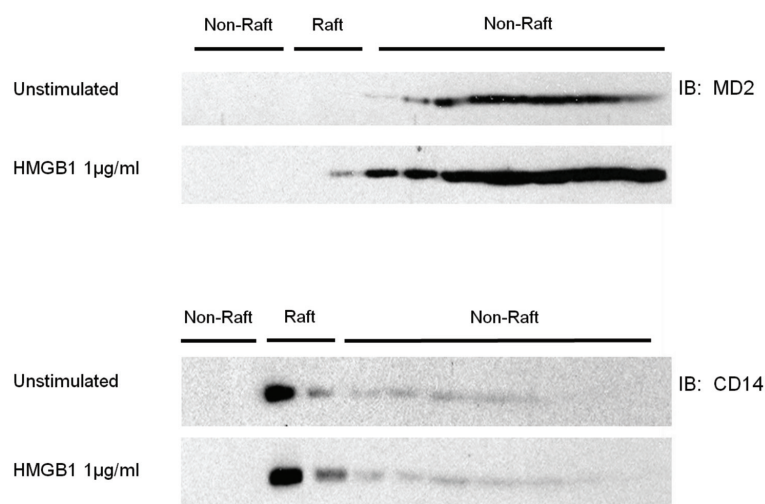


Figure 5. HMGB1 promotes localization of TLR4 coreceptors within the lipid raft. RAW264.7 macrophages were treated with 1 µg/mL HMGB1 for 60 min, and lysates were subjected to sucrose gradient fractionation. Lysates were then drawn off in 12 separate fractions and run on SDS-PAGE before immunoblot (IB) with flotillin-1 to identify raft-enriched fractions. Fractions were then probed for MD2 or CD14. Images shown are representative of at least three independent experiments.

tivated in a CD14-dependent manner, we measured the levels of nuclear phosphorylated mitogen-activated protein (MAP) kinases (pp38, pERK and pJNK) in HMGB1- or LPS-treated peritoneal macrophages by imaging cytometry. Peritoneal macrophages from WT, TLR4^{-/-} and CD14^{-/-} mice were treated with HMGB1 (5 µg/mL) or LPS (10 ng/mL) in Opti-MEM for 30 min, 1 h and 4 h. After

HMGB1 or LPS treatment, activation of pp38 (Figure 7A), pERK (Figure 7B) and pJNK (Figure 7C) were markedly increased compared with nontreated macrophages in WT by 30 min. This increase persisted to 4 h for pERK and pJNK and 2 h for pp38. The activation of all three MAP kinases was markedly diminished in both TLR4^{-/-} and CD14^{-/-} macrophages in HMGB1- and LPS-treated

cells. Taken together, these studies of macrophage activation and signaling indicate that the cytokinelike activation of macrophages by HMGB1 requires CD14.

DISCUSSION

The capacity of HMGB1 with cysteine 106 in the thiol state to activate TLR4 is now well established (9,13). TLR4/MD2 recognition of LPS is facilitated by CD14, which is thought to transfer LPS to the TLR4/MD2 complex (22,34). The goal of the study reported here was to determine if TLR4-dependent activation of macrophages by HMGB1 also requires CD14. We provide strong evidence in both cell lines, as well as primary macrophages, that CD14 is necessary for the recognition of HMGB1 by TLR4. Furthermore, our findings suggest that CD14 could play a role in the formation of a signaling complex within lipid rafts. We also extend previous findings to show that HMGB1-TLR4 signaling involves not only MyD88 but also TRIF and extends to MCP-1, IP-10 and MIP-1α in addition to TNF-α.

The explanation for inconsistencies in the capacity of HMGB1 to trigger specific cellular receptors was recently explained by a series of reports that defined the structural requirements for cytokinelike and chemokinelike activities of HMGB1. Yang *et al.* (9,13) demonstrated that cysteine 106 in the B-box domains of HMGB1 must be in the nonreduced and nonoxidized state to trigger signaling through TLR4 by binding to MD2. Recently, these observations were carried even further to show that not only must cysteine 106 be in the thiol state, but the other two cysteines at positions 23 and 45 must form a disulfide bond (35,36). If all of these cysteines are in the thiol state, HMGB1 stimulates the production of and interacts directly with CXCL12 to activate the chemokine receptor CXCR4 (15). For all of our experiments, we used HMGB1 in the C23–C45 disulfide, C106-thiol conformation (a gift from Kevin Tracey).

There is considerable evidence indicating that HMGB1–TLR4 interactions are

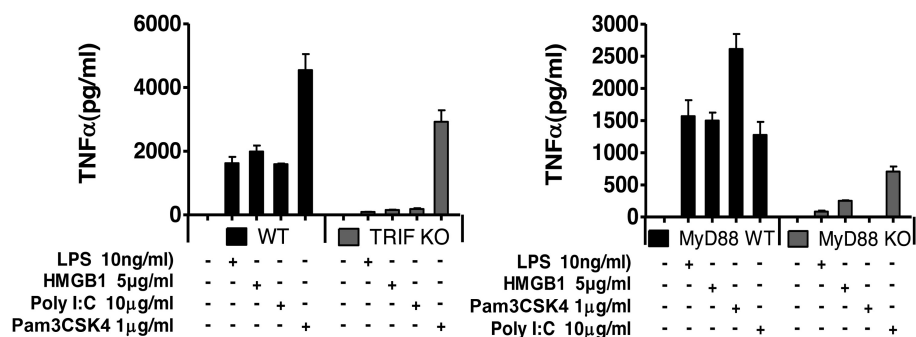


Figure 6. HMGB1-induced TNF-α release depends on MyD88/TRIF. Peritoneal macrophages from WT, TLR4^{-/-}, CD14^{-/-}, TRIF^{-/-} and MyD88^{-/-} mice were treated with HMGB1 (5 µg/mL) or LPS (10 ng/mL) in Opti-MEM for 18 h. Supernatants were then tested for TNF-α by ELISA. We found that the TNF-α release induced by HMGB1 and LPS is TRIF (A) and MyD88 (B) dependent. Data are representative of three independent experiments in triplicate (mean ± SEM).

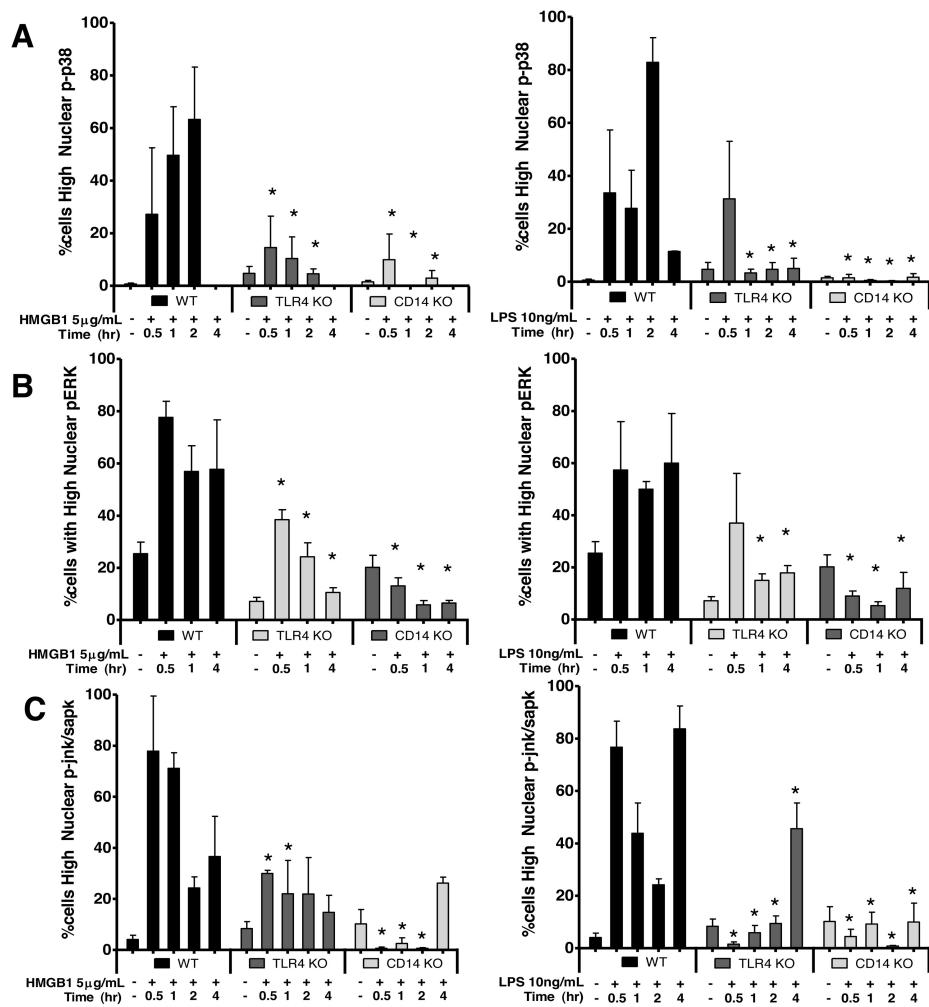


Figure 7. HMGB1-induced MAP kinase activation depends on TLR4/CD14. Peritoneal macrophages from WT, TLR4^{-/-} and CD14^{-/-} mice were treated with HMGB1 (5 μg/mL) or LPS (10 ng/mL) in Opti-MEM for the time points up to 4 h. pp38 (A), pERK (B) and pJNK (C) activation were measured by imaging cytometry. Data are representative of three independent experiments in triplicate (mean ± SEM, *P < 0.05 versus WT at the same time point).

important for both acute and chronic inflammation-associated pathobiology (10,11,37). We first showed this relationship *in vivo* in liver ischemia/reperfusion (I/R) (8,38), hemorrhagic shock (39,40) and cold cardiac I/R (41). Similar observations were noted in the kidney (42, 43) and cerebral (44, 45) models of I/R injury. Islet cell injury (46), inflammation-associated seizures (47) and skin cancer (48) as well as nonsteroidal antiinflammatory drug (NSAID)-induced intestinal damage (49) have all been linked experimentally to HMGB1-TLR4 interactions.

Whether any of these *in vivo* conditions also require CD14 recognition of HMGB1 is uncertain. We also recently found that CD14^{-/-} mice are protected from I/R-induced liver damage to a similar level to that observed in TLR4^{-/-} mice.

Most of our knowledge of TLR4 structure and function comes from experiments involving its prototypic ligand LPS. We know that LPS does not interact directly with TLR4, but instead binds first to CD14, which enables subsequent binding of LPS with MD2 (16,50). Because neither CD14 nor MD2 contain

transmembrane domains, signaling depends on activation of TLR4 when LPS triggers a conformational change in the TLR/MD2 complex (16,34). Members of this signaling apparatus accumulate within the lipid raft on stimulation with LPS, facilitating efficient signal transduction and promoting a subsequent proinflammatory response (7,51). In addition, multiple intracellular signaling proteins may also migrate into the raft scaffolding following stimulation with LPS (16).

Here we show that recombinant HMGB1 also requires CD14 to trigger signaling through TLR4. We demonstrated that on stimulation with HMGB1, both TLR4 and MD2 migrate to the lipid raft and closely associate with the raft protein GM1. CD14 was found within the lipid raft domain before stimulation to a large degree, but increased after HMGB1 stimulation. These findings suggest a requirement for both MD2 and CD14 within the raft and suggest that some interaction between these coreceptors may take place on the cell surface on stimulation with HMGB1. Because CD14 resides largely within the lipid rafts at baseline, it seems reasonable to suggest that this interaction may take place within the raft. Furthermore, the requirement for CD14 may be to facilitate the association of other proteins within the lipid raft complex (something worthy of additional study). In addition to showing that HMGB1-induced TNF-α production is TLR4 and CD14 dependent, we show that other mediators known to be produced after TLR4 ligation are also produced in a TLR4- and CD14-dependent manner by HMGB1 stimulation. HMGB1 induced robust production of MCP-1, IP-10 and MIP-1α in a TLR4- and CD14-dependent manner. Furthermore, we also demonstrated that HMGB1-induced TNF-α is TRIF and MyD88 dependent, consistent with what is known regarding LPS downstream signaling. Therefore, it is not surprising that downstream-signaling events including MAP kinases in response to HMGB1 were markedly diminished in the absence of TLR4 and CD14.

CONCLUSION

We demonstrate that CD14 is critically important in efficient HMGB1-dependent TLR4 activation. A detailed mechanistic understanding of HMGB1–TLR4 interaction and the downstream signaling pathways in the pathophysiology of inflammation could have significant implications for designing therapeutics to limit HMGB1-mediated inflammation.

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DISCLOSURE

The authors declare that they 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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