Supporting Information

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SI Materials and Methods

Generation of Floxed-*Hmgb1* Mice. The linealized targeting vector was electroporated into E14.1 embryonic stem (ES) cells. The G418 resistant clones were screened for homologous recombination by PCR. The correctly targeted clones were confirmed by Southern blot analysis with the probes indicated in Fig. S1. One of the targeted clones was then transiently transfected with a pIC-cre–expressing construct to delete the pGK-*neo* gene flanked by two loxP sites. The clones that became sensitive to G418 were subjected to Southern blot analysis to detect *Hmgb1* flox clones. Three *Hmgb1* flox ES clones were microinjected into C57BL/6 blastocysts to generate chimeric mice. Two chimeric mice successfully transmitted into the germ line. F_1 (*Hmgb1*$^{f/+}$) mice were intercrossed to generate *Hmgb1*$^{f/f}$ mice. *Cre*-recombinase–expressing mice were mated with *Hmgb1*$^{f/f}$ mice to generate *Cre*-expressing *Hmgb1*$^{f/f}$ mice.

Immunoblot Analysis. Cell extracts were prepared with RIPA lysis buffer [25 mM Tris·HCl (pH 7.6), 150 mM NaCl, 1% (vol/vol) Nonidet P-40, 1% (wt/vol) sodium deoxycholate, 0.1% (wt/vol) SDS] as described previously (1, 2). Samples were separated by 15% (wt/vol) SDS/PAGE and transferred to PVDF membrane. Anti-HMGB1 antibody (ab128129), anti-LC3 antibody (PM036), and anti–β-actin antibody (A5441) were purchased from Abcam, MBL International, and Sigma, respectively. Band density was measured by ImageJ.

Macrophage Differentiation Study. Bone-marrow–derived macrophages (BMMs) were seeded 2.5×10^5 cells per well in 48-well plates overnight. Cells were treated as follows (3): for M1 macrophage differentiation, LPS (50 ng/mL) and IFN-γ (100 ng/mL; R&D Systems); for M2a differentiation, IL-4 (20 ng/mL; Peprotech); for M2b differentiation, LPS (50 ng/mL) and immune complex [150 μg/mL anti-OVA monoclonal antibody preincubated with 15 μg/mL OVA protein (Sigma) at 37 °C for 30 min]; and for M2c differentiation, IL-10 (20 ng/mL). Seven hours after the treatment, cells were harvested and total RNA was extracted.

ELISA. Mouse TNF-α, IL-6, IL-12p40, IL-1β, IL-18, and HMGB1 were measured by ELISA with kits from R&D Systems (TNF-α, IL-6, IL-12p40, IL-1β, and IL-18) or from Shino-Test (HMGB1), according to the manufacturer's protocol.

RNA Analysis. Total RNA isolation and cDNA synthesis were performed as described previously (2). Quantitative real-time PCR analysis was carried out using LightCycler 480 and the SYBR Green system (Roche). The primer sequences for *Gapdh*, *Tnfa*, *Il6*, *Il12b*, *Il1b*, *iNOS*, *Il10*, and *Hmgb1* mRNA have been described (1, 2). The following primers for *Arg1* were used: 5′-gcaacctgtgtcctttctcc-3′ (sense) and 5′-gcaagccaatgtacacgatg-3′ (antisense). All data are presented as relative expression units after normalization to *Gapdh* mRNA expression level.

Cell Viability. Cell viability was determined by the crystal violet staining method, as described previously (4). In brief, BMMs were plated in 48-well plates at a density of 2×10^5 cells per well. Cells were treated with LPS (500 ng/mL) in the presence of z-VAD-fmk (15 μM or 20 μM). After a 24-h incubation, viable cells were fixed and stained with 4% (vol/vol) formaldehyde and 0.5% (wt/vol) crystal violet for 15 min at room temperature. Plates were then washed four times with tap water. After drying, cells were lysed with methanol, and dye uptake was measured at 595 nm using a 96-well plate reader. Cell viability was calculated from relative dye intensity compared with untreated samples.

Flow Cytometry Analysis. Mouse peripheral blood cells, splenocytes, bone marrow cells, and peritoneal exudate cells were stained with Pacific blue (PB)-conjugated anti-NK1.1, anti-CD3ε, and anti-CD19 antibodies, APC-conjugated anti-CD11b antibody, FITC-conjugated anti-Ly6C and anti-CD19 antibodies, PE-Cy7–conjugated anti-Ly6G antibody, or PerCP-Cy5–conjugated anti-F4/80 antibody. Subsequently, cells were analyzed using a LSRII Fortessa flow cytometer (5) (BD Biosciences). Antibodies were purchased from BioLegend.

Bacterial Infection. *Listeria monocytogenes* were prepared as described previously (5). Peritoneal macrophages were infected with *L. monocytogenes* for the indicated time periods in RPMI supplemented with 10% (vol/vol) FCS. For the in vivo analysis of *L. monocytogenes* infection, mice were injected with *L. monocytogenes* (5×10^5 cfu) intraperitoneally and monitored every 24 h. Whole spleens and livers were extracted 3 d after infection for colony formation assay.

Colony Formation Assay. Spleens and livers from *L. monocytogenes*-infected mice were homogenized in water. Serial dilutions of homogenates were plated on brain heart infusion agar plates and colonies were counted after incubation at 37 °C.

Fluorescence Microscopy. RAW264.7 cells (2×10^5) were cultured on glass coverslips. Cells were fixed in 4% (wt/vol) paraformaldehyde for 15 min, permeabilized in 0.1% (vol/vol) Triton X-100 for 5 min, followed by blocking in 3% (wt/vol) BSA for 30 min. Then, samples were stained with anti-HMGB1 antibody (1 μg/mL) for 1 h. An Alexa Fluor 488-conjugated anti-rabbit IgG was used as secondary antibody. Nuclei were stained with DAPI. Microscopy analysis was performed using an Olympus FV-1000 confocal microscope. Images were acquired using a sequential acquisition mode to avoid cross-excitation.

Liver Ischemia–Reperfusion. A nonlethal model of segmental (70%) hepatic warm ischemia was used (6). Under sodium pentobarbital (40 mg/kg, i.p.) anesthesia, a midline laparotomy was performed. With the use of an operating microscope, the liver hilum was dissected free of surrounding tissue. All structures in the portal triad (hepatic artery, portal vein, bile duct) to the left and median liver lobes were occluded with a microvascular clamp for 90 min; reperfusion was initiated by removal of the clamp. Throughout the ischemic interval, evidence of ischemia was confirmed by visualizing the pale blanching of the ischemic lobes. The clamp was removed and gross evidence of reperfusion that was based on immediate color change was assured before closing the abdomen with a polypropylene suture. Temperature was monitored by rectal temperature probe and was maintained at 37 °C by means of a warming pad and heat lamp. At the end of the observation period following reperfusion, the mice were anesthetized and killed by exsanguination.

1. Negishi H, et al. (2005) Negative regulation of Toll-like-receptor signaling by IRF-4. *Proc Natl Acad Sci USA* 102(44):15989–15994.

2. Yanai H, et al. (2009) HMGB proteins function as universal sentinels for nucleic-acid-mediated innate immune responses. *Nature* 462(7269):99–103.

3. Luo C, Chen M, Madden A, Xu H (2012) Expression of complement components and regulators by different subtypes of bone marrow-derived macrophages. *Inflammation* 35(4):1448–1461.
4. Takaoka A, et al. (2003) Integration of interferon-α/β signalling to p53 responses in tumour suppression and antiviral defence. *Nature* 424(6948):516–523.
5. Negishi H, et al. (2012) Cross-interference of RLR and TLR signaling pathways modulates antibacterial T cell responses. *Nat Immunol* 13(7):659–666.
6. Abe Y, et al. (2009) Mouse model of liver ischemia and reperfusion injury: Method for studying reactive oxygen and nitrogen metabolites in vivo. *Free Radic Biol Med* 46(1): 1–7.

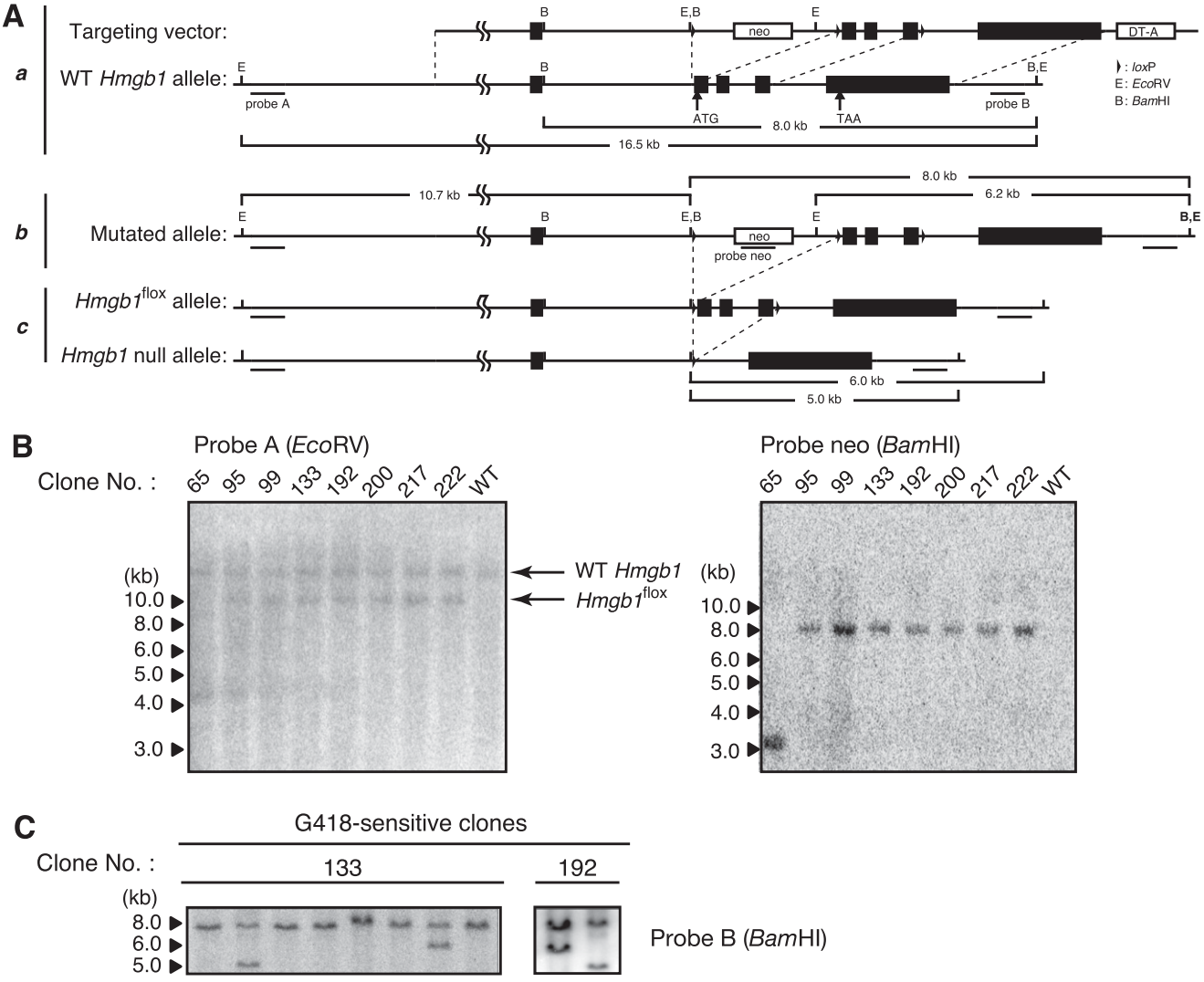

Fig. S1. Generation of *Hmgb1* gene conditional knockout mice. (*A*) Construction of the targeting vector. (*A*, *a*) Genomic structure of the mouse *Hmgb1* gene and the targeting vector. Exons 2–4 were flanked by two loxP sequences. A third loxP sequence was introduced to flank the *neo* gene. (*A*, *b*) The targeted allele contains three loxP sequences and the *neo* gene. (*A*, *c*) Cre-mediated deletion was expected to produce a *Hmgb1*^{flox} allele and *Hmgb1* null alleles. Solid triangles denote loxP sequences. E, *Eco*RV; B, BamHI. (*B*) Southern blot analysis of ES cells. Genomic DNA was isolated from target-vector–inserted ES cells, electrophoresed, transferred to nylon membrane, and hybridized with the probes indicated in *A*. Data obtained by using probe A (*Left*) and probe neo (*Right*) are shown. (*C*) Southern blot analysis of G418-sensitive ES cells. ES cells obtained in *B* were transiently transfected with pIC-cre vector. Genomic DNA was extracted and subjected to Southern blot analysis.

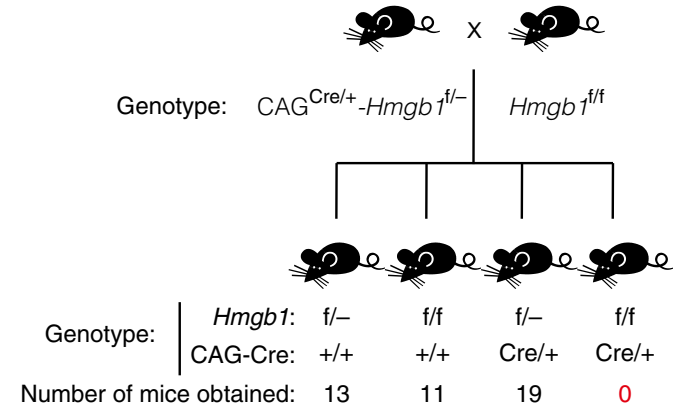

Fig. S2. Lethal effect of HMGB1 deficiency in the whole body. CAG$^{Cre/+}$-*Hmgb1*$^{f/-}$ mice were crossed with *Hmgb1*$^{f/f}$ mice. The numbers of mice of the indicated genotypes obtained are shown.

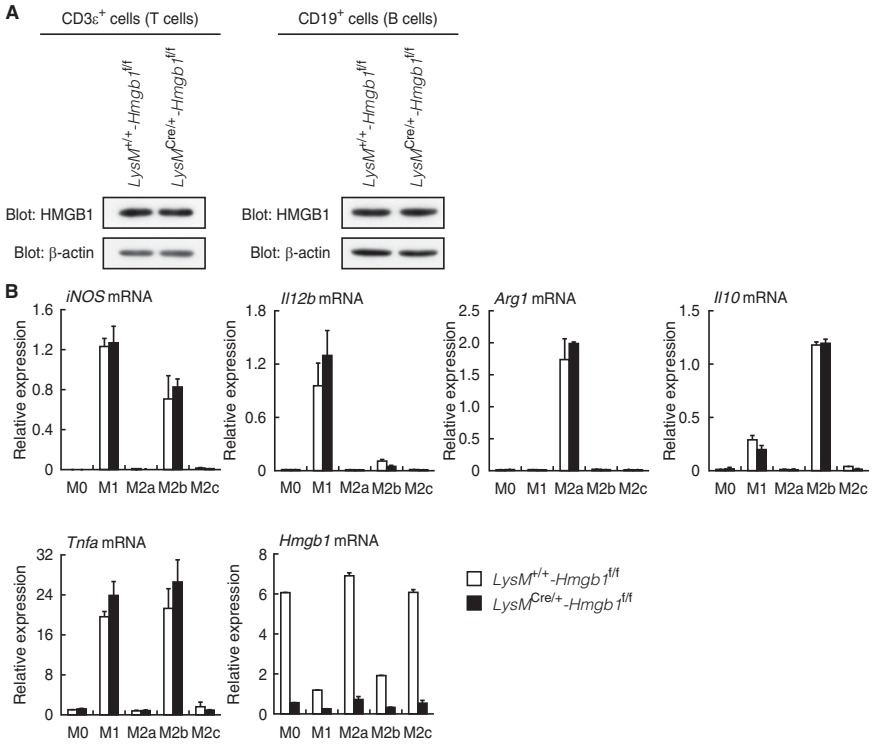

Fig. S3. Effect of HMGB1 deficiency on T- and B-cell populations and on macrophage differentiation in *LysM*$^{Cre/+}$-*Hmgb1*$^{f/f}$ mice. (*A*) CD3ε-positive T cells (*Left*) and CD19-positive B cells (*Right*) were prepared from spleen of *LysM*$^{+/+}$-*Hmgb1*$^{f/f}$ or *LysM*$^{+/Cre}$-*Hmgb1*$^{f/f}$ mice. Whole cell extracts (50 μg) were prepared and subjected to immunoblot analysis with anti-HMGB1 and β-actin antibodies. (*B*) Bone marrow cells were prepared from *LysM*$^{+/+}$-*Hmgb1*$^{f/f}$ or *LysM*$^{Cre/+}$-*Hmgb1*$^{f/f}$ mice. Bone marrow macrophages were cocultured with following mitogens for 7 h to differentiate into macrophages (M0, M1, M2a, M2b, and M2c as described previously (3). *iNOS*, *Il12b*, *Arg1*, *Il10*, *Tnfa*, and *Hmgb1* mRNA expression levels were measured by quantitative RT-PCR analysis. Data are shown as means ± SD of triplicate determinants.

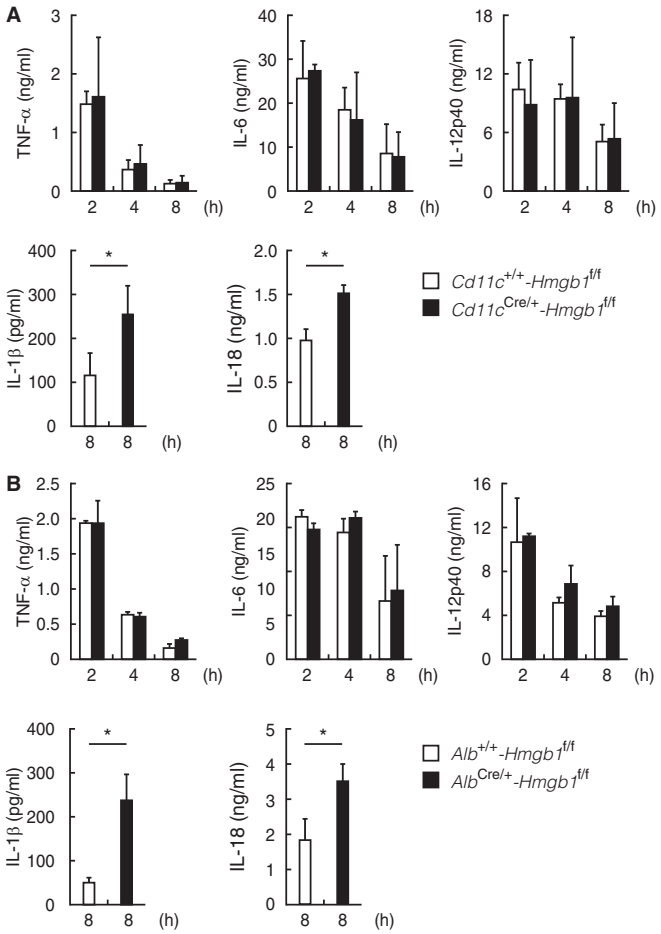

Fig. S4. LPS-induced cytokines in *Cd11c*$^{Cre/+}$-*Hmgb1*$^{f/f}$ and *Alb*$^{Cre/+}$-*Hmgb1*$^{f/f}$ mice. (*A* and *B*) *Cd11c*$^{+/+}$-*Hmgb1*$^{f/f}$ ($n = 3$) or *Cd11c*$^{Cre/+}$-*Hmgb1*$^{f/f}$ ($n = 4$) mice (*A*), or *Alb*$^{+/+}$-*Hmgb1*$^{f/f}$ ($n = 3$) or *Alb*$^{Cre/+}$-*Hmgb1*$^{f/f}$ ($n = 3$) mice (*B*) were intraperitoneally injected with LPS (17.5 mg/kg). TNF-α, IL-6, IL-12p40 (*Upper*), IL-1β, and IL-18 (*Lower*) production levels in sera were determined by ELISA. Data are shown as means ± SD; $*P < 0.05$.

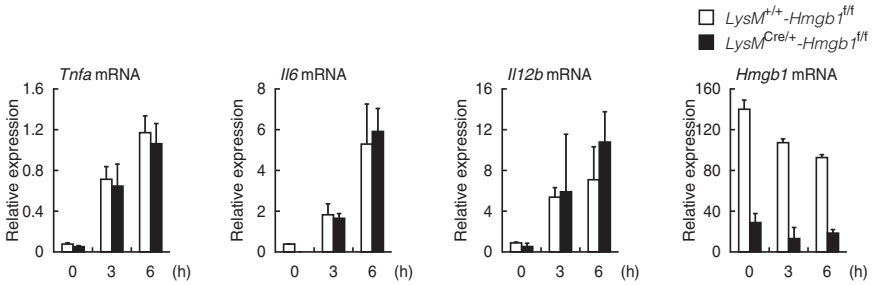

Fig. S5. Normal induction of *Tnfa*, *Il6*, *and Il12b* mRNA in *Hmgb1*-deficient peritoneal macrophages upon *L. monocytogenes* infection. Peritoneal macrophages from *LysM*$^{+/+}$-*Hmgb1*$^{f/f}$ or *LysM*$^{Cre/+}$-*Hmgb1*$^{f/f}$ mice were infected with *L. monocytogenes* (MOI of 10) for 3 or 6 h. *Tnfa*, *Il6*, *Il12b*, and *Hmgb1* mRNA expression levels were examined by quantitative RT-PCR analysis. Data are shown as means ± SD of triplicate determinants.

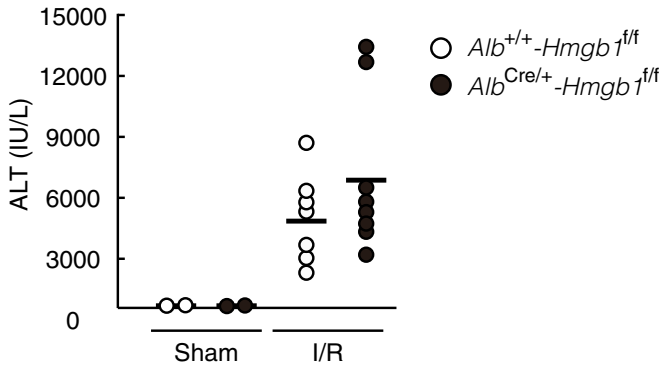

Fig. S6. Role of hepatocyte HMGB1 in hepatic ischemia–reperfusion injury. *Alb*$^{+/+}$-*Hmgb1*$^{f/f}$ ($n = 7$) or *Alb*$^{Cre/+}$-*Hmgb1*$^{f/f}$ ($n = 8$) mice were subjected to hepatic ischemia for 90 min, followed by reperfusion for 8 h. The level of plasma alanine aminotransferase (ALT) was measured. Horizontal bars represent the mean.