SUPPLEMENTAL FIGURES AND LEGENDS

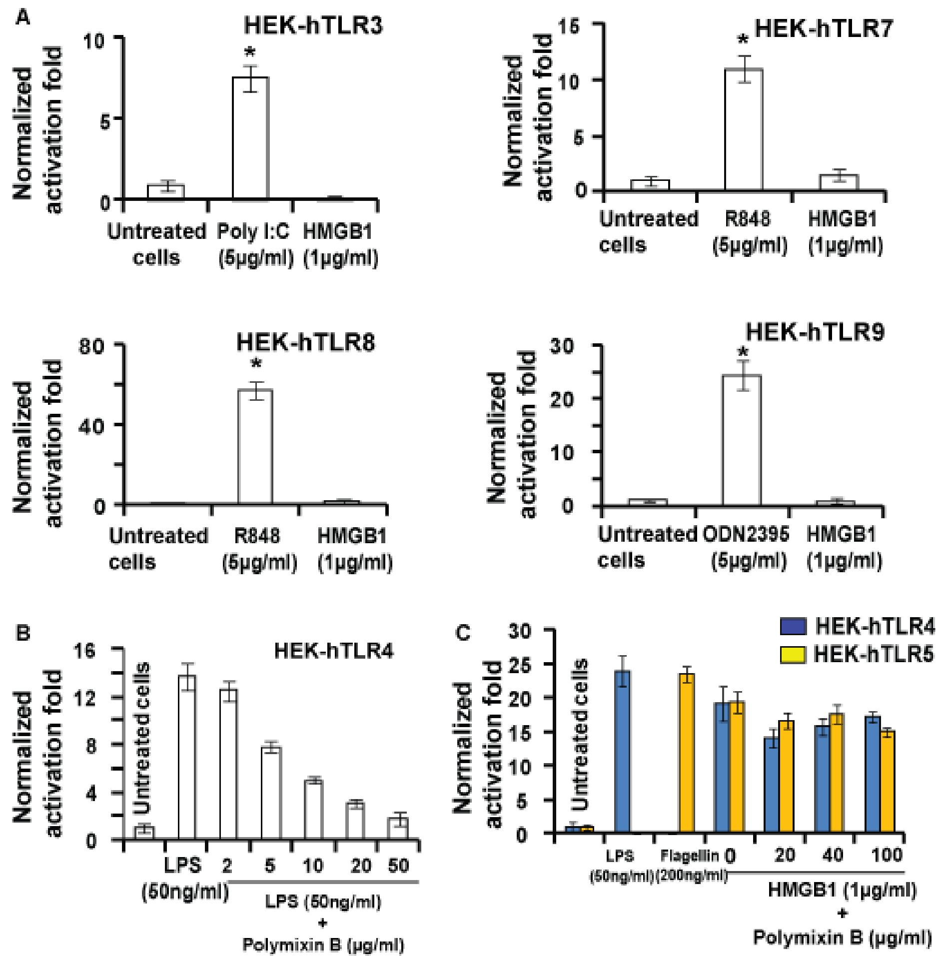

Figure S1. Determining the role of endotoxin free HMGB1 towards various TLRs, Related to Figure 1. (A) HEK-TLR3, TLR7, TLR8 and TLR9 cells were incubated with various TLR ligands (ligands and doses as indicated) along with HMGB1 for 24 h, and activation was evaluated by SEAP secretion in the culture supernatants by the QUANTI-Blue SEAP reporter assay. (B) HEK-TLR4 cells were incubated with 50 ng/ml LPS, followed by addition of PMB (2 to 50 μg/ml) for 24 h. (C) HEK-TLR4 and TLR5 cells were incubated with 50 ng/ml LPS, 200 ng/ml flagellin and 1 μg/ml HMGB1, followed by addition of PMB (20 to 100 μg/ml) for 24 h. All data are represented as mean ± SD, n=3. * *P*< 0.05 (unpaired student's *t*-test) relative to untreated cells.

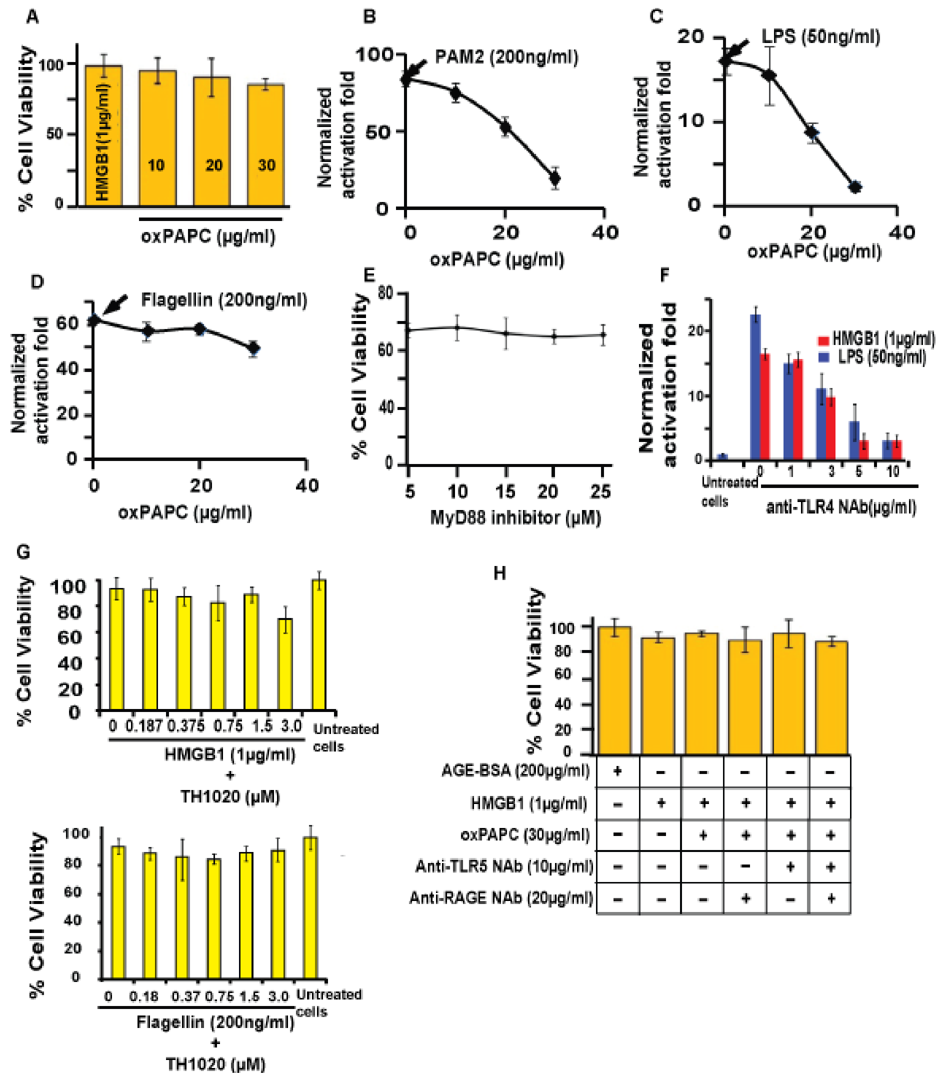

Figure S2. Characterization of inhibitory and cytotoxic profiles of oxPAPC, MyD88 inhibitor, anti-TLR5/RAGE neutralizing antibodies and TH1020 TLR5 inhibitor in mammalian cells, Related to Figure 1, 2 and 4. (A) Measurement of cellular toxicity in RAW 264.7 cells upon oxPAPC treatment by WST-cell viability assay. Dose-dependent effect of oxPAPC on (B) PAM2 (200 ng/ml) and (C) LPS (50 ng/ml) mediated TLR2 and TLR4 signaling in HEK-TLR2 and 4 cells. (D)No effect of oxPAPC on TLR5 inhibition is demonstrated in HEK-TLR5 cells activated by 200 ng/ml flagellin. (E) WST-cell viability assay shows no apparent cytotoxic effect of MyD88 inhibitor on HEK-TLR5 cells treated with HMGB1 (1µg/ml). (F) HEK-TLR4 cells were treated with LPS (50 ng/ml) and HMGB1 (1 µg/ml) followed by a dose-dependent inhibition of TLR4 mediated NF-κB activation by anti-TLR4 NAb. (G) Measurement of cellular toxicity in HEK-TLR5 cells treated with HMGB1 and flagellin followed by the addition of TH1020, a TLR5 inhibitor (based on Figure 2D). (H) Measurement of cellular toxicity in THP-1 cells used for proinflammatory cytokine release and ELISA tests (based on Figure 5D). Cellular toxicity data was normalized as (raw data-20% DMSO)/ (untreated cells-20% DMSO) such that untreated cells are 100% survival, and 20% DMSO is 0% survival. All data are represented as mean ± SD, n=3.

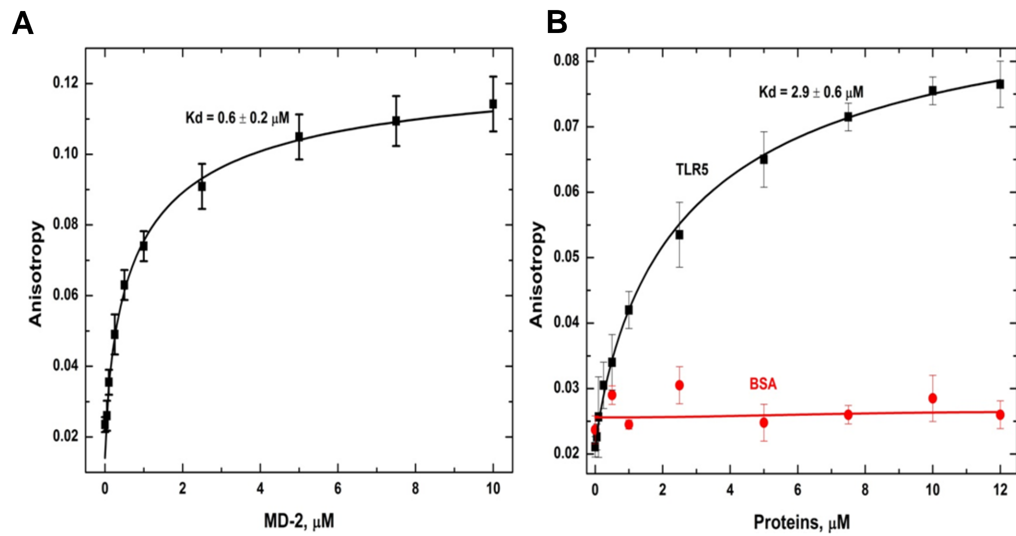

Figure S3. Characterization of HMGB1 and *dr*TLR5 ECD binding by fluorescence anisotropy assay, Related to Result section "C-terminal tail of HMGB1 interacts with TLR5". The binding affinity between: (A) 200 nM HMGB1 and MD-2 protein and (B) 200 nM HMGB1 and TLR5 ECD protein were performed by incubating fluorescein-labeled HMGB1 with varying amounts of the target protein (MD-2 [as positive control] and TLR5 ECD) for 30 min at room temperature in binding buffer (40 mM HEPES, pH 7.5 and 150 mM NaCl). Data are represented as mean ± SD, n=3.

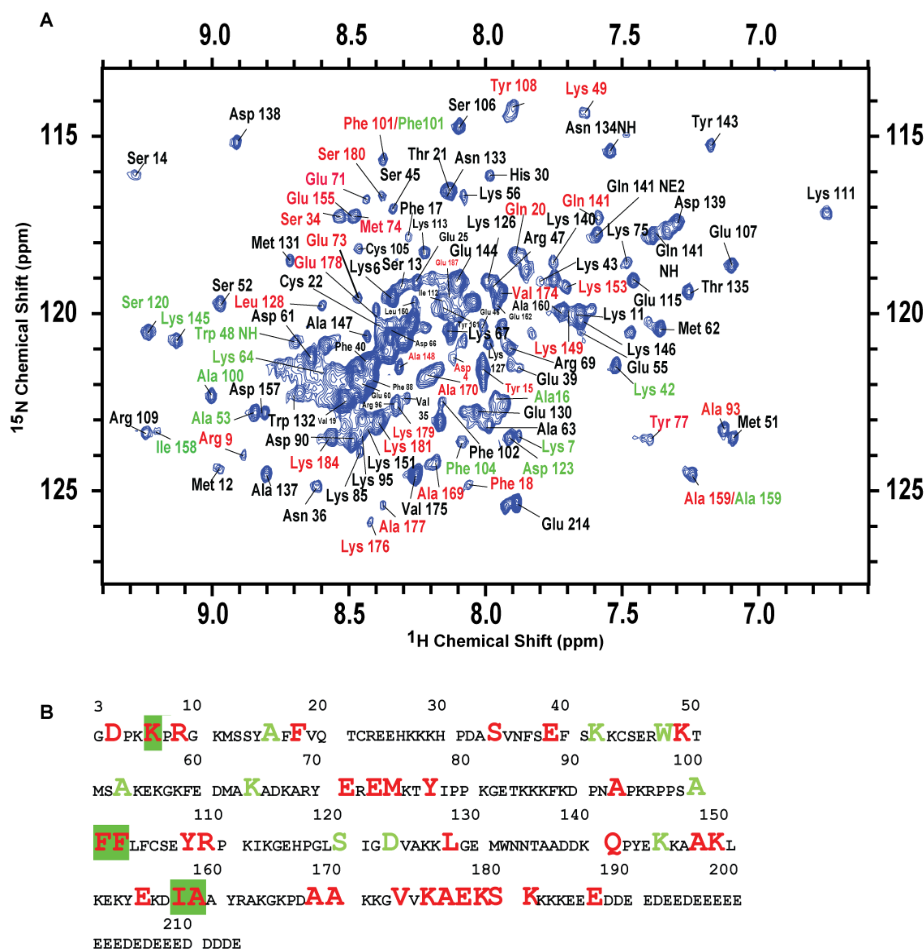

Figure S4. Backbone resonance assignment of rat HMGB1 with N-terminal CBP tag, Related to Figure 5. (A) ^{1}H-^{15}N HSQC spectra showing the sequence specific backbone amide N assignments. The previously deposited chemical shift table of HMGB-1 under BioMagResBank (BMRB) accession number 15502 (Watson et al., 2007) was used for chemical shift mapping on this spectra. Residues labeled in black are those which have been assigned by chemical shift mapping. Residues labeled in red are shifted upon TLR5 ECD addition. Residues labeled in green are shifted in the tailless mutants of HMGB-1 (apo-tailless HMGB1). (B) Primary sequence of HMGB1 showing residues shifted upon TLR5 ECD addition to full-length HMGB1 (red color). Residues which are shifted in the tailless HMGB1 mutant are labeled in green. The common residues which are shifted upon TLR5 ECD in full length and tailless HMGB1 mutant are shown in green backgrounds as shown for K, FF and IA.

A Rat HMGB1 Full length with N terminal CBP Tag

```
        10         20         30         40         50         60
       NFI AVSAANRFKK ISSSGALLVP RGSIEGRMGK GDPKKPRGKM SSYAFFVQTC
        70         80         90        100        110        120
REEHKKKHPD ASVNFSEFSK KCSERWKTMS AKEKGKFEDM AKADKARYER EMKTYIPPKG
       130        140        150        160        170        180
ETKKKFKDPN APKRPPSAFF LFCSEYRPKI KGEHPGLSIG DVAKKLGEMW NNTAADDKQP
       190        200        210        220        230        240
YEKKAAKLKE KYEKDIAAYR AKGKPDAAKK GVVKAEKSKK KKEEEDDEED EEDEEEEEEE
       250
EDEDEEEDDD DE
```

Human HMGB1 Full length with N terminal 6X HisTag

```
                      10         20         30         40         50
6X-His Tag-MGKGDPKKPR GKMSSYAFFV QTCREEHKKK HPDASVNFSE FSKKCSERWK
                      60         70         80         90        100
           TMSAKEKGKF EDMAKADKAR YEREMKTYIP PKGETKKKFK DPNAPKRPPS
                     110        120        130        140        150
           AFFLFCSEYR PKIKGEHPGL SIGDVAKKLG EMWNNTAADD KQPYEKKAAK
                     160        170        180        190        200
           LKEKYEKDIA AYRAKGKPDA AKKGVVKAEK SKKKKEEEED EEDEEDEEEE
                     210
           EDEEDEDEEE DDDDE
```

Human HMGB1 Tail less Mutant with N terminal 6X HisTag

```
                      10         20         30         40         50
6X-His Tag-MGKGDPKKPR GKMSSYAFFV QTCREEHKKK HPDASVNFSE FSKKCSERWK
                      60         70         80         90        100
           TMSAKEKGKF EDMAKADKAR YEREMKTYIP PKGETKKKFK DPNAPKRPPS
                     110        120        130        140        150
           AFFLFCSEYR PKIKGEHPGL SIGDVAKKLG EMWNNTAADD KQPYEKKAAK
                     160        170        180
           LKEKYEKDIA AYRAKGKPDA AKKGVVKAEK SKKKK
```

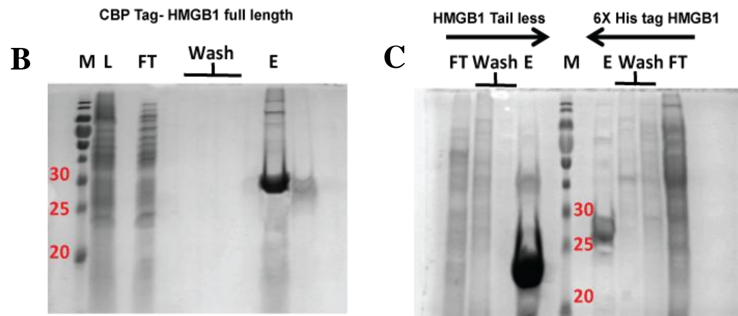

Figure S5. Amino acid sequences and expression, purification of different constructs of HMGB1 proteins, Related to Experimental Procedures section. (A) Amino-acid sequences of three different constructs of HMGB1 used for protein expression and purification. The sequence of the expressed rat HMGB1 with an N-terminal CBP tag shown in red, followed by thrombin cleavage site shown in green connected to the full-length rat HMGB-1 protein by a linker (blue color). The sequence of the expressed human HMGB1 with an N-terminal 6X-His tag. Only two amino acids in the tail region differ in the human and rat full-length HMGB1 protein sequence. Residue 189 is "D" in rat and "E" in human and residue 202 is "E" in rat and "D" in human HMGB1. The expressed sequence of human HMGB1 tailless mutant with an N-terminal 6X-His tag. The tailless mutant lacks the 30 amino-acid acidic C terminal tail of the full-length HMGB1 protein.12% SDS-PAGE gel showing products of the expression and purification steps of (B) rat HMGB1 with an N-terminal CBP tag and (C) human HMGB1 tailless mutant with an N-terminal 6X-His tag, human HMGB1 with an N-terminal 6X-His-tag. M: molecular weight markers; L: whole cell lysate; FT: flow through from the nickel column; Washes: two consecutive washing steps; and E: elution from the nickel column.

Table S1. Experimentally observed chemical shifts table of full-length HMGB1, Related to Figure 5. In columns 5 and 6, residues whose chemical shifts were changed upon TLR5 ECD addition are shown and cited as "TLR5 bound". Similarly, chemical shifts changed in the tailless mutant of HMGB1 are called "Tailless". Some residues showed chemical shift changes in both TLR5 bound and tailless mutant. In these cases, first sub column shows the chemical shift change for TLR5 bound and the second sub column describes the chemical shift change in the tailless mutant of HMGB1.

$^{\xi}$ Those chemical shifts which were observed in our spectra are only present in this table. Discontinuity in assignment is shown by gaps in between the tables.

† Backbone resonance assignments were performed by chemical shift mapping in (Watson et al., 2007).

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

SEAP reporter gene assay, Related to Experimental Procedure "QUANTI-Blue SEAP Assay"

HEK-293 cells stably transfected with human TLRs 2, 3, 4, 5, 7, 8 and 9 (HEK-hTLR) along with an inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene and appropriate TLR accessory proteins were purchased from Invivogen (San Diego, CA) The *SEAP* reporter gene was placed under the control of an IL-12 p40 minimal promoter fused with five NF-κB and activating protein 1 (AP1) binding sites. Stimulation with a TLR ligand activates NF-κB and AP1, which induces the production of SEAP in the cell culture supernatant. Enhancement in SEAP signaling correlates to specific TLR activation and was monitored by the QUANTI-Blue SEAP assay. All concentrations used for the treatments here for the SEAP signaling assay are: 200 ng/ml PAM2 or PAM3 (TLR2), 5 μg/ml Poly I:C (TLR3), 50 ng/ml LPS (TLR4), 200 ng/ml flagellin (TLR5), 5 μg/ml R848 (TLR7/8), 1 μg/ml HMGB1, 200 μg/ml AGE-BSA (RAGE), and 5 μg/ml ODN2395 (TLR9) according to the manufacturer's protocol (InvivoGen, CA). Neutralizing antibodies, including anti-hTLR5 NAb and anti-hTLR4 NAb (0 to 10 μg/ml) (InvivoGen, CA) were utilized to determine receptor involvement in HMGB1 signaling. Various inhibitors were used to monitor the NF-κB signaling pathway as follows: a small molecule MyD88 inhibitor (0-25 μM), polymyxinB (2-50 μg/ml), a peptide TIRAP inhibitor DRQIKIWFQNRRMKWKKLQLRDAAPGGAIVS (12.5-50 μM), small molecule TLR5 inhibitor TH1020 (0.187-3 μM), and NF-κB inhibitor triptolide (10-100 nM). After treatment with compounds, cells were incubated overnight at 37°C for 18-24 h. To detect SEAP activity, 20 μl of media was removed from each well and transferred to a transparent 96-well plate (Thermo Scientific, MA) containing 180 μl of QUANTI-Blue reagent (InvivoGen, CA) and incubated at 37°C (in the dark) for 30 min to 2 h. The QUANTI-Blue is a colorometric enzymatic assay in which the presence of alkaline phosphatase changes the media color from pink to blue and can be monitored at 620 nm using a plate reader (Beckman-Coulter DTX 880 Multimode Detector). Data were normalized as (well raw data untreated cells)/(ligand + solvent control-untreated cells) such that ligand + solvent is 100% activation, and untreated cells are 0% activation. Experiments were conducted with a minimum of three biological replicates (triplicate).

***In vitro* NO Activation assay with RAW 264.7 cells, Related to Experimental Procedures "NO Activation assay with RAW 264.7 cells"**

RAW 264.7 cells were seeded on day 1 at 70,000 cells/well in a tissue culture grade 96-well plate. Cells were supplemented with RPMI-1640 medium (10% fetal bovine serum, 1% L-gluamine, 1% penicillin/streptomycin) (complete growth media) and incubated at 37°C. On day 2, supplemented media was removed, and unsupplemented RPMI media was added to the cells. Cells were treated with 200 ng/ml PAM2, 50 ng/ml LPS, 200 ng/ml flagellin (InvivoGen, CA), 1 μg/ml HMGB1, 20 to 200 μg/ml AGE-BSA (Abcam, MA) and 2 to 20 μg/ml anti-RAGE NAb (R&D systems, MN), as well as oxPAPC (10 to 30 μg/ml) (InvivoGen, CA). On day 3, 90 μL media was transferred to a black 96-well plate (Thermo Scientific, MA) and 10 μL of 0.05 mg/mL 2, 3-diaminonapthalene (Sigma Aldrich, MO) in 0.62 M HCl added to the media. The plate was covered in aluminum foil and shaken at room temperature for 15 min. A Beckman Coulter DTX 880 Multimode detector was used to quantify results. Samples were excited at 360 nm and emission was measured at 430 nm. Data was normalized as (well raw data-untreated cells)/(ligand + solvent control untreated cells) such that ligand + solvent is 100% activation, and untreated cells are 0% activation. Experiments were conducted with a minimum of three biological replicates (triplicate).

Procedure to prepare Jurkat-T cell sensitive to TLR5 ligand only, Related to "Jurkat-T cell transfection and NF-κB–GFP reporter assay"

Human Jurkat cells [American Type Culture Collection (ATCC) TIB-152] were grown and maintained in RPMI-1640 medium containing 10% FBS and 1% penicillin/streptomycin. An NF-κB–GFP reporter was stably inserted using the commercially available pGreenFire lentiviral plasmid (System Bioscience, CA). Briefly, HEK 293T cells (ATCC CRL-3216) were transfected using a 6:1 polyethylenimine:DNA ratio with the pGreenFire vector (4.33 μg), pREV (4.33 μg), pMDL (4.33 μg), and pVSVg (2 μg) viral packaging plasmids. Viral particles were harvested from the medium 48 to 72 h after transfection and concentrated using 8.5% PEG-8000 (polyethylene glycol) and 10 mM NaCl. The concentrated virus and polybrene (8 μg/ml) were added to Jurkat cells for 48 h. Growth medium supplemented with puromycin (1 mg/ml) was used to select for stably transfected cells. After complete selection, the cells were sorted for GFP expression using a MoFlo Cytomation (Beckman Coulter, CA) fluorescence-activated cell sorter. After sorting for insertion, cells were treated with flagellin (100 ng/ml) (InvivoGen, CA) and sorted for activation. The top 10% of activated cells were collected for each sort until no further peak separation was achieved between untreated and treated cells. The sorted cells were seeded in six-well plates at 1×10^6 cells per well with 3 ml of complete growth medium [RPMI 1640 medium, supplemented with 10% FBS, 1% penicillin/streptomycin] and TLR ligands (200 ng/ml of PAM2 and PAM3, 5μg/ml Poly I:C, 50 ng/ml LPS, 200 ng/ml flagellin, 5μg/ml R848, 1 μg/ml HMGB1 and 200

μg/ml AGE-BSA) for 24 h at 37°C in a 5% CO_2 humidified incubator. After 24 h, the cells in each well were mixed and 200 μl cells containing medium were stained by propidium iodide for 10 min before flow cytometry analysis.

HMGB1 protein preparation, Related to Experimental Procedures "HMGB1 protein expression and purification"

Protein expression

The plasmids for full-length HMGB1 with N-terminal CBP and 6X His tags were the generous gifts from Prof. Kevin J. Tracey and Prof. Patrick C Swanson labs respectively (Figure S5A). Full-length proteins were purified using previously published protocols (Li et al., 2004). Briefly, the plasmids of CBP tag ^{15}N-HMGB1 and 6X His tag ^{15}N-HMGB1 full-length and tailless were transformed into BL21(DE3) and BL21(DE3)PLys *E. coli* strains for large-scale protein expression. A single colony was picked to inoculate four 50 ml precultures of LB media with 100 μg/ml ampicillin antibiotic in each. Precultures were grown overnight at 37°C in a shaker incubator and transferred to four 1 L of LB media with the same ampicillin antibiotic (100 μg/ml). The 4 L of LB media were grown first at 37ºC until OD_{600} reached 1.0-1.5. Cells were harvested by centrifugation, washed twice with M9 media, and transferred to 1 L M9 media with 1 g $^{15}NH_4Cl$ stable isotopes. The M9 media was incubated for 30 min at 37ºC in a shaker incubator followed by adding 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to induce HMGB1 full-length and tailless protein overexpression. For CBP tag-HMGB1, protein overexpression was continued at 37 ºC for 3 h, while for 6x His tag HMGB1 and tailless protein, overexpression was continued at 30°C for 5-6 h. After OD_{600} reached 4.0-5.0, the cells were harvested by centrifugation at 5,000-6,000xg for 10 min at room temperature. The cell pellet for CBP tag-^{15}N HMGB1 (full-length) was resuspended in 20 ml of 20 mM Tris–HCl, pH 8.6, 150 mM NaCl, 2.5 mM $CaCl_2$ (Buffer A), while His tag-^{15}N HMGB1 full-length and HMGB1 tailless were resuspended in 40 mM Tris–HCl, pH 7.5, 500 mM NaCl and stored at -80°C.

Protein purification

Modified protocols of previously published procedures were used to purify CBP tag-^{15}N isotope labeled HMGB1 (full-length), 6XHis tag- ^{15}N isotope labeled HMGB1 (full-length) and 6X His tag- ^{15}N HMGB1 tailless proteins (Figure S5B and S5C). ^{15}N isotope labeling method for overexpressing protein in bacterial culture was adapted from (Das et al., 2015).

CBP tag-^{15}N HMGB1 (full-length)

Cells harvested from 1 L M9 media were thawed and mixed with 20 μl benzonase (Novagene) and 0.25 mg lysozyme at room temperature (RT). 20% (vol/vol) lysis buffer (20 mM Tris, pH 8.0, 1% TritonX-100, 8 M urea) was added to the mixture. The ratio between Buffer A and lysis buffer was 4:1 vol/vol with a total volume of 60 ml. The solution was mixed for 30 min at room temperature and passed through a syringe with a 0.1 mm needle three times (Das et al., 2015). The solution was centrifuged at 10,000xg for 45 min at 8 ºC to remove bacterial debris. Supernatant was mixed gently with 5 ml Calmodulin Affinity Resin (Agilent Technologies) at 4ºC for 2 h by continuous shaking. The column was washed with 20 column volume (CV) Buffer A (Wash 1) followed by 40μl benzonase in Buffer B (40 mM Tris–HCl, pH 7.8, 6mM $MgCl_2$) (Wash 2) for 2 h at RT with gentle shaking. The column was again washed with 10 CV Buffer A (Wash 3). CBP-tagged HMGB1 was eluted with 50 mM Tris-HCl, pH 7.8, 2 mM EGTA, 200 mM NaCl (elution). The protein yield was >35mg/L M9 media. All fractions were checked by 12% SDS-PAGE gel (Figure S5B). Purified protein was dialyzed against 40 mM Tris-HCl, pH 7.8, 1 mM DTT overnight, then concentrated. 80-100 μM purified protein in 270 μl with 3% D_2O was used for NMR spectroscopy.

6X His tag-^{15}N full-length HMGB1 and tailless HMGB1

The procedure up to the lysis step and separating the cellular debri by centrifugation was exactly same as for CBP tag-^{15}N HMGB1 purification. For protein purification, the supernatant was transferred to a 5 ml His TrapTM FF Nickel Column (GE Healthcare) equilibrated with buffer 1 (40 mM Tris, pH 7.8, 10 mM Imidazole, 300 mM NaCl) and purified by AKTApurifier UPC10 FPLC (GE Healthcare). Two consecutive wash steps were performed. Wash step 1 was performed with 4 CV of buffer 1 and step 2 with buffer 2 (40 mM Tris, pH 7.8, 60 mM imidazole, 100 mM NaCl). Finally, protein was eluted with 40 mM Tris pH 7.8, 400 mM imidazole, 100 mM NaCl at 4°C. The elution fraction absorbance was monitored at 280 nm by Nanodrop. The yield of purified His tag-^{15}N HMGB1 full-length and His tag-^{15}N HMGB1 tailless proteins was 25-30 mg/L of M9 media. Protein purity was checked by 12% SDS-PAGE gel (Figure S5C).