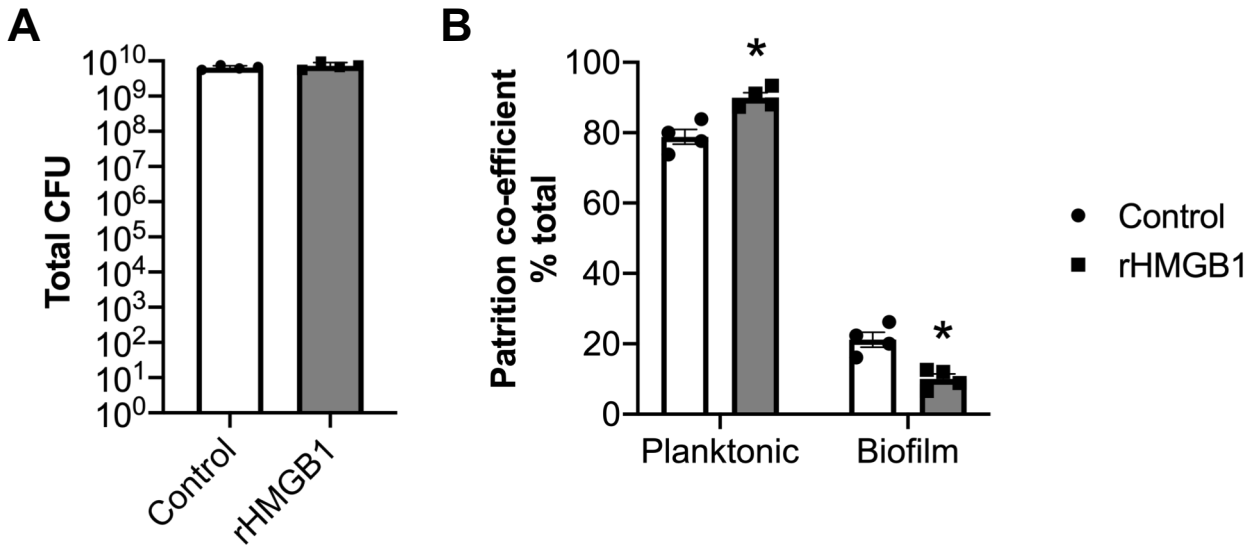

Figure S1. HMGB1 disrupted biofilms and released biofilm-resident bacteria into the planktonic state. 24 h UPEC biofilms were incubated with rHMGB1 (200 nM) for 16 h, then bacteria in the planktonic (conditioned media) versus biofilm state (adherent bacteria) were enumerated. (A) Total CFU (planktonic + biofilm) was plotted. (B) Relative percent of total bacteria in the planktonic versus the biofilm state was plotted. Bars represent the SEM. *P<0.05 via paired *t* test. Note that rHMGB1 had no bactericidal effect, however it induced the partitioning of bacteria from biofilm residence into the planktonic state.

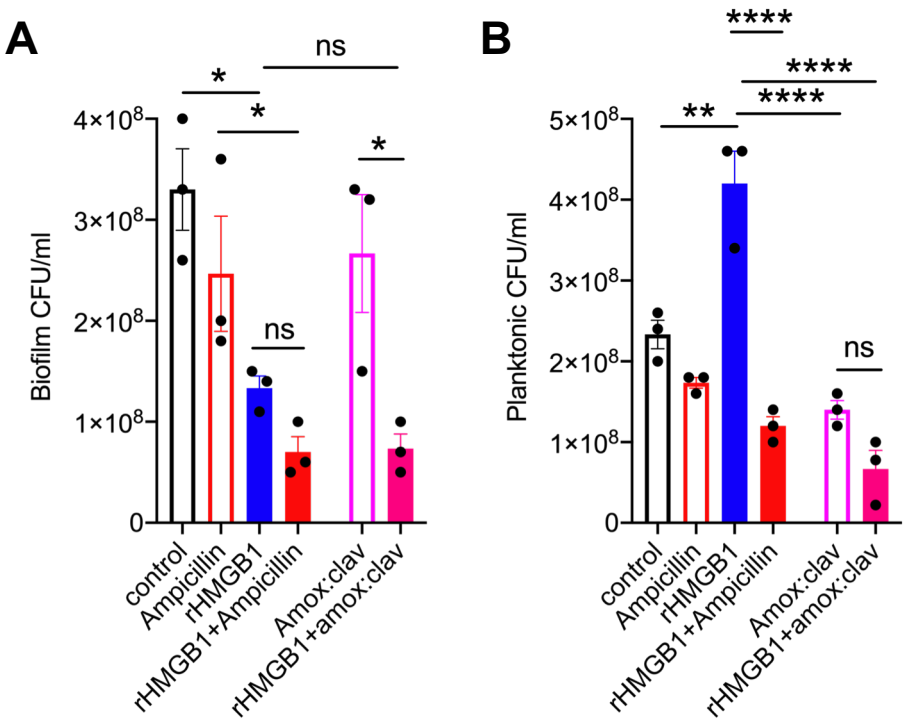

Figure S2. rHMGB1 synergized with antibiotics *in vitro* in the eradication of planktonic and biofilm-resident bacteria. 24 h NTHI biofilms were incubated with rHMGB1 (200 nM) alone or in combination with ampicillin (32 μg/ml) or amoxicillin-clavulanate (1 μg/ml) for 16 h, then bacteria in the planktonic (conditioned media) versus biofilm state (adherent bacteria) were enumerated. (A) Biofilm CFU and (B) planktonic CFU were plotted. Bars represent the SEM. *P<0.05, **P<0.01, ****P<0.0001 via One-way ANOVA. Note that rHMGB1 had no bactericidal effect, however when delivered in combination with antibiotics, rHMGB1 promoted killing of both planktonic and biofilm-resident bacteria.

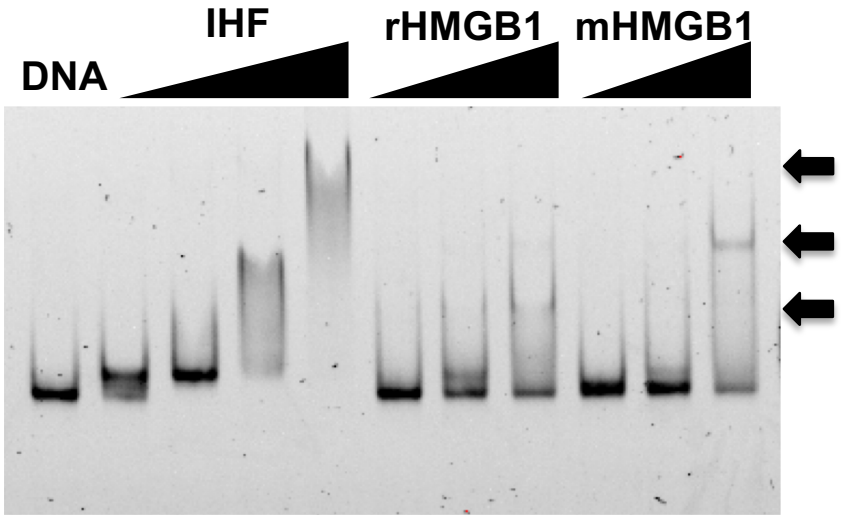

Figure S3. Engineered single amino acid variant mHMGB1 retained its ability to bind to HJ DNA. 5' end labeled 6-carboxyfluorescein HJ DNA (20 nM) was incubated with increasing concentrations (50 – 500 nM) of IHF, rHMGB1 or mHMGB1, then resolved by non-denaturing PAGE. Arrows indicate the HJ DNA-protein complex. Note that mHMGB1 maintained its ability to bind HJ DNA.

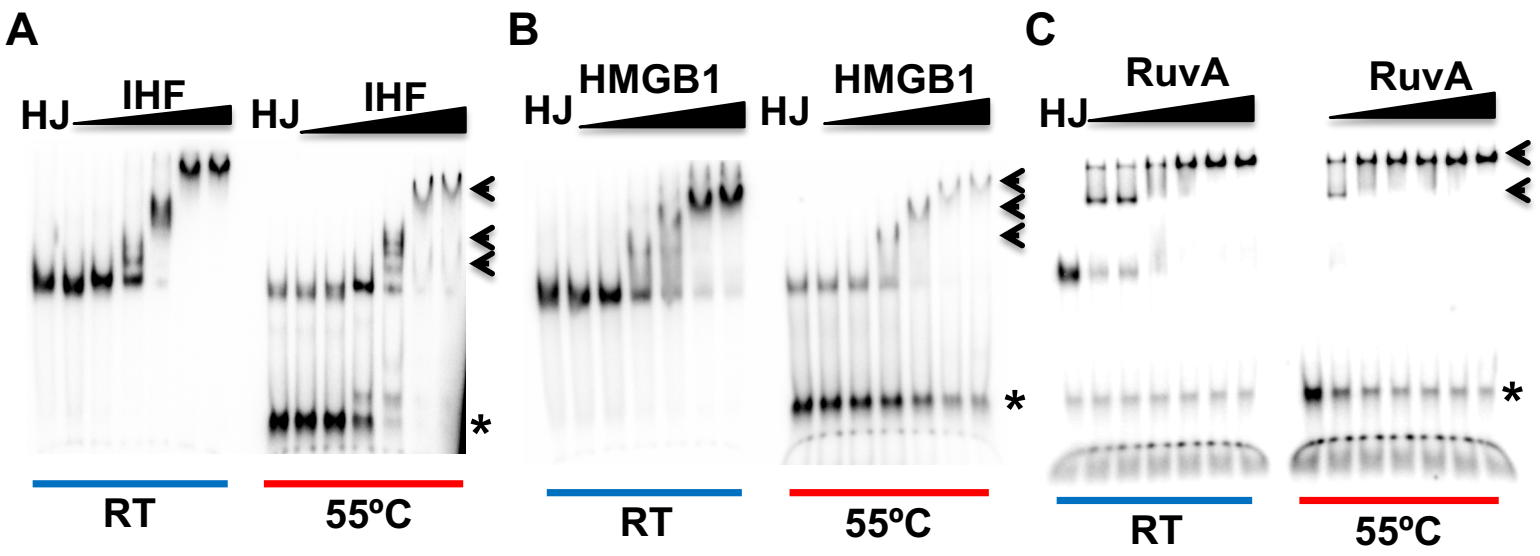

Figure S4. HMGB1 bound to HJ DNA but was unable to stabilize the HJ DNA. 5' end-labeled ^{32}P HJ DNA was incubated with increasing concentrations (25 – 500 nM) of (A) IHF, (B) rHMGB1 or (C) RuvA either at room temperature (RT) or at 55ºC for 10 min, then resolved on a 6% non-denaturing PAGE. Asterisk indicates melted oligos and arrows indicate DNA-protein complex. Whereas the DNA-HMGB1 complex was stable at RT, it was unstable at 55ºC, which resulted in the increase in abundance of the melted constituent oligos. This outcome contrasts with that observed with both IHF and the prototypic HJ DNA binding protein, RuvA.

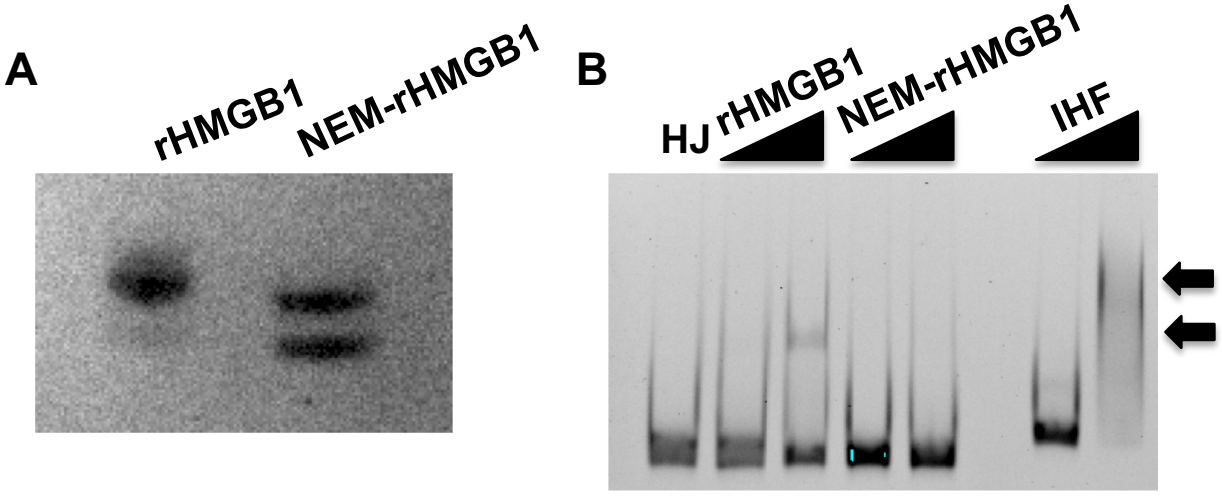

Figure S5. NEM-rHMGB1 does not bind to HJ DNA. (A) NEM-modified rHMGB1 was confirmed by triton acetic acid urea gel (TAU gel). (B) 5' end-labeled 6-carboxyfluorescein HJ DNA (20 nM) was incubated with increasing concentrations (250 - 500 nM) of IHF, rHMGB1 or NEM-rHMGB1, then resolved by non-denaturing PAGE. Arrows indicate the HJ DNA-protein complex. Note that NEM-rHMGB1 lost its ability to bind HJ DNA.

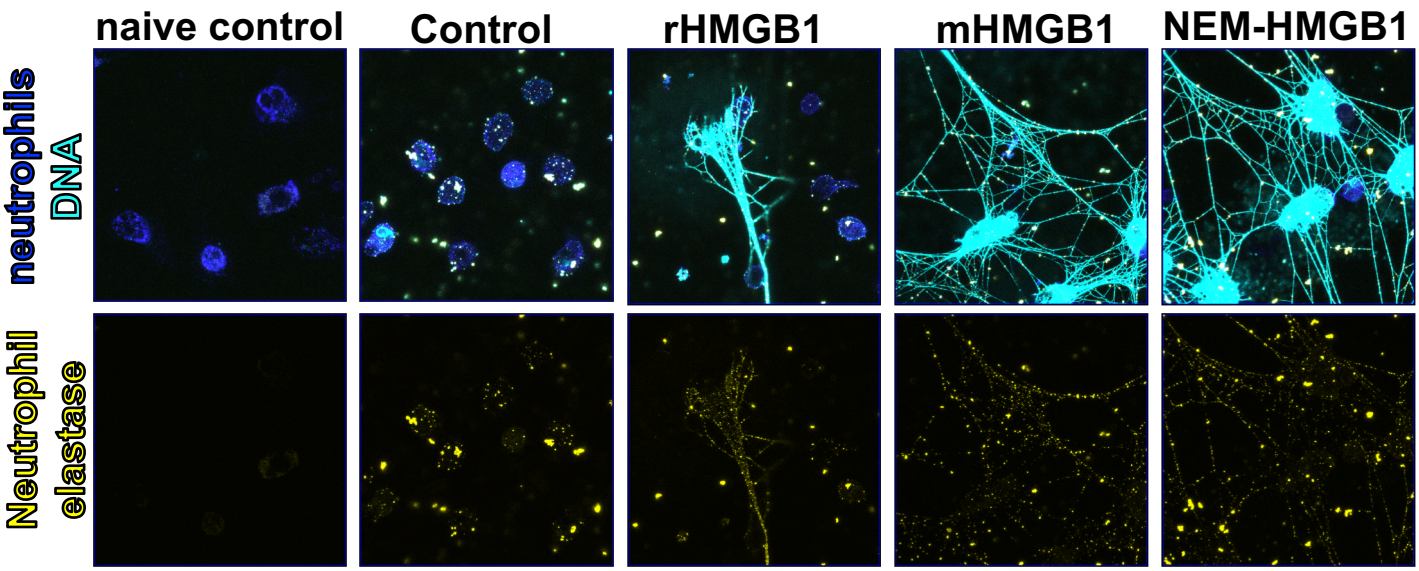

Figure S6. HMGB1 isoforms induced varied degrees of neutrophil-mediated NETs formation, *in vitro*. Neutrophils were incubated with the indicated protein (200 nM) for 3.5 h. Neutrophils were fixed, then incubated with α-dsDNA monoclonal antibody and α-neutrophil elastase antibody, then incubated with goat α-mouse IgG conjugated to AlexaFluor 488 (teal) and goat α-rabbit IgG conjugated to AlexaFluor 594 (yellow). Neutrophils were stained with wheat germ agglutinin (WGA) conjugated to AlexaFluor 350 (blue). NETs were visualized by CLSM. Note the intertwined NETs specifically formed upon incubation of neutrophils with rHMGB1, mHMGB1 and NEM-rHMGB1 albeit to variable degrees. Scale bars represent 10 μm.

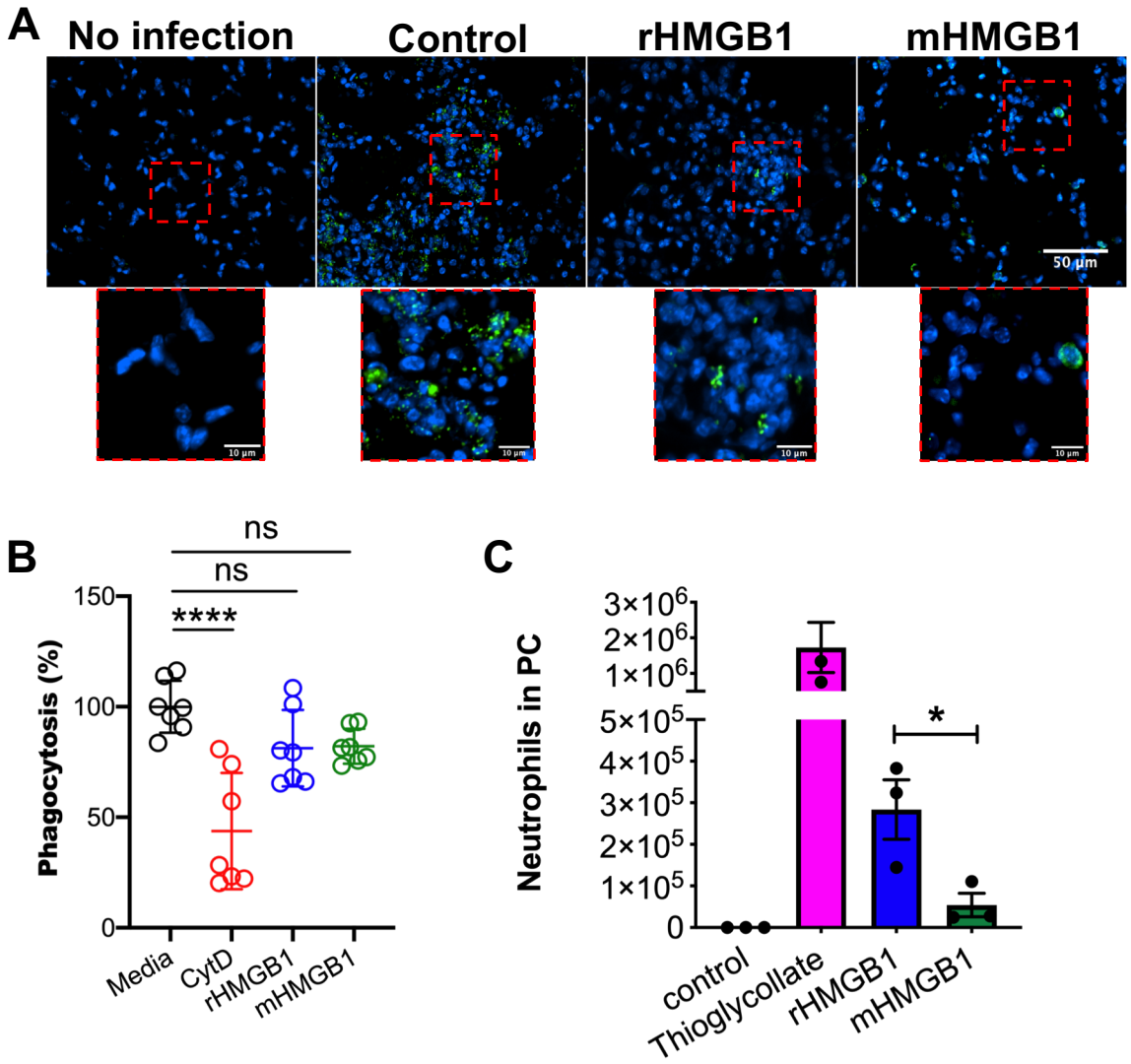

Figure S7. mHMGB1 cleared *B. cenocepacia* from the murine lungs despite reduced phagocytosis and exhibited an attenuated neutrophil recruitment to the peritoneal cavity. (A) C57BL/6 mice were challenged i.t. with 10^7 CFU *B. cenocepacia* and treated 24 hours later with 0.2 nmol of rHMGB1 or mHMGB1. 48 h after treatment, mice were sacrificed, and sections of lung were labeled with *E. coli* α-EF-Tu monoclonal antibody to label *B. cenocepacia* (green) and with DAPI to detect neutrophils (blue). (B) Macrophages were treated with 5 μg/ml rHMGB1, 5 μg/ml mHMGB1 or 10 μM cytochalasin D for 2 h, then pHrodo red *E. coli* bioparticles were added and incubated for 2 h. Any excess bioparticles were removed by washing the cells with HBSS, the phagocyted bioparticles were measured by plate reader (560/585nm). (C) C57BL/6 mice were injected i.p. with the indicated HMGB1 isoform, then 24 h later, influx of neutrophils into the peritoneal cavity was determined by flow cytometry with α-CD45, CD11b, and Ly-6G antibodies. n=3. Bars represent the SD. *P<0.05, ****P<0.0001 as assessed by One-way ANOVA. Whereas treatment with rHMGB1 induced significant migration of neutrophils into the peritoneal cavity, treatment with mHMGB1 significantly attenuated this pro-inflammatory response albeit with a modest reduction in phagocytosis.

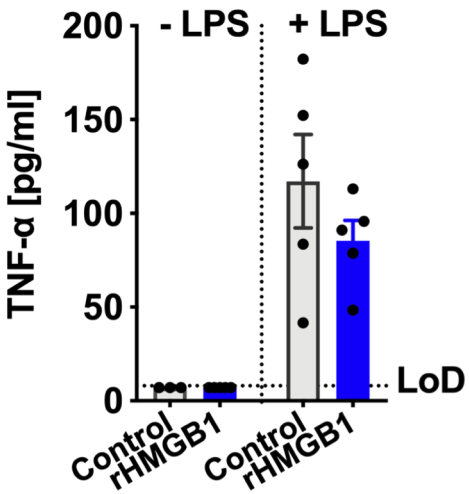

Figure S8. rHMGB1 used to treat biofilms *in vivo* did not induce dysregulated host response to infection associated with septic shock in mice. Mice were injected i.p. with either 0.2 nmol endotoxin-free HMGB1, 5 mg/kg LPS, or both then monitored for 24 h for signs of septic shock. Serum TNF-α was measured by ELISA after 24 h. Bars represent SD. LoD: limit of detection. Note that rHMGB1 at the same concentration that was used to treat *in vivo* biofilms did not induce septic shock as indicated by an increase in TNF-α.

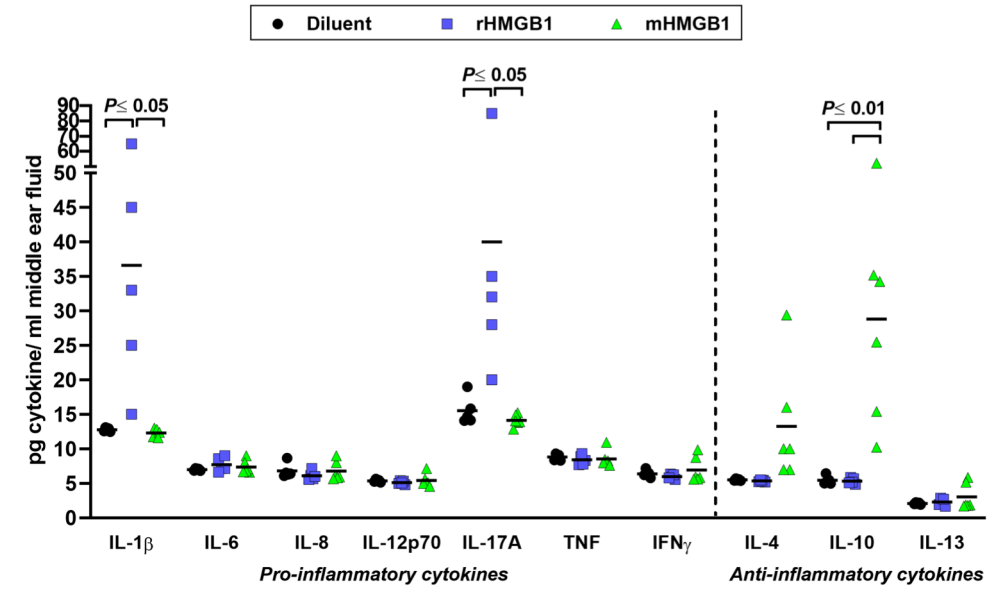

Figure S9**.** Pro-inflammatory cytokines IL-1β and IL-17A were significantly more abundant in middle ear fluids recovered from chinchillas treated with rHMGB1, whereas anti-inflammatory cytokines predominated in the mHMGB1-treated cohort. Six days after NTHI challenge (one day after completion of treatment), middle ear fluids were recovered and screened for relative quantity of a panel of pro-inflammatory and anti-inflammatory cytokines by cytometric bead array. Each data point represents an individual middle ear fluid and the mean for each cohort is indicated by the horizontal bar. Note significantly elevated concentration of pro-inflammatory cytokines IL-1β and IL-17A in rHMGB1-treated chinchillas ($p<0.05$), whereas a significantly elevated concentration of the anti-inflammatory cytokine IL-10 was observed in the cohort treated with mHMGB1 ($P<0.01$).

Protein purification

Tagless recombinant HMGB1 was generated with the IMPACT kit (New England Biolabs) as previously described (1). Human HMGB1 was PCR amplified from a plasmid that contained human HMGB1 tagged with haemagglutinin with the oligonucleotides 5'- ggtggttgctcttccaacatgggcaaaggag - 3' and 5' – ggtggtccatggtcattattcatcatcatc - 3'. The PCR products were cloned into the pTXB1 vector as described (1) to generate tagless human rHMGB1. The constructs were transformed into the *E. coli* expression strain ER2566 (New England Biolabs) and selected on lysogeny broth (LB) agar that contained 100 μg/ml ampicillin. rHMGB1 with a single amino acid mutation C45S (mHMGB1) was generated by following the Agilent QuickChange II Site Directed Mutagenesis Method. HMGB1 was PCR amplified from pSG899 with PAGE purified oligos 5'-ggtcttccacctctctgaactcttcttagaaaactctgag-3' and 5'-ctcagagttttctaagaagagttcagagaggtggaagacc-3' (Integrated DNA Technologies) and Phusion DNA polymerase (New England BioLabs) according to the manufacturer's instructions for a total of 16 cycles. The PCR product was then digested with DpnI, followed by transformation into NEB® Stable Competent *E. coli* following the manufacture's protocol and selection on LB agar that contained 100 μg/ml ampicillin. Random transformants were selected, plasmids were purified and the C45S mutation was confirmed via sequencing. Constructs that contained the C45S mutation were further transformed into the expression strain *E. coli* ER2566. rHMGB1 and mHMGB1 were overexpressed and purified on a chitin resin column as described (1). The eluted proteins were dialyzed in heparin binding buffer [10mM Na-Phosphate buffer (pH 7.0), 200 mM NaCl] overnight at 4°C. The dialyzed protein was loaded onto an AKTA PURE FPLC using a 1 ml HI-Trap Heparin-Sepharose column (GE Healthcare) equilibrated in binding buffer. The column was washed with 20 column volumes of binding buffer, and the bound protein was eluted with 30 column volumes of elution buffer [10 mM phosphate buffer (pH 7), 2M NaCl] with a linear gradient and were fractionated by an AKTA F9-C fraction collector. Each protein was further purified (if <95% purity by SDS-PAGE analysis) by FPLC using a 1ml Hi-Trap DEAE-Sepharose resin column (GE Healthcare) equilibrated in 40 mM Tris pH 8.5, 2 mM EDTA. The bound protein was eluted with 30 column volumes of a linear gradient of elution buffer that contained 40

mM Tris pH 8.5, 2 mM EDTA and 1M NaCl. The fractions were analyzed by SDS-PAGE, pooled, and dialyzed in storage buffer [50 mM Tris (pH 7.4), 600 mM KCl, 1mM EDTA]. The proteins were concentrated in a centrifugal filter (3000 MWCO), quantified using Pierce BCA Protein assay kit (Thermo Scientific) with bovine serum albumin as the standard, as recommended by the manufacturer. The protein was purified to approximately 95% homogeneity. Lastly, proteins were supplemented with 10% glycerol and stored at -80°C.

Visualization of HMGB1 and DNABII proteins within biofilms formed *in vivo*. Bullae from chinchillas which had been transbullarly challenged with NTHI were used. Upon sacrifice, bullae were removed, aseptically opened, any fluid collected, washed with sterile saline and filled with OCT compound then snap frozen over liquid nitrogen and stored at -80 C until use. Prior to sectioning, OCT filled bullae were placed on bed of dry ice while the external bone of the middle ear was carefully chipped away. Bulla was then split in the plane through the tympanic membrane and re-embedded in OCT for orientation. Ten micron serial sections were cut and placed on slides (Mercedes Medical, Lakewood Ranch, FL). Slides were either stained with H&E for morphology or by immunofluorescence using standard laboratory techniques. The following commercial antibodies were used: murine monoclonal antibody against human neutraphil elastase (NE-elastase) monoclonal antibody (NOVUS), a-HU and a-HMGB1, AlexaFluor 488 Goat anti- guinea Pig, AlexaFluor 546 Goat anti-Mouse and AlexaFluor 594 Goat anti-Rabbit secondary antibodies (Invitrogen). Slides were allowed to air dry at room temp, fixed in ice cold acetone and blocked with ITX-image enhancer (Invitrogen, Carlsbad, CA) and Super Block (Scytek, West Logan, UT) per manufacturer's instructions. Sections were incubated with a cocktail of primary antibodies [(mouse anti-Elastase and rabbit anti-NTHI OMP P5) or (mouse-anti-HMGB1 and guinea pig anti-HU)] for 1 hour at room temp, washed, incubated with fluorescently tagged secondary antibodies (Invitrogen, Carlsbad, CA) for 30 minutes, and washed prior to coverslipping using Prolong Gold plus DAPI (Invitrogen, Carlsbad, CA). Low magnification images were capture using a Zeiss Axioskop microscope and Zeiss Zen software. High

magnification 3D images were captured on a Zeiss LSM 8000 confocal microscope with airyscan.

Oxidation and NEM modification of rHMGB1

rHMGB1 was purified as described above in 'Protein Purification'. Purified rHMGB1 was oxidized with hydrogen peroxide as described (2). rHMGB1 was purified as described previously. Pooled fractions of rHMGB1 were incubated with 10 mM DTT (Dithiothreitol) for 15 minutes at 4°C and immediately followed by the addition of NEM (N-Ethylmaleimide) at a final concentration of 50 mM for 5 minutes as described (3). NEM-HMGB1 was dialyzed overnight in 50 mM Tris-HCl pH 7.4 with 600 mM KCl. The protein was quantified by BCA assay (Pierce).

Phagocytosis assay

Human monocytes were purified from peripheral blood by using α-CD14 antibodies and magnetic beads (Miltenyi Biotec). In order to differentiate the monocytes to macrophages (MΦ), the cells were cultured for 5 days in plates coated with collagen (Corning) and RPMI media supplemented with 10% FBS. MΦ were detached by using collagenase IV (Worthington) and seeded in 96 well plates at density of 80,000 cells per well and cultured for 48 h in RPMI supplemented with 10% FBS. MΦ were treated with 5μg/ml rHMGB1, 5μg/ml mHMGB1 or 10μM cytochalasin D for 2 h, then pHrodo red *E. coli* bioparticles (Thermofisher) were added according to the manufacturer's instructions and incubated for 2 h. Any excess bioparticles were removed by washing the cells with HBSS, the phagocyted bioparticles were measured by plate reader (560/585nm) and by fluorescent microscopy (Nikon Eclipse Ti).

Assay of neutrophil influx into the peritoneal cavity of mice

C57BL/6 mice were injected *i.p.* with one of the following: 2 ml of 4% thioglycollate (Fluka Analytical), or 0.2 nmol of rHMGB1 or mHMGB1. Four hours later, peritoneal cells were harvested in PBS plus 1 mM EDTA and stained with α-CD45 brilliant violet 421 (Biolegend), α-CD11b Alexa Fluor 700 (Biolegend) and α-Ly6G PerCP-Cy5.5 (Biolegend) antibodies and LIVE/DEAD blue discriminator (Invitrogen, Eugene OR). Cells were

acquired with a LSR II flow cytometer (BD, Franklin Lakes, NJ). The number of neutrophils were calculated based on the number of neutrophils in peritoneal cavity (Live, $CD45^{+}$, $CD11b^{high}$, $Ly6G^{high}$) and the total counts performed by hemocytometer.

Mouse model of endotoxemia C57BL/6 mice were injected *i.p.* with 5mg/kg of *E. coli* lipopolysaccharide (Sigma), or 0.2 nmol rHMGB1 alone or in combination with LPS. Animals were euthanized 24 h later, serum was collected, and TNF-α was quantified by ELISA (ThermoFisher).

Visualization of eDNA lattice structure within biofilms formed by *K. pneumoniae*

K. pneumoniae biofilms were established for 24 hours as described above in 'Disruption of bacterial biofilms by various isoforms of HMGB1' and then incubated with one of the following: LB broth (control), rHMGB1 (200 nM), or mHMGB1 (200 nM) for 16 hours at 37°C, 5% CO_2. eDNA lattice structure was visualized by immunofluorescence as described (4).

Surface plasmon resonance

To determine the affinity of HMGB1 and the DNABII proteins IHF_{NTHI} and HU_{NTHI}, surface plasmon resonance using a Biacore 3000 instrument (GE Healthcare Life Sciences) was performed. All experiments were conducted at 25°C and 10 mM HEPES (pH 7·4)- 150 mM NaCl- 3 mM EDTA supplemented with 5mg/ml BSA served as the running buffer. Via amine couple chemistry and at a flow rate of 5 µl/ min, mHMGB1 were immobilized to a flow cell of a CM5 sensor chip (GE Healthcare) to 250 resonance units. Next, native IHF_{NTHI} or HU_{NTHI} was suspended in running buffer plus NSB reducer (GE Healthcare) and serial two-fold dilutions from 50 nM to 3.1 nM, including a buffer-only sample, were injected across the antibody-bound and reference surface at a flow rate of 30 µl/ min, 3 min injection time, 3 min dissociation time using KINJECT. All data was reference cell and buffer control subtracted. BiaEvaluation software (GE Healthcare) was used to align sensorgram curves and determine K_D values.

Quantitation of cytokines in middle ear fluids

To quantitate cytokines in middle ear fluids (MEF), a BD™ Cytometric Bead Array was performed with fluids collected at the time of animal sacrifice. With BD™ human-specific Flex Sets, each MEF was individually examined for relative quantity of IL-1β, IL-4, IL-6, IL-8, IL-10, IL12-p70, IL-13, IL-17A, TNF or IFNγ according to manufacturer's instructions. MEFs from each animal were assayed individually. Data were captured on a BD Accuri™ C6 cytometer (BD Biosciences) and analyzed with FlowJo software (FlowJo, LLC). The concentration of cytokines in each MEF was determined using a standard curve and calculated using GraphPad Prism software.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed as previously described (4).

Visualization of NETs structure upon incubation of neutrophils with various isoforms of HMGB1

Human neutrophils were isolated from blood using the EasySep™ isolation kit (Stemcell Technologies Inc.). Neutrophils ($1x10^5$) were allowed to attach to chambered coverglass followed by incubation with 200nM rHMGB1, mHMGB1 or NEM-rHMGB1 at 37°C for 3.5 hours. NETs were fixed in formalin, blocked with 10% normal goat serum (Life Technologies), and labeled with α-dsDNA monoclonal antibody [1 µg; Abcam (catalog no. ab27156)], and α–neutrophil elastase polyclonal antibody [1 µg; Abcam (catalog no. ab68672)] or naive rabbit and mouse isotype controls [1 µg; Abcam (catalog no. ab37415 and ab18415)] in 200 µl PBS for 16h hours at 4°C. NETs were washed with PBS and incubated for 1h with 1:200 dilution goat α-rabbit IgG conjugated to AlexaFluor® 594 (Invitrogen A11032), goat α-mouse IgG conjugated to AlexaFluor® 488 (Invitrogen A11001), and 1:500 dilution wheat germ agglutinin conjugated to AlexaFluor® 350 (PMN membrane stain). NETs were imaged with a Zeiss LSM 800 confocal microscope (Carl Zeiss Inc.) and rendered with Zeiss Zen software.

1. Devaraj A, Buzzo J, Rocco CJ, Bakaletz LO, and Goodman SD. The DNABII family of proteins is comprised of the only nucleoid associated proteins required for nontypeable Haemophilus influenzae biofilm structure. *MicrobiologyOpen.* 2017.

2. Liu A, Fang H, Dirsch O, Jin H, and Dahmen U. Oxidation of HMGB1 causes attenuation of its pro-inflammatory activity and occurs during liver ischemia and reperfusion. *PLoS One.* 2012;7(4):e35379.
3. Kwak MS, Kim HS, Lkhamsuren K, Kim YH, Han MG, Shin JM, et al. Peroxiredoxin-mediated disulfide bond formation is required for nucleocytoplasmic translocation and secretion of HMGB1 in response to inflammatory stimuli. *Redox Biol.* 2019;24:101203.
4. Devaraj A, Buzzo JR, Mashburn-Warren L, Gloag ES, Novotny LA, Stoodley P, et al. The extracellular DNA lattice of bacterial biofilms is structurally related to Holliday junction recombination intermediates. *Proceedings of the National Academy of Sciences of the United States of America.* 2019.