Supporting Information

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SI Materials and Methods

Peptides and siRNA. RVG-9dR (YTIWMPENPRPGTPCDIFTNSRGKRASNGGGGRRRRRRRRR) and RV-MAT-9dR (MNLLRKIVKNRRDEDTQKSSPASAPLDGGGGRRRRRRRRRR) peptides were synthesized and purified by high performance liquid chromatography (HPLC) at the Tufts University Core Facility (Boston, MA). In RVG-9dR and RV-MAT-9dR peptides, the carboxyl-terminal 9-arginine residues were D-arginine. siRNAs targeting firefly Luci (siLuc; 5′-CUUACGCUGAGUACUUCGAdTdT-3′), human CyPB (siCyPB; 5′-TGTCTTGGTGCTCTCC A CCdTdT-3′), human TNF-α (siTNF; 5′-5′-GGCGUGGAGCUGAGAGAUAdTdT-3′), mouse TNF-α (siTNF; 5′-GACAACCAACUAGUGGUGCdTdT-3′), and human siHMGB1 smart pool siRNAs were all purchased from Dharmacon. For some experiments, FITC-labeled Luci siRNA (at the 3′ end of the sense strand) was used.

Generation of MDDCs, MDMs, and PHA-Induced T-Cell Blasts. Human PBMCs were isolated from blood obtained from healthy donors by centrifugation over a Ficoll-Paque Plus (GE Healthcare) density gradient according to standard protocols. Monocytes were selected from PBMCs by immunomagnetic separation using human monocyte-enrichment kit (Stemcell Technologies). For generation of MDDCs, monocytes were seeded at 5×10^5 cells per well in 24-well plates in RPMI medium 1640 (Gibco BRL) supplemented with 10% heat-inactivated FBS, 50 U/mL penicillin, 50 μg/mL streptomycin, 2 mM L-glutamine, and human recombinant cytokines GM-CSF (1,000 U/mL; R&D Systems) and IL-4 (500 U/mL; R&D Systems) for 6 d at 37 °C in a CO_2 incubator. For generation of MDMs, human PBMCs (5×10^6 cells per well) were seeded in 24-well plates at 37 °C for 16 h, nonadherent cells were removed by repeated gentle washing with warm medium, and the adherent population was allowed to differentiate for an additional 5 d in the presence of recombinant human M-CSF (50 ng/mL; R&D Systems). For generation of PHA-induced T-cell blasts, human PBMCs (5×10^5 cell per well) were seeded in 24-well plates, and cells were cultured with 5 μg/mL PHA at 37 °C for 2 d.

siRNA Transduction and Gene Silencing in Vitro. RVG-9dR/siRNA complexes, prepared by incubating 200 pmol of the indicated siRNA with RVG-9dR peptide at a 10:1 peptide:siRNA ratio in serum-free medium for 15 min, were added to the cells. After incubation for 4 h at 37 °C, the cells were for tested for FITC siLuci uptake by flow cytometry. For the cyclophilin B silencing, after incubation for 4 h, the medium was replaced with fresh medium supplemented with 10% FBS (Invitrogen), and the cells were cultured for an additional 24 h before being examined by qRT-PCR. Transfection with Lipofectamine 2000 (Invitrogen) was performed in accordance with the manufacturer's instructions.

Generation of Humanized BLT Mice. Immunodeficient NOD.cg-*Prkdc*scidIL2rgtm/Wjl/Szj (NOD/SCID/IL2R$\gamma^{-/-}$) mice at 6–10 wk of age were purchased from The Jackson Laboratory and housed in specific pathogen-free microisolator cages. Humanized BLT mice preparation was performed as described previously (1). In brief, human fetal thymus and liver tissues of gestational age of 17–20 wk were obtained from Advanced Bioscience Resources. Mice were conditioned with sublethal (1-2 Gy) whole-body irradiation. Human fetal thymus and liver fragments measuring about 1 mm^3 were then implanted under the recipient kidney capsule. After implantation, mice received $CD34^+$ fetal liver cells (1–5 $\times 10^5$/mouse, intravenously) purified from the same donor on the day of human thymus/liver implantation. $CD34^+$ fetal liver cells were isolated by the magnetic-activated cell sorter separation system using$CD34^+$ positive-selection kit (Stemcell Technologies). Transplanted mice were tested for engraftment 12 wk later by flow cytometric analysis as described below. Protocols involving the use of human tissues and animals were approved by the Institutional Review Board and Institutional Animal Care and Use Committee (IACUC) at Texas Tech University. Blood was drawn from volunteers after obtaining an informed consent.

CLP Procedure. CLP was performed essentially as described in ref. 2. Briefly, mice were anesthetized with 3% isoflurane using a vaporizer (E-Z ANESTHESIA; Euthanex), and a 1-cm midline incision was made in the skin. After gently separating the skin, an incision was made on the linea alba to expose the cecum with adjoining intestine. The cecum was exteriorized and tightly ligated with a silk suture at 50% of its length and punctured through-and-through once with a 26-gauge needle midway between the ligation and the tip. The cecum was then gently squeezed to extrude a small amount of feces from the perforation sites and returned to the peritoneal cavity. The abdomen was closed in two layers (suture for muscle and clip for skin), and the animals were resuscitated by injecting 1 mL of prewarmed 0.9% saline s.c. Buprenorphine was used for postoperative analgesia, but no antibiotics were used. For control sham CLP, abdomen was opened and closed without ligation or puncture of cecum.

siRNA Delivery in Vivo. For all siRNA delivery experiments in vivo, peptide (RVG-9R or RVGMAT-9R)/siRNA complexes (at a peptide-to-siRNA molar ratio of 10:1) were prepared in 200 μL of 5% glucose, incubated at room temperature for 10 min, and then injected i.v. at 50 μg of siRNA per mouse per injection.

Quantitative RT-PCR. Total RNA was isolated from MDDCs, MDMs, and PMBCs using an RNeasy Minikit (Qiagen). RNA was reverse-transcribed with the SuperScript VILO cDNA Synthesis Kit (Invitrogen) in accordance with the manufacturer's protocol. RT-PCR was performed using 3 μL of cDNA with the SYBR green PCR Master Mix Kit (Applied Biosystems) according to the manufacturer's instructions. Amplification conditions were as follows: 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min using the 7900HT Fast Real-Time PCR system (Applied Biosystems). Primers used were for cyclophilin B (forward, 5′-CATGGACAAGATGCCAGGAC-3′; reverse, 5′-GTACCTGTTCTACGGTCCTG-3′), β-actin (forward, 5′-TGAGTCTGACGTGGACATC-3′; reverse, 5′-ACTCGTCATACTCCTGCTTG-3′), IFN-β (forward, 5′-AGCTGAAGCAGTTCCAGAAG-3′; reverse, 5′-AGTCTCATTCCAGCCAGTGC-3′), STAT-1 (forward, 5′-CGGTTGAACCCTACACGAAG-3′; reverse, 5′-ACTTTCCAAAGGCATGGTC-3′), and 2,5-oligoadenylate synthetase (OAS-1) (forward, 5′-GCAGAAAGAGGGCGAGTTC-3′; reverse, 5′-TACTGAGGTGGCAGCTTCC-3′). Relative mRNA expression was calculated using the ΔC_T method.

Flow Cytometry. Cell surface antigen staining was performed by 20-min incubation on ice with pertinent antibodies. The following monoclonal antibodies were used: rabbit polyclonal anti–nicotinic AchR α7 (Abcam); FITC-conjugated anti-rabbit IgG (Abcam); anti-human CD45, CD14, CD3, CD4, CD8, CD11c, and CD19; and isotype control mAbs (all purchased from BD Pharmingen). Intracellular staining was performed with Cytofix/Cytoperm

Fixation/Permeabilization Kit (BD Bioscience) according to the manufacturer's protocol. The following antibodies were also used: FITC-conjugated rabbit anti-active caspase-3 (BD Pharmingen) and PE-conjugated Milli-Mark anti-HMGB1 (Millipore). Data were acquired by BD FACS Canto II and analyzed on BD FACS Diva software.

Silencing LPS-Induced TNFα Production in Humanized Mice. BLT mice that were generated as described above were divided into three treatment groups: siLuc/RVG-9dR; siTNF-α/RVG-9dR; and siTNF-α/RVMAT-9dR. Each group had equal human $CD45^+$, $CD14^+$ cell percentages. Mice were injected i.v. with TNF siRNA/RVG-9dR complex 18 and 6 h before LPS injection i.p. One hour after LPS injection, total RNA isolated from PBMCs was tested for TNF-α mRNA levels by qRT-PCR, and serum was tested for human TNF-α protein levels by ELISA (Biolegend).

Silencing HMGB1. HMGB1 siRNA /RVG-9R complex was injected 15 h before CLP and was repeated 2 and 24 h after surgery. Twenty hours after CLP, blood was collected for testing the HMGB1 level by ELISA (Chondrex) and murine and human inflammatory cytokine levels by cytometric bead array (CBA) in accordance with the manufacturer's protocol. PBMCs were stained with CD45-PE, CD3–allophycocyanin (APC), and active caspase-3-FITC. The HMGB1 level in human $CD45^+$, $CD14^+$ double-positive splenocytes were tested at 20 h after CLP surgery by flow cytometry. Mice were monitored until 8 d for survival.

Post–CLP Treatment with HMGB1 siRNA/RVG-9R. CLP was induced in BLT mice, and siRNA treatment was started 10 h after CLP, when the mice showed clinical signs of sepsis, such as ruffling of hair, lethargy, and hunching. Serum HMGB1 and cytokines were tested as described above. Mice were monitored for 12 d for survival.

Detection of the Physical Presence of siRNA Within the Cells. Human $CD14^+$ and $CD3^+$ cells from the septic humanized mice spleen were isolated using magnetic beads (Stemcell Technologies), and total RNA, including small RNAs, was extracted with the miRNeasy Minikit (Qiagen). The RNAs were poly(A)-tailed by NCode microRNA (miRNA) first-strand cDNA synthesis and qRT-PCR kit (Invitrogen) and subjected to RT-PCR as described above. The primers used were: for siHMGB1 (5′- AGTTTGTA-AATGTAAGTGG-3′) and for U6 (5′- ATGACACGCAAATT-CGTGAAGC-3′).

Bacteria Count. Ten-fold dilutions of mouse blood collected at different times after induction of sepsis were plated (20 μL per plate) onto an antibiotic-free LB plate, cultured overnight, and counted for bacterial colonies.

Depletion of Neutrophils. Neutrophils were depleted by a single i.p. injection of 1.0 mg of anti-Ly6G mAb, clone 1A8 (Bio X Cell) 3 d before CLP surgery. Neutrophil depletion was confirmed by staining the cells for antigranulocyte receptor-1 (Gr-1) and Ly6G antibodies.

1. Melkus MW, et al. (2006) Humanized mice mount specific adaptive and innate immune responses to EBV and TSST-1. *Nat Med* 12(11):1316–1322.

2. Rittirsch D, Huber-Lang MS, Flierl MA, Ward PA (2009) Immunodesign of experimental sepsis by cecal ligation and puncture. *Nat Protoc* 4(1):31–36.

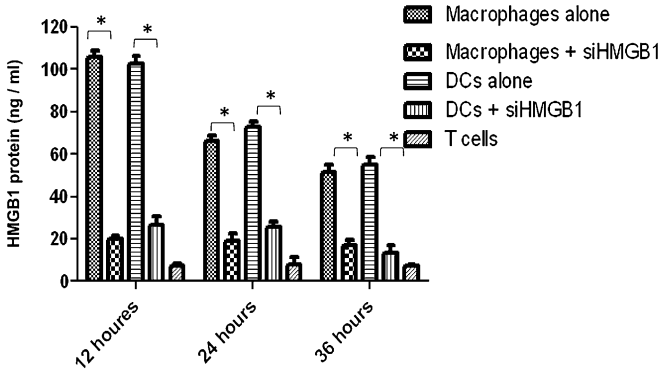

Fig. S1. HMGB1 is secreted by human macrophages and DCs, and siRNA silences HMGB1. In vitro–cultured human macrophages and DCs were either untreated or treated with HMGB1 siRNA 24-h before stimulation with LPS, and at the indicated times after stimulation, the culture supernatants were tested for secreted HMGB1 by ELISA. T cells were stimulated with PHA (n = 3 wells per group).

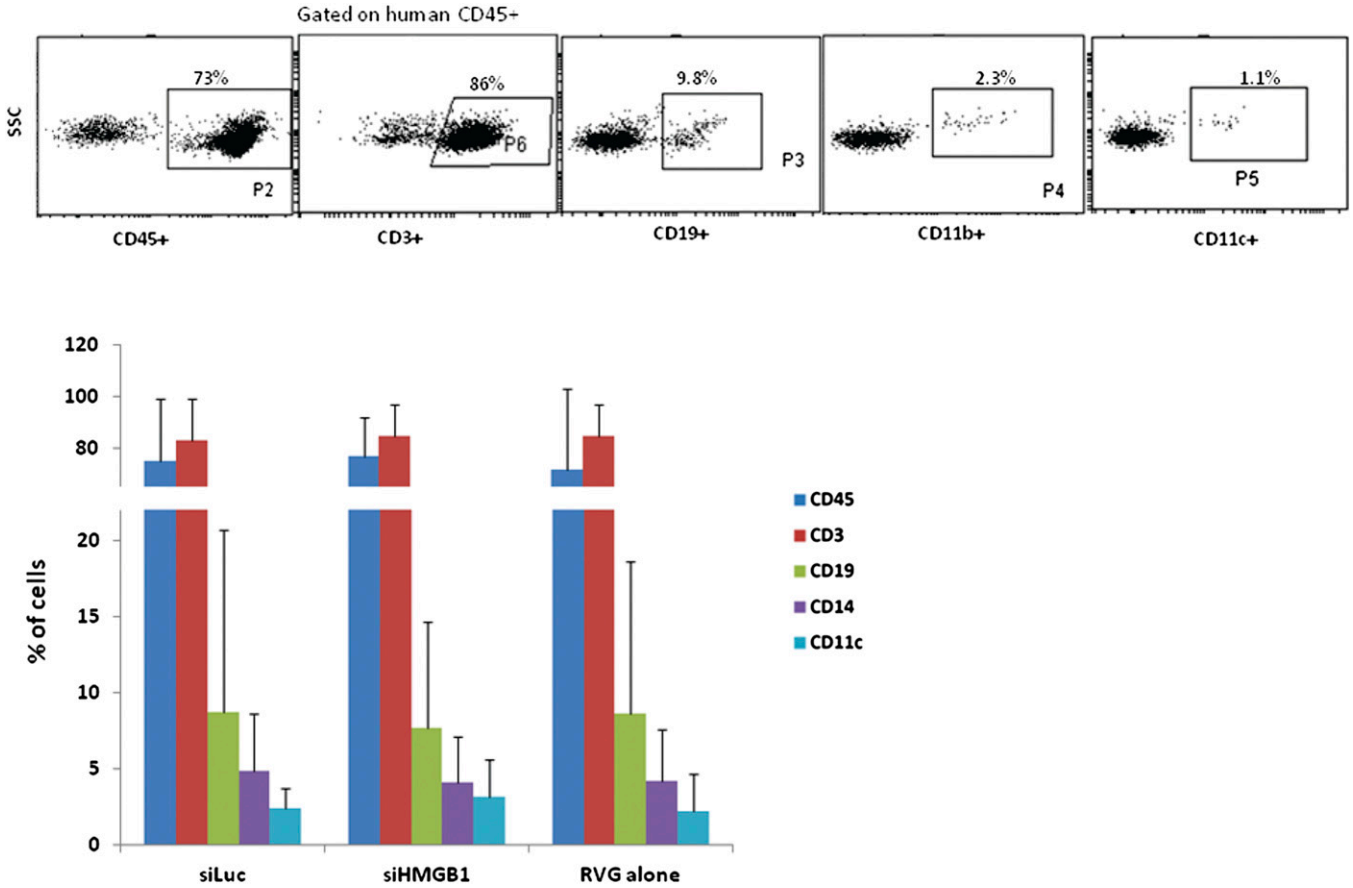

Fig. S2. Comparable hematopoietic cell reconstitution in humanized mice. Humanized mice were tested for the indicated immune-cell subset reconstitution by flow cytometry. Representative flow-cytometric results (*Upper*) and cumulative data from seven mice for each group used for treatment with control siRNA, RVG peptide alone, and HMGB1 siRNA treatment (*Lower*) are shown. Human $CD45^+$ cells as a percentage of total cells, as well as CD3, CD19, CD14, and $CD11c^+$ cells, as a percentage of $CD45^+$ cells (gated on $CD45^+$ cells) are shown.

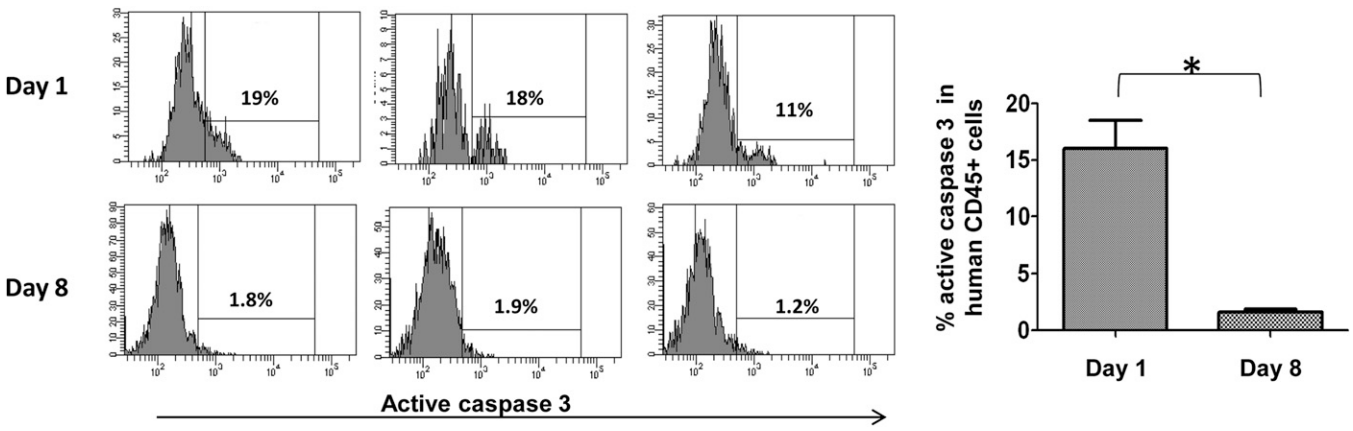

Fig. S3. Active caspase-3^+ T cells decrease over time in HMGB1 siRNA–treated mice. PBMCs obtained on days 1 and 8 from HMGB1 siRNA/RVG-9R–treated mice in Fig. 3 were tested for the active caspase-3 positivity in human $CD45^+$ cells by flow cytometry. Representative flow-cytometric data (*Left*) and cumulative data from three mice (*Right*) are shown.

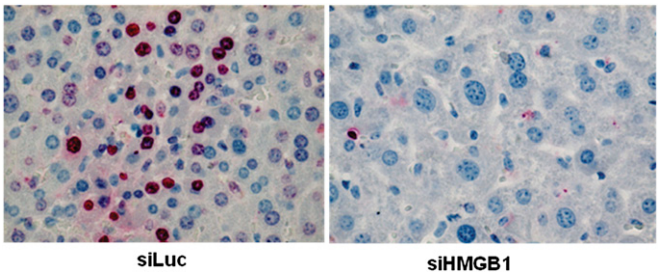

Fig. S4. CLP-induced apoptosis is reduced after treatment with RVG-9R/HMGB1 siRNA. Representative TUNEL-stained liver sections obtained from control and HMGB1 siRNA–treated mice (in Fig. 3) are shown.

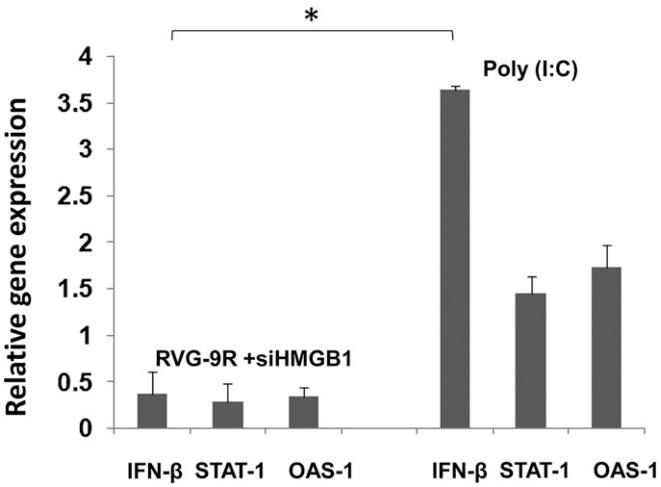

Fig. S5. RVG-9R/HMGB1 siRNA does not activate IFN response. Human PBMCs were treated with RVG-9R/HMGB1 siRNA or poly(I:C) as a positive control, and 4 h later, the cellular RNA was tested for up-regulation of IFN-β, STAT-1, and OAS-1 mRNAs by qRT-PCR (n = 3).

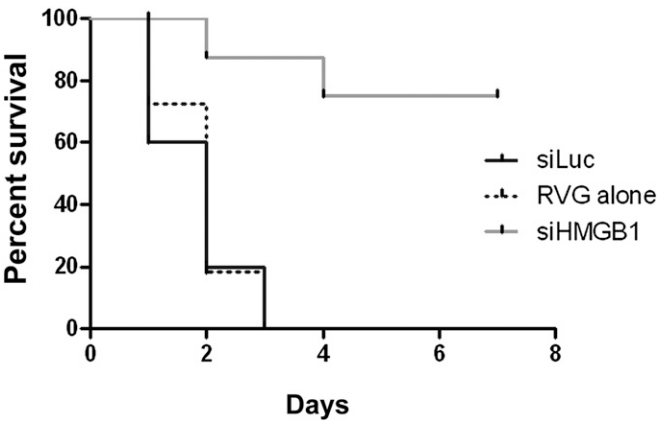

Fig. S6. RVG peptide alone does not protect from CLP-induced mortality. Humanized mice were treated with RVG peptide alone, control Luci siRNA, or HMGB1 siRNA complexed with RVG-9R before and after CLP as described in *SI Materials and Methods* (*Silencing HMGB1*) and followed for survival over time.

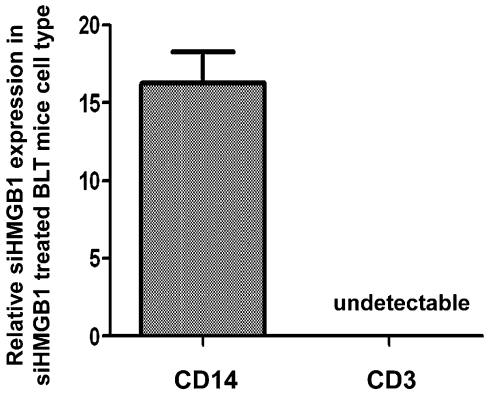

Fig. S7. Physical presence of HMGB1 siRNA within $CD14^+$ cells in the siRNA-treated humanized mice. Humanized mice were treated with RVG-9R/HMGB1 siRNA, and 24 h later, $CD14^+$ cells and $CD3^+$ cells isolated from the spleen were tested for the presence of HMGB1 siRNA by qRT-PCR (n = 3 mice).