Figure S1. HMGB1 binds and enables extracellular LPS to trigger pyroptosis. Related to Figure 1.

(A) Reduced HMGB1 recombinant protein was immobilized to the chips. The binding between LPS and reduced HMGB1 was assessed by surface plasmon resonance.

(B) Mice received i.p. biotin-labeled LPS (5mg/kg) or saline. 2 hours later, peritoneal lavage fluid was incubated with streptoavidin-coated beads. The soluble LPS binding molecules isolated using these beads was characterized by Mass spectrometry.

(C) HMGB1 promotes extracellular LPS-induced LDH release. LDH assay for mouse peritoneal macrophages stimulated with indicated LPS-interacting proteins (LIPs) and LPS (1μg/ml).

(D) ELISA to detect TNF in the culture medium of mouse peritoneal macrophages of indicated genotypes upon exposure to the necrotic Hmgb1-/- or Hmgb1+/+ MEFs (106 cells/mL) in the presence or the absence of LPS (1μg/ml) for 16h (the ratio of necrotic cells to macrophages is 1:1). Graphs show the mean ± SD of technical replicates and are representative of three independent experiments.

Figure S2. HMGB1 enables extracellular LPS to activate caspase-11 and downstream caspase-1. Related to Figure 1.

(A-B) ELISA for IL-1β release (A) and Immuno-blots (B), pro-IL-1β cleavage and caspase-1 activation in the supernatants (SN), as well as concentrations of pro-IL-1β, pro-caspase-1 and β-actin in the cell lysates (Cell) of mouse peritoneal macrophages from mice of indicated genotypes stimulated with either LPS alone (1μg/ml) (L) or LPS (1μg/ml) + HMGB1 (400ng/ml) (LH) for 16h.

(C-D) ELISA for IL-1β release (C) and Immuno-blots (D), pro-IL-1β cleavage and caspase-1 activation in the supernatants (SN), as well as concentrations of pro-IL-1β, pro-caspase-1 and β-actin in the cell lysates (Cell) of WT mouse peritoneal macrophages stimulated with LPS (1μg/ml) or HMGB1 (400ng/ml) in the presence of indicated concentration of MCC950 for 16h.

(E) LDH assay for WT mouse peritoneal macrophages stimulated with LPS (1μg/ml) or HMGB1 (400ng/ml) in the presence of indicated concentration of LPS-RS for 16h.

(F) Recombinant full-length HMGB1 proteins or truncated HMGB1 forms containing only A-box or B-box were immobilized to the chips. The binding between these recombinant proteins and LPS was assessed by surface plasmon resonance.

(G-H) LDH assay and ELISA to assess pyroptosis and IL-1α released from WT mouse peritoneal macrophages stimulated with full-length HMGB1 (HMG, 1μg/ml), truncated HMGB1 forms containing A-box (A box, 1μg/ml), or truncated HMGB1 forms containing B-box (B box, 1μg/ml) in the presence or the absence of LPS (1μg/ml).

(I-L) LDH or IL-1α released from WT or Hmgb1-/- mouse macrophages stimulated with LPS (1μg/mL) + cholera toxin B (10μg/mL) or lipofectamine 3000. Graphs show the mean ± SD of technical replicates and are representative of three independent experiments.

Figure S3. HMGB1 delivers extracellular LPS into the cytosol through RAGE and lysosomal rupture. Related to Figure 2 and Figure 3.

(A) The physical interaction between HMGB1 and LPS was visualized as red spots using the proximity-ligation assay (PLA) in mouse macrophages stimulated with LPS alone or LPS+HMGB1. Scale bar: 10μm.

(B) Using the same experimental conditions, co-localization of HMGB1/LPS complexes with EEA1 or LAMP1 (indicated by white arrows) is shown by immunostaining and confocal microscopy. The proportion of HMGB1/LPS complexes that co-localize with EEA1 or LAMP1 is shown in the right panel. Scale bar: 10μm.

(C) RAGE blocking peptide (32μM) markedly reduced the number of HMGB1/LPS complexes in the cells as shown by PLA. Scale bar: 10μm.

(D) Deletion of RAGE diminished the number of HMGB1/LPS complexes in the cells as shown by PLA. Scale bar: 10μm.

(E) The physical interaction between caspase-11 and LPS were visualized as the red spots by PLA in WT or Ager-/- mouse peritoneal macrophages primed with 100ng/ml LPS for 4h and then stimulated with LPS alone (L, 5μg/ml), HMGB1 alone (10μg/ml) (H), or LPS (5μg/ml)+HMGB1 (10μg/ml) (LH) for 4h. Scale bar: 10μm.

(F) The expression of Caspase1, Caspase11, IL-1α , pro-IL-1β and GAPDH in WT or Ager-/- mouse peritoneal macrophages after 4h of LPS stimulation (1μg/ml).

(G) ELISA for IL-1β in the supernatants of Pam3CSK4-primed mouse peritoneal macrophages from mice of indicated genotypes or unprimed WT mouse peritoneal macrophages stimulated with increasing concentration of HMGB1 for 16h.

(H) Whole-cell patch-clamp recording of truncated HMGB1 forms containing A-box induced an inward current across the cytoplasmic membrane of HEK293 cells at acidic conditions (pH=5.1). Graphs show the mean ± SD of technical replicates and are representative of three independent experiments.

Figure S4. Hepatocyte-released HMGB1 and RAGE are essential for caspase-11-dependent pyroptosis and lethality in endotoxemia and bacterial sepsis. Related to Figure 4, Figure 5 and Figure 6.

(A) Plasma IL-1β concentrations from WT or Casp11-/- mice injected with low dose of LPS (400μg/kg) for 6h and then challenged with high dose LPS (10mg/kg) for 4h.

(B) Immuno-blot to detect HMGB1 expression in hepatocytes or peritoneal macrophages isolated from mice of indicated genotypes; shown in the Low panel are plasma IL-1β concentrations from mice of indicated genotypes injected with low dose of LPS (400μg/kg) for 6h and then challenged with high dose LPS (10mg/kg) for 4h.

(C) Plasma IL-1β concentrations from WT mice injected with low dose of LPS (400μg/kg) for 6h and then subjected to administration of high dose LPS (10mg/kg) with monoclonal HMGB1 neutralizing antibodies (150μg per mouse) or isotype control IgG (150μg per mouse) for 4h.

(D) ELISA for serum IL-1α, IL-1β concentrations of WT mice following intraperitoneal injection with LPS (400μg/kg) and HPep1 (Pep1, 200μg per mouse) or control peptides (Ctrl, 200μg per mouse) for 6h and then challenged by a high dose LPS (10mg/kg) for 4h.

(E) Kaplan–Meier survival plots for WT mice injected with a low dose of LPS (400 μg/kg) for 6h and then challenged by a high dose LPS (10mg/kg) with or without LPS-RS (5mg/kg).

(F) Kaplan–Meier survival plots for WT mice injected with either high dose of LPS (54mg/kg) or high dose of HMGB1 (1mg per mouse).

(G) Plasma IL-1β concentrations from WT or Ager-/- mice injected with low dose of LPS (400μg/kg) for 6h and then challenged with high dose LPS (10mg/kg) for 4h.

(H) Kaplan–Meier survival plots for mice with indicated genotypes injected with low dose of LPS (400μg/kg) for 6h and then challenged with high dose LPS (10mg/kg) with or without soluble RAGE (sRAGE, 5μg/mouse).

(I) Plasma IL-1α and IL-1β concentrations from WT and Ager-/- mice subjected to either cecum ligation and puncture (CLP) or sham operation for 18 hours.

(J) Immuno-blot to detect the expression of Casp11 and GAPDH in spleen or kidney of the WT or Caspase-11 KO mice subjected to either CLP or sham operation for 18h. C: sham control.

(K) Cell death in the spleens of WT and Ager-/- mice was assessed by TUNEL staining. Scale bar: 20μm.

(L) Flow cytometry to assess the proportion of TMR- and CD11b-positive cells in the peritoneal cavity of mice subjected to either CLP or sham operation. Graphs show the mean ± SD. *$P < 0.05$; ***$P < 0.001$ (Student's t-test)

Figure S5. Hepatocyte TLR4 is important for HMGB1 release and caspase-11-dependent pyroptosis. Related to Figure 7.

(A) Primary WT hepatocytes co-cultured with WT or Casp11-/- mouse bone marrow derived macrophages were stimulated with LPS (1μg/ml) for 18h.

(B) Primary WT hepatocytes co-cultured with WT mouse bone marrow derived macrophages were stimulated with LPS (1μg/ml) in the presence of HMGB1 neutralizing monoclonal antibodies or isotype control IgG for 18h.

(C) Primary hepatocytes isolated from mice of indicated genotypes co-cultured with WT mouse bone marrow derived macrophages. IL-1α released from macrophages after stimulation of LPS (1μg/ml) for 18h was measured by ELISA.

(D) Plasma IL-1β concentrations from mice of indicated genotypes injected with a low dose of LPS (400μg/kg) for 6h and then challenged with a high dose LPS (10mg/kg) for 4h.

(E-G) Plasma HMGB1, IL-1α and IL-1β concentrations from mice of indicated genotypes subjected to either cecum ligation and puncture (CLP) or sham operation for 18 hours. Circles represent individual mice. *P＜0.05; **P＜0.01; ***P＜0.001 (Student's t-test).

Figure S6. Hepatocyte caspase-11 and GSDMD mediate HMGB1 release from live cells. Related to Figure 7.

(A) Immuno-blot for Na+-K+ ATPase, Rab7, Lamp1 and β-actin in the cytosolic and residual fraction from LPS-stimulated primary mouse hepatocytes.

(B) LPS activity assay of the cytosolic fraction from LPS (0.1μg/ml)-stimulated mouse primary hepatocytes from mice of indicated genotypes.

(C) Primary hepatocytes from mice of indicated genotypes were stimulated by LPS (0.1μg/ml). HMGB1 release from hepatocytes was assessed by ELISA.

(D) LPS activity assay of the cytosolic fraction from LPS (0.1μg/ml)-stimulated WT or Hmgb1-/- mouse primary hepatocytes.

(E) LPS activity assay of the cytosolic fraction from LPS (0.1μg/ml)-stimulated WT mouse hepatocytes in the presence of different concentrations of extracellular HMGB1.

(F) HMGB1 release (ELISA) of primary WT, Casp11-/- or Gsdmd-/- hepatocytes stimulated by LPS (0.1μg/ml) for 18h.

(G-H) Cell death (Zombie staining) of primary WT hepatocytes stimulated by LPS (0.1μg/ml) for 18h or 20% of ethanol (EtOH, positive control) for 5min.

(I) Immuno-blot for cleaved GSDMD (p30), pro-GSDMD, caspase1 and GAPDH in the cell lystes of primary WT, Casp11-/- or Gsdmd-/- hepatocytes stimulated by LPS (0.1μg/ml) for 18h.

(J) Hepatocytes isolated from mice of indicated genotypes were cultured in anti-HMGB1 antibodies pre-coated plates and then stimulated with LPS (0.1μg/ml) for 16h. The number of

HMGB1-releasing cells was then measured by ELISPOT and shown in the left panel. Data of ELISpot assays and trypan blue staining were shown in the right panel.

(K) Primary hepatocytes were transfected with plasmids encoding HMGB1-EGFP for 24h. After LPS (1μg/mL) stimulation for 3h, time-lapse images were collected with a scanning confocal microscope. Figure shows a single basal confocal plane from the 3D time lapse series shown in movie. Panel A shows the image of the entire cell at T=0 (3 hours after treatment with LPS). The box highlighted on the right hand side of the image is at the attenuated leading edge of the cell. HMGB1 puncta are highlighted in the box (Scale bar =10μm). Panels B-F are time sequenced images of the insets in panel A at 15 minute intervals showing the gradual progressive loss of signal of HMGB1 puncta as HMGB1 are released from the cell. There is no possibility that the HMGB1 puncta moved to a different axial plane as the entire attenuated leading edge of the cell is contained within this image (Scale bar =2μm for panels B-F). Graphs show the mean ± SD of technical replicates and are representative of three independent experiments.

Figure S7. Hepatocyte caspase-11 mediates HMGB1 release and is important for pyroptosis in endotoxemia and bacterial sepsis. Related to Figure 7.

(A) Kaplan–Meier survival plots for WT mice underwent BM irradiation and transplantation with WT or Casp11-/- BM to reconstitute the hematopoietic compartment.

(B) Kaplan–Meier survival plots for Casp11-/- mice or WT mice underwent BM irradiation and transplantation with WT BM to reconstitute the hematopoietic compartment. All mice were injected with low dose of LPS (400μg/kg) for 6h and then challenged with high dose LPS (10mg/kg).

(C) The reconstitution of hematopoietic compartment was confirmed by flow cytometric analysis of the expression of CD45.1 and CD45.2 on hematopoietic cells.

(D) Immuno-blot to detect Caspase11 expression in hepatocytes or peritoneal macrophages isolated from mice of indicated genotypes.

(E) Plasma IL-1β and LTB4 concentrations from mice of indicated genotypes injected with a low dose of LPS (400μg/kg) for 6h and then challenged with a high dose LPS (10mg/kg) for 4h.

(F) Plasma HMGB1, IL-1α, IL-1β and LTB4 concentrations from mice of indicated genotypes subjected to either cecum ligation and puncture (CLP) or sham operation for 18 hours. Circles represent individual mice. Graphs show the mean ± SD. *P＜0.05; **P＜0.01; ***P＜0.001 (Student's t-test). The survival of these mice was shown in the Kaplan–Meier survival plots. *P ＜0.05, ***P＜0.001 (Log-rank test).

Figure S1

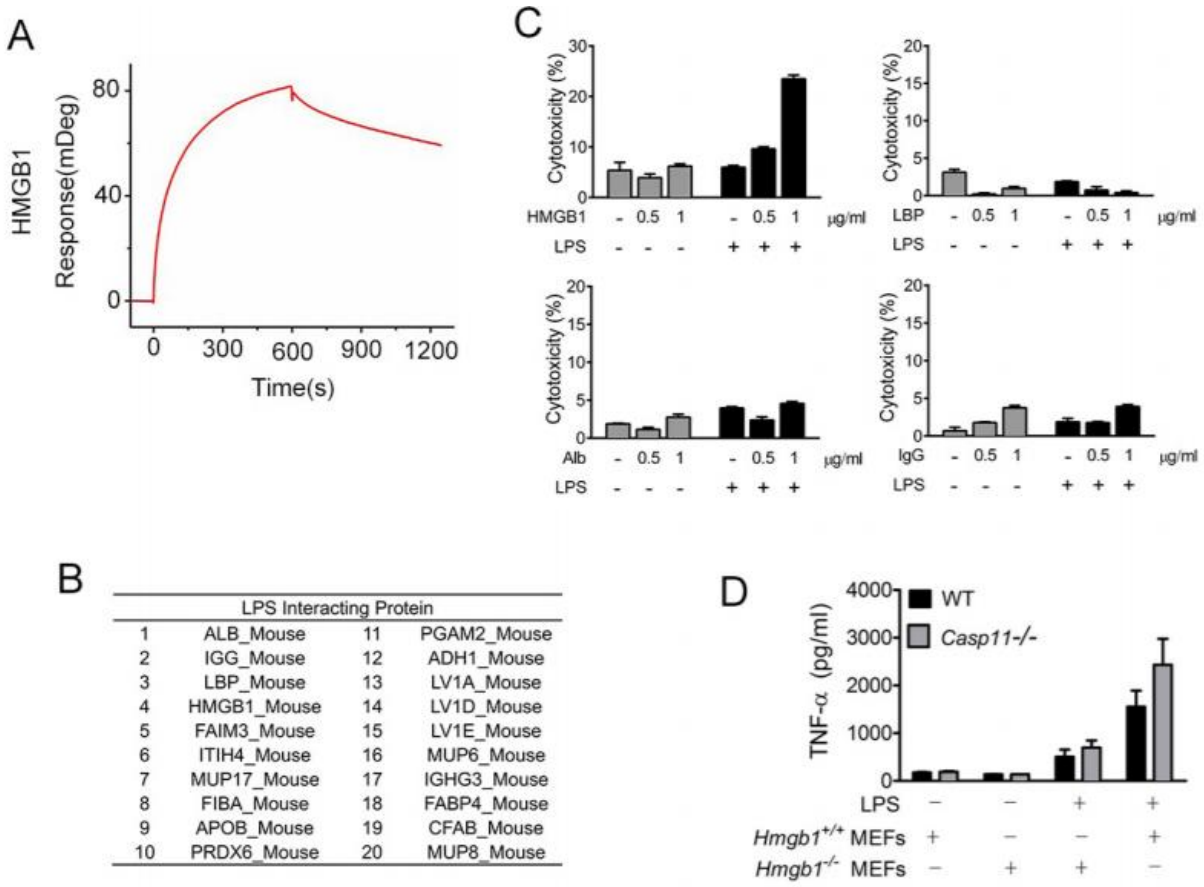
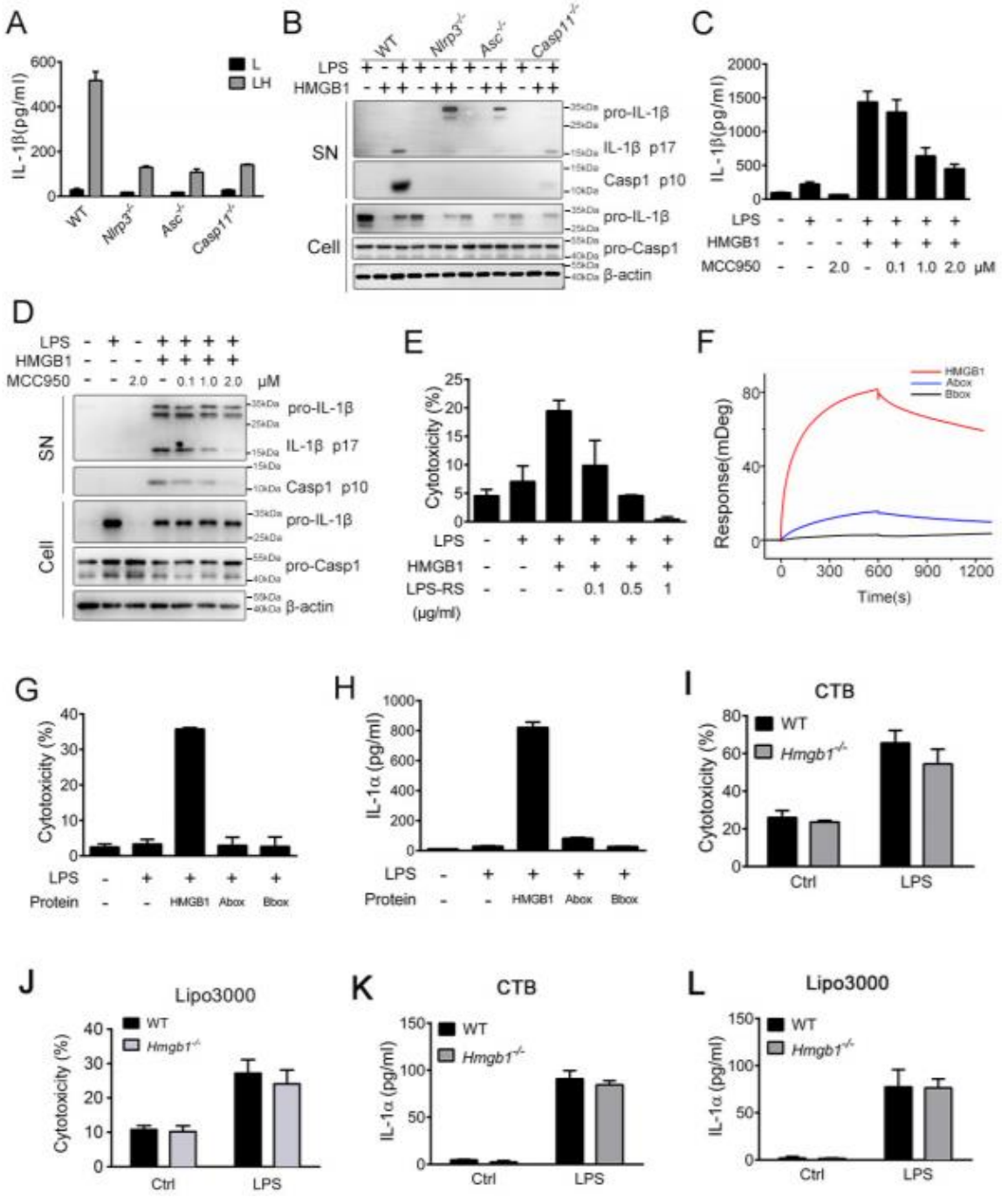

A
HMGB1
Response(mDeg)
80
40
0
0
300
600
900
1200
Time(s)
C
Cytotoxicity (%)
HMGB1 - 0.5 1 - 0.5 1 μg/ml
LPS - - - + + +
LBP - 0.5 1 - 0.5 1 μg/ml
LPS - - - + + +
Alb - 0.5 1 - 0.5 1 μg/ml
LPS - - - + + +
IgG - 0.5 1 - 0.5 1 μg/ml
LPS - - - + + +
B
LPS Interacting Protein
1 ALB_Mouse 11 PGAM2_Mouse
2 IGG_Mouse 12 ADH1_Mouse
3 LBP_Mouse 13 LV1A_Mouse
4 HMGB1_Mouse 14 LV1D_Mouse
5 FAIM3_Mouse 15 LV1E_Mouse
6 ITIH4_Mouse 16 MUP6_Mouse
7 MUP17_Mouse 17 IGHG3_Mouse
8 FIBA_Mouse 18 FABP4_Mouse
9 APOB_Mouse 19 CFAB_Mouse
10 PRDX6_Mouse 20 MUP8_Mouse
D
TNF-α (pg/ml)
4000
3000
2000
1000
0
WT
Casp11-/-
LPS - - + +
Hmgb1+/+ MEFs + - - +
Hmgb1-/- MEFs - + + -

Figure S2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IL-1β(pg/ml)
L
LH
WT
Nlrp3-/-
Asc-/-
Casp11-/-
B
LPS
HMGB1
SN
Cell
pro-IL-1β
IL-1β p17
Casp1 p10
pro-Casp1
β-actin
C
IL-1β(pg/ml)
LPS
HMGB1
MCC950
μM
D
E
Cytotoxicity (%)
LPS-RS (μg/ml)
F
HMGB1
Abox
Bbox
Response(mDeg)
Time(s)
G
Cytotoxicity (%)
Protein
H
IL-1α (pg/ml)
I
CTB
Hmgb1-/-
Ctrl
LPS
J
Lipo3000
K
CTB
L
Lipo3000

Figure S3

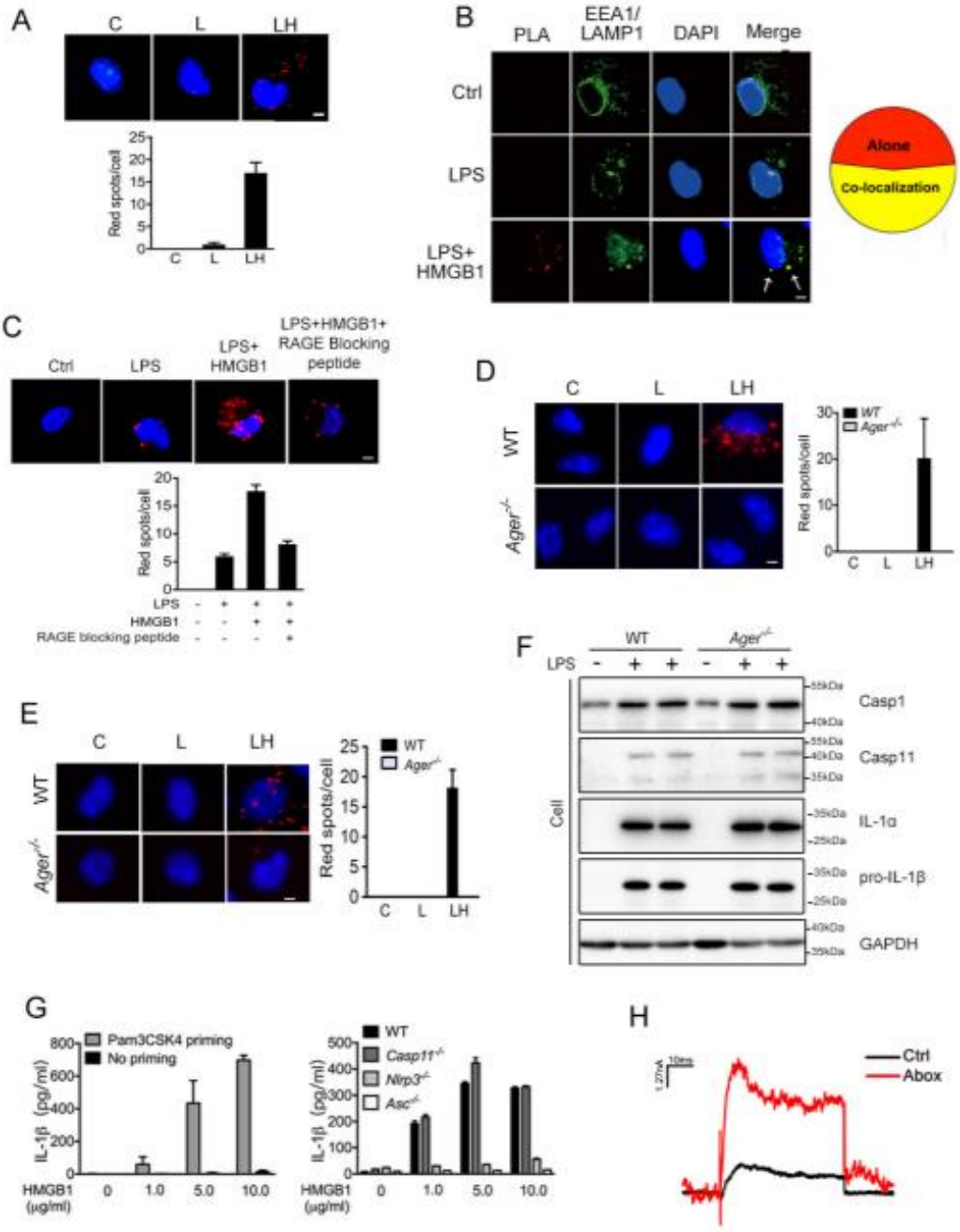

A
C
L
LH
Red spots/cell
B
PLA
EEA1/ LAMP1
DAPI
Merge
Ctrl
LPS
LPS+ HMGB1
Alone
Co-localization
C
Ctrl
LPS
LPS+ HMGB1
LPS+HMGB1+ RAGE Blocking peptide
Red spots/cell
LPS
HMGB1
RAGE blocking peptide
D
C
L
LH
WT
Ager-/-
Red spots/cell
E
C
L
LH
WT
Ager-/-
Red spots/cell
F
WT
Ager-/-
LPS
Cell
Casp1
Casp11
IL-1α
pro-IL-1β
GAPDH
G
Pam3CSK4 priming
No priming
IL-1β (pg/ml)
HMGB1 (μg/ml)
WT
Casp11-/-
Nlrp3-/-
Asc-/-
IL-1β (pg/ml)
HMGB1 (μg/ml)
H
Ctrl
Abox

Figure S4

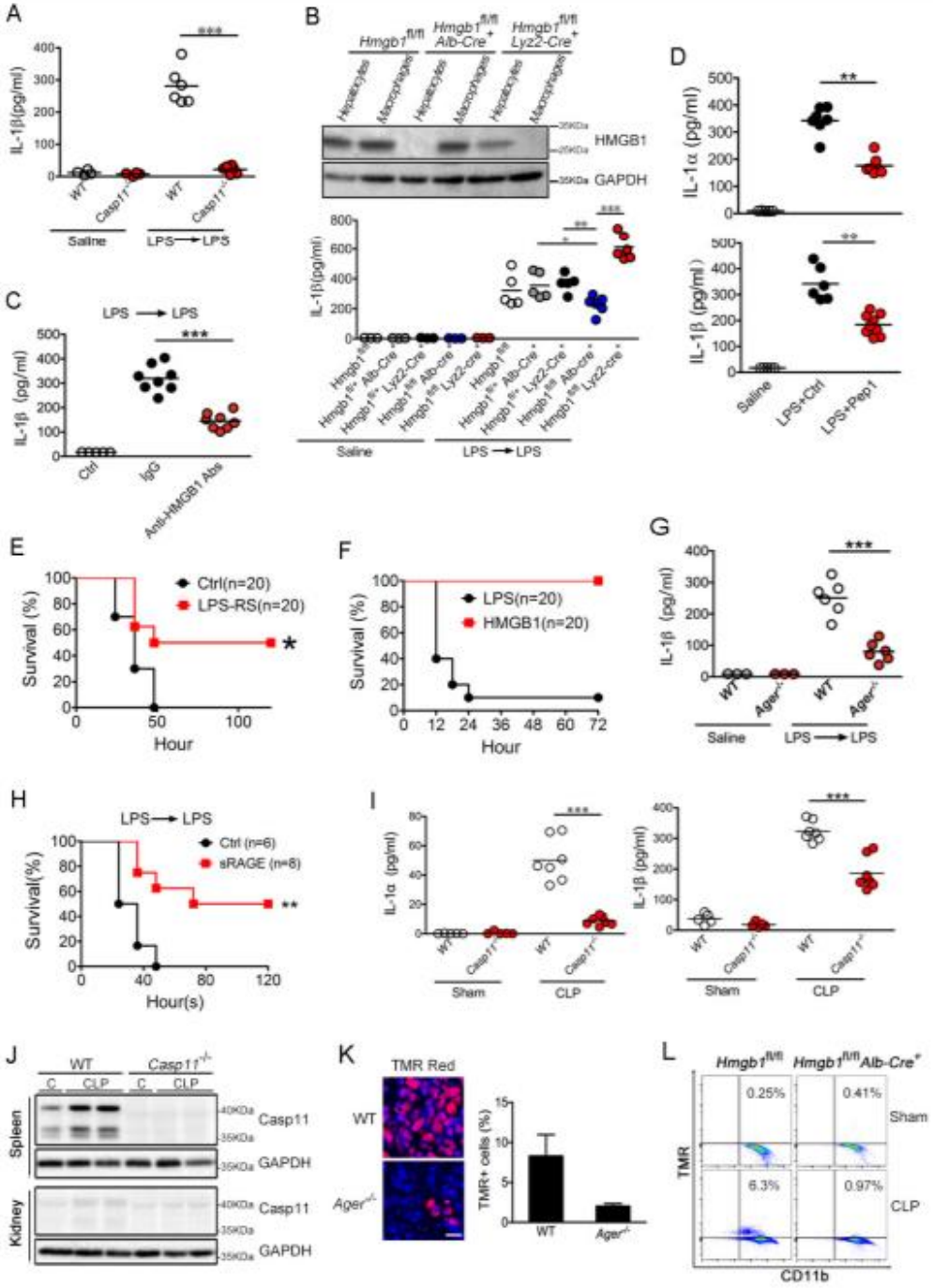

A
IL-1β(pg/ml)
WT
Casp11-/-
Saline
LPS→LPS
B
Hmgb1fl/fl
Hmgb1fl/fl Alb-Cre+
Hmgb1fl/fl Lyz2-Cre+
Hepatocytes
Macrophages
HMGB1
GAPDH
IL-1β(pg/ml)
Saline
LPS→LPS
C
LPS → LPS
IL-1β (pg/ml)
Ctrl
IgG
Anti-HMGB1 Abs
D
IL-1α (pg/ml)
IL-1β (pg/ml)
Saline
LPS+Ctrl
LPS+Pep1
E
Survival (%)
Ctrl(n=20)
LPS-RS(n=20)
Hour
F
Survival (%)
LPS(n=20)
HMGB1(n=20)
Hour
G
IL-1β (pg/ml)
WT
Ager-/-
Saline
LPS→LPS
H
LPS→LPS
Survival(%)
Ctrl (n=6)
sRAGE (n=6)
Hour(s)
I
IL-1α (pg/ml)
IL-1β (pg/ml)
Sham
CLP
J
WT
Casp11-/-
C
CLP
Spleen
Kidney
Casp11
GAPDH
K
TMR Red
WT
Ager-/-
TMR+ cells (%)
L
Hmgb1fl/fl
Hmgb1fl/fl Alb-Cre+
0.25%
0.41%
6.3%
0.97%
Sham
CLP
TMR
CD11b

Figure S5

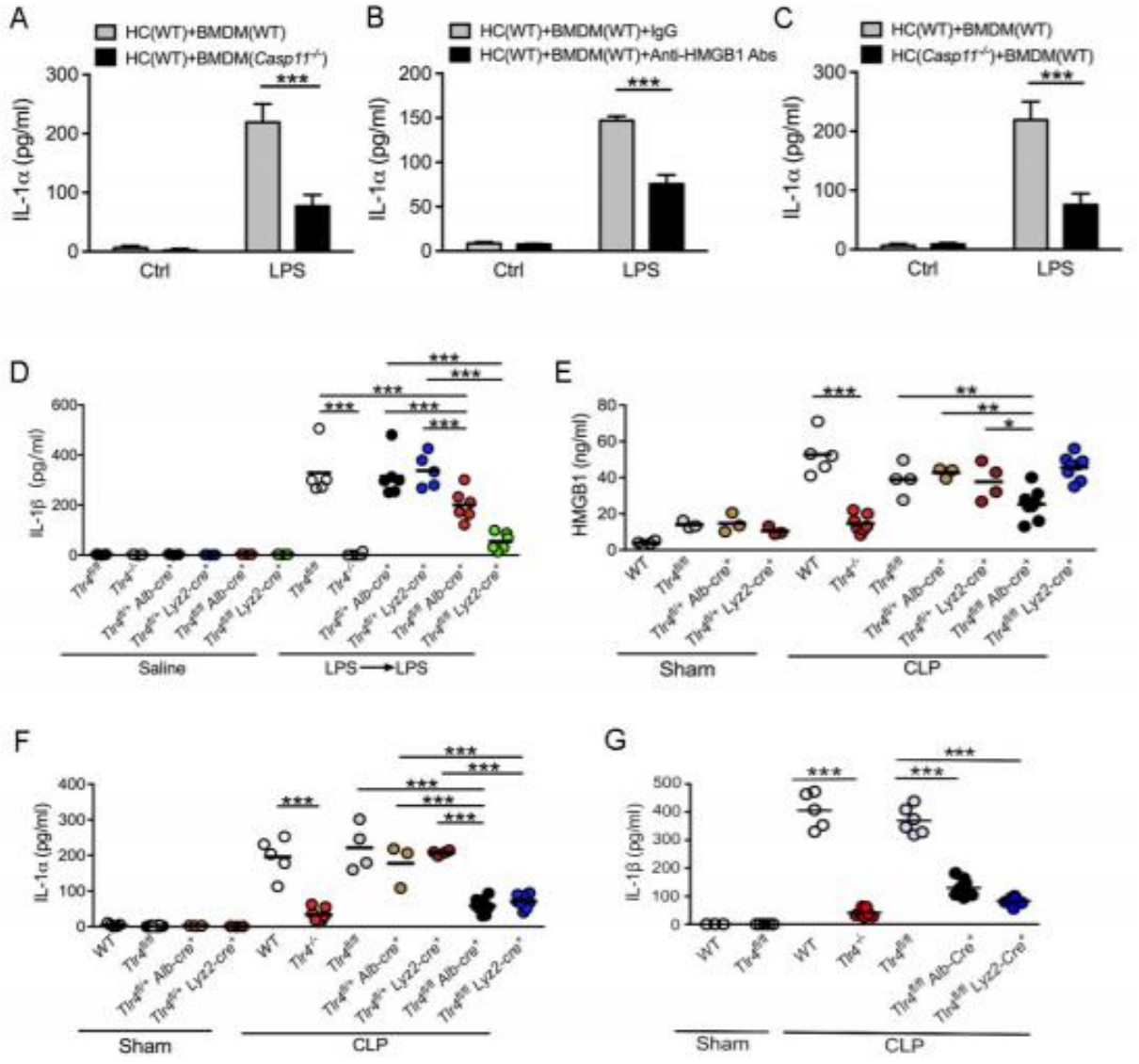

A
HC(WT)+BMDM(WT)
HC(WT)+BMDM(Casp11-/-)
IL-1α (pg/ml)
Ctrl
LPS
B
HC(WT)+BMDM(WT)+IgG
HC(WT)+BMDM(WT)+Anti-HMGB1 Abs
C
HC(WT)+BMDM(WT)
HC(Casp11-/-)+BMDM(WT)
D
IL-1β (pg/ml)
Saline
LPS→LPS
E
HMGB1 (ng/ml)
Sham
CLP
F
IL-1α (pg/ml)
G
IL-1β (pg/ml)

Figure S6

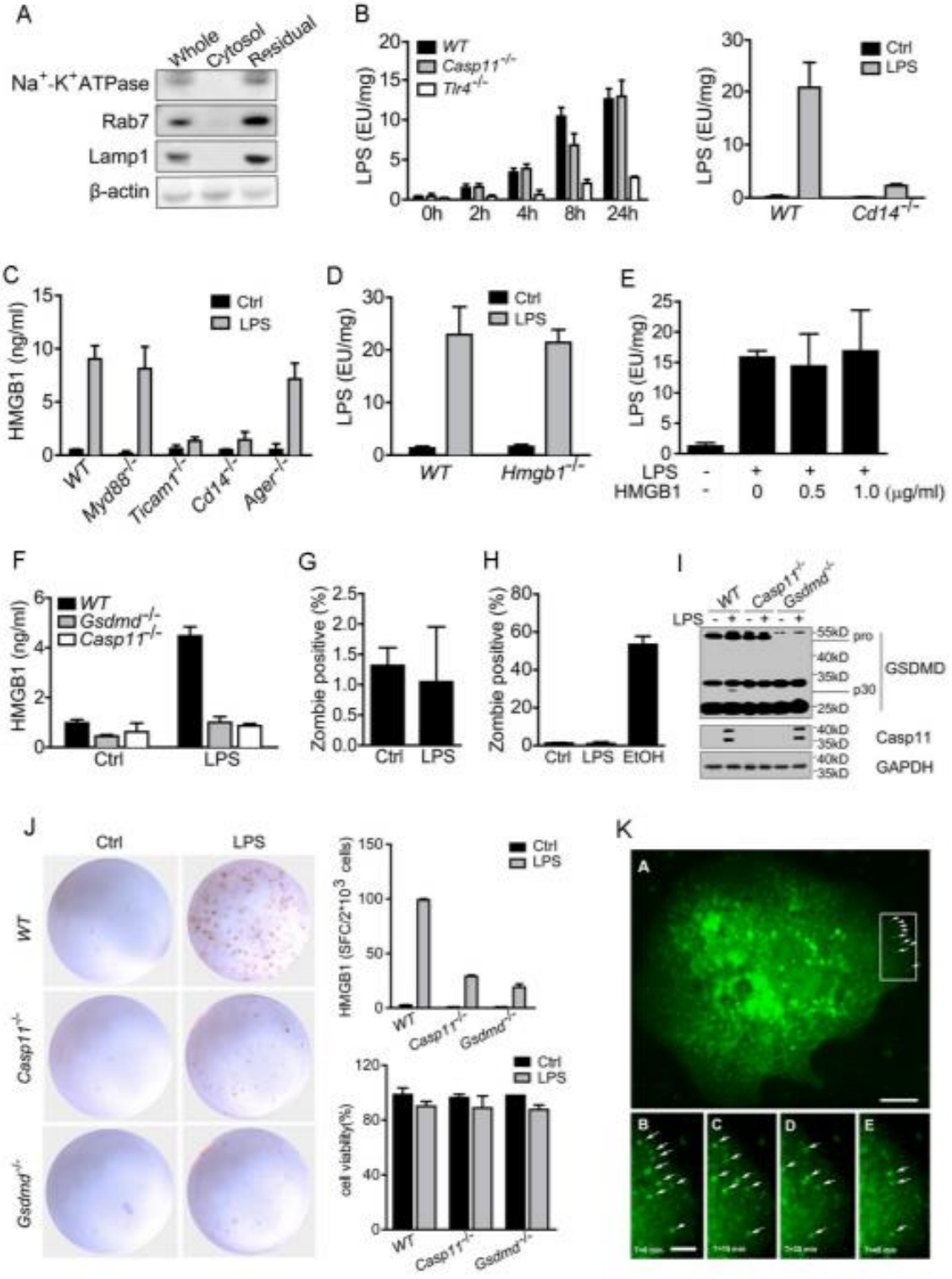
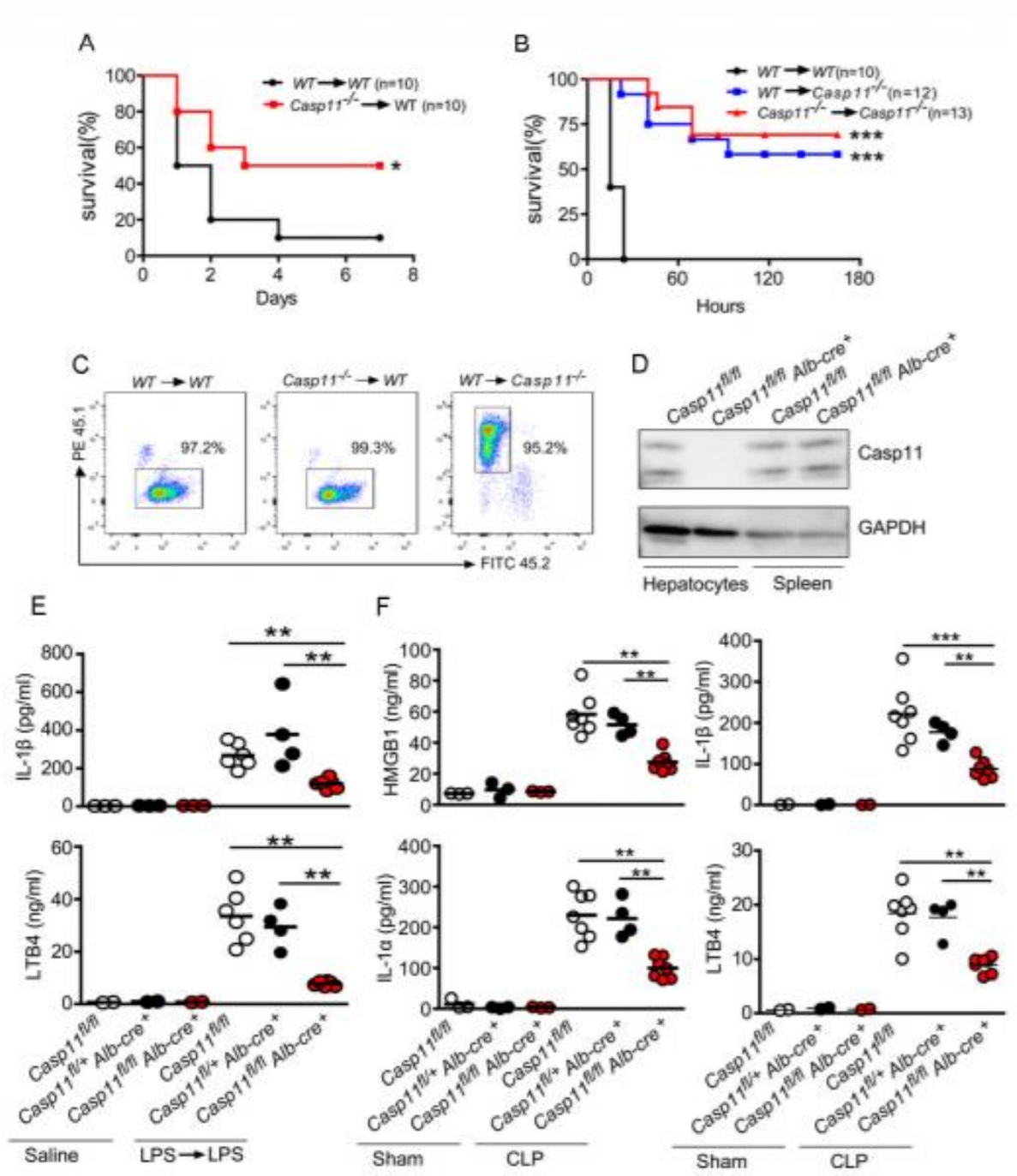

A
Whole
Cytosol
Residual
Na+-K+ATPase
Rab7
Lamp1
β-actin
B
WT
Casp11-/-
Tlr4-/-
LPS (EU/mg)
0h
2h
4h
8h
24h
Ctrl
LPS
WT
Cd14-/-
C
HMGB1 (ng/ml)
Ctrl
LPS
WT
Myd88-/-
Ticam1-/-
Cd14-/-
Ager-/-
D
LPS (EU/mg)
Ctrl
LPS
WT
Hmgb1-/-
E
LPS (EU/mg)
LPS
HMGB1
0
0.5
1.0 (μg/ml)
F
HMGB1 (ng/ml)
WT
Gsdmd-/-
Casp11-/-
Ctrl
LPS
G
Zombie positive (%)
Ctrl
LPS
H
Zombie positive (%)
Ctrl
LPS
EtOH
I
WT
Casp11-/-
Gsdmd-/-
LPS
55kD pro
40kD
35kD
p30
25kD
GSDMD
40kD
35kD
Casp11
40kD
35kD
GAPDH
J
Ctrl
LPS
WT
Casp11-/-
Gsdmd-/-
HMGB1 (SFC/2*10^3 cells)
cell viability(%)
K
A
B
C
D
E

Figure S7


A
survival(%)
Days
WT → WT (n=10)
Casp11-/- → WT (n=10)
B
survival(%)
Hours
WT → WT(n=10)
WT → Casp11-/-(n=12)
Casp11-/- → Casp11-/-(n=13)
C
WT → WT
Casp11-/- → WT
WT → Casp11-/-
PE 45.1
FITC 45.2
97.2%
99.3%
95.2%
D
Casp11fl/fl
Casp11fl/fl Alb-cre+
Casp11fl/fl
Casp11fl/fl Alb-cre+
Casp11
GAPDH
Hepatocytes
Spleen
E
IL-1β (pg/ml)
LTB4 (ng/ml)
Saline
LPS → LPS
F
HMGB1 (ng/ml)
IL-1β (pg/ml)
IL-1α (pg/ml)
LTB4 (ng/ml)
Sham
CLP
Sham
CLP