Immunity, Volume *54*

Supplemental Information

Heparin prevents caspase-11-dependent septic lethality independent of anticoagulant properties

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Supplemental information

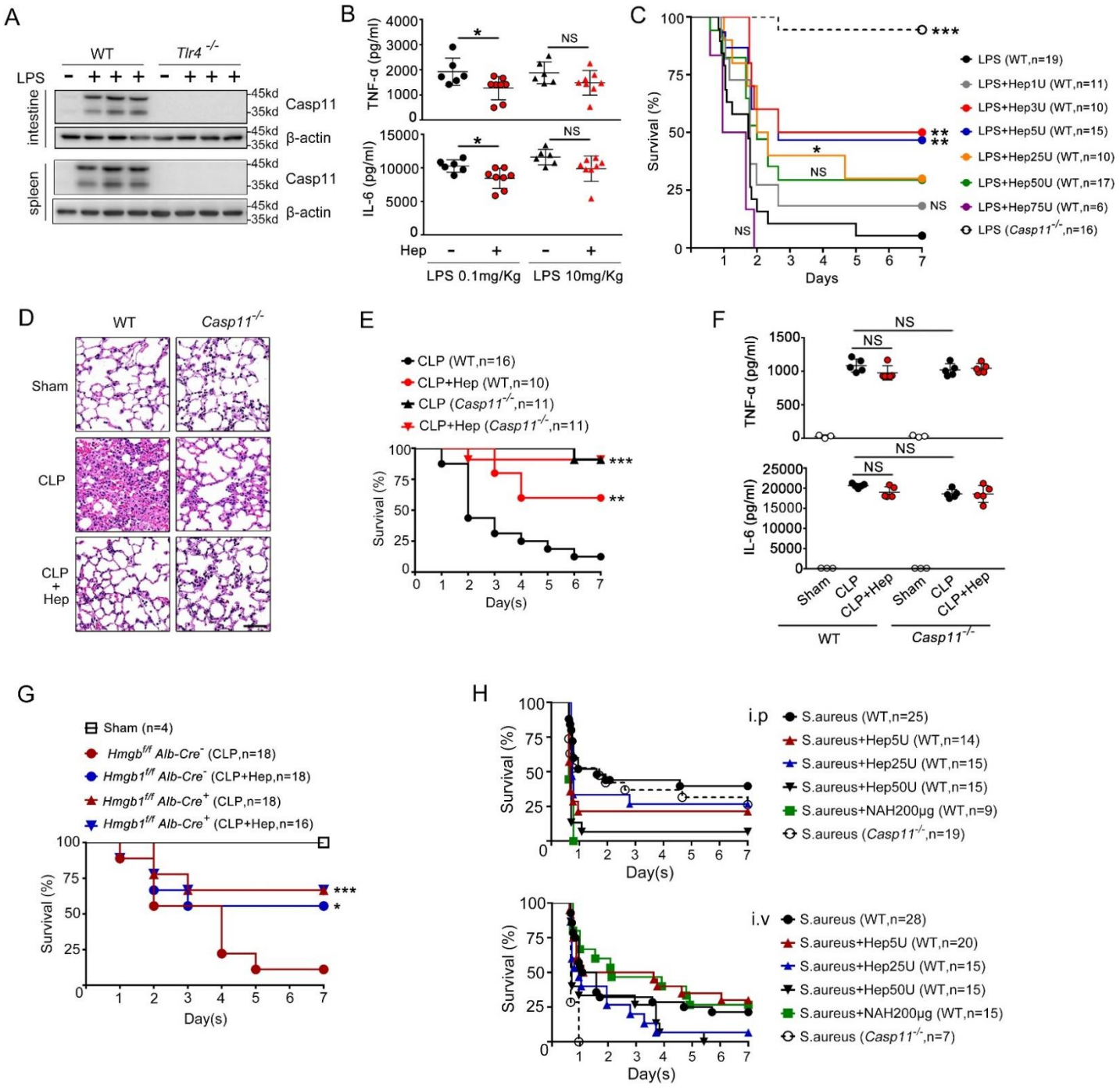

Figure S1. Heparin treatment prevents caspase-11-dependent immune responses and lethality in sepsis. Related to Figure 1.

(A) Immuno-blot to detect the casepase11 (Casp11) expression in intestine or spleen from WT or *Tlr4*$^{-/-}$ mice injected intraperitoneally with LPS (25mg/kg) or saline.

(B) Serum TNF-α and IL-6 concentrations from mice injected intraperitoneally with LPS 0.1mg/kg or 10mg/kg. Heparin (Hep, 5units/mouse) was administered subcutaneously 30min after LPS injection.

(C) Kaplan Meier survival curves from WT or *Casp11*$^{-/-}$ mice injected intraperitoneally with LPS (25mg/kg). Indicated dose of heparin (Hep) was administered subcutaneously 30min after LPS injection.

(D-F) Histopathological images of lung tissues *(D)*, Kaplan Meier survival curves *(E)* and Serum TNF-α and IL-6 concentrations (*F*) from WT or *Casp11*$^{-/-}$ mice subjected to either cecum ligation and puncture (CLP) or sham operation. Heparin (Hep, 5units/mouse) was administered subcutaneously 2h and 16h after CLP. Scale bar represents 50 μm.

(G) Kaplan Meier survival curves from mice of indicated genotypes subjected to either cecum ligation and puncture (CLP) or sham operation. Heparin (Hep, 5units/mouse) was administered subcutaneously 2h and 16h after CLP.

(H) Kaplan Meier survival curves from WT or *Casp11*$^{-/-}$ mice injected intraperitoneally (i.p left panel) with Staphylococcus aureus (S.aureus, 3×10^8 cfu) or intravenously (i.v right panel) with Staphylococcus aureus (S.aureus, 2×10^8 cfu). Indicated dose of heparin (Hep) or NAH was administered immediately after LPS injection.

Circles represent individual mice or patient. *P < 0.05; **P < 0.01; ***P < 0.001; NS: not significant (Two-way ANOVA test or Student's t-test and log-rank test for survival).

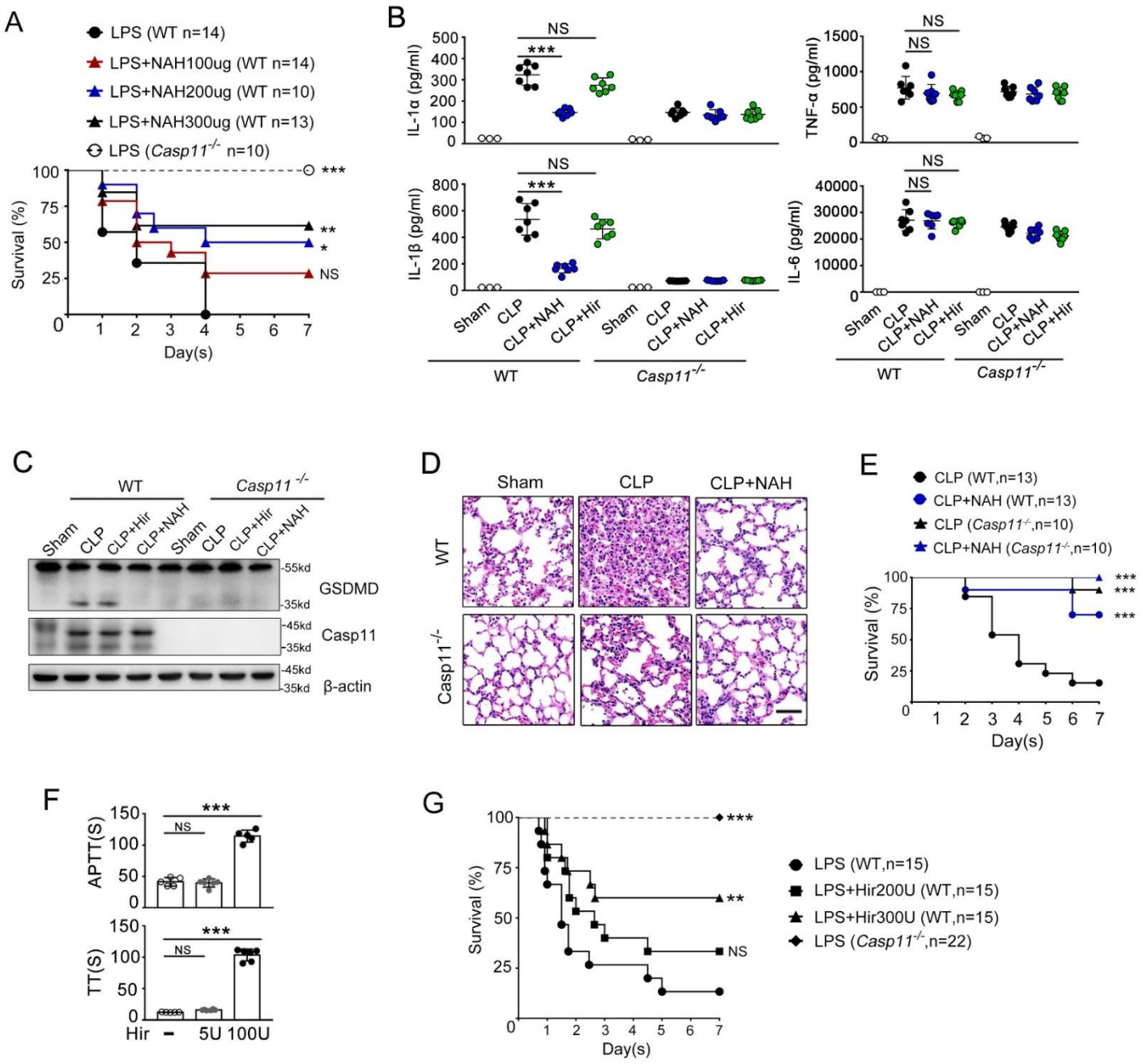

Figure S2. Heparin treatment inhibits caspase-11 activation in sepsis independent of its anticoagulant properties. Related to Figure 2.

(A) Kaplan Meier survival curves from WT or *Casp11*$^{-/-}$ mice injected intraperitoneally with LPS (25mg/kg). Indicated dose of NAH was administered subcutaneously 30min after LPS injection.

(B) Serum IL-1α, IL-1β, TNF-α and IL-6 concentrations from WT or *Casp11*$^{-/-}$ mice subjected to either cecum ligation and puncture (CLP) or sham operation. NAH (200μg

/mouse) was administered subcutaneously 1h after CLP. Hirudin (Hir, 300units/mouse) was administered subcutaneously 2h and 16h after CLP.

(C) Immuno-blot to detect the GSDMD cleavage and Casp11 expression in lung, from WT or *Casp11*$^{-/-}$ mice subjected to either cecum ligation and puncture (CLP) or sham operation. NAH was administered subcutaneously 1h after CLP. Hirudin was administered subcutaneously 2h and 16h after CLP.

(D-E) Histopathological images of lung tissues *(D)* and Kaplan Meier survival curves *(E)* from WT or *Casp11*$^{-/-}$ mice subjected to either cecum ligation and puncture (CLP) or sham operation. NAH (200μg /mouse) was administered subcutaneously 1h after CLP. Scale bar represents 50 μm.

(F) Plasma activated partial thromboplastin time (APTT) and thromboplastin time (TT) from WT mice administered subcutaneously with hirudin (5units/mouse or 100 units/mouse) for 12h.

(G) Kaplan Meier survival curves from WT or *Casp11*$^{-/-}$ mice injected intraperitoneally with LPS (25mg/kg). Indicated dose of Hirudin (Hir) was administered immediately after LPS injection.

Circles represent individual mice or patient. *P < 0.05; **P < 0.01; ***P < 0.001; NS: not significant (Two-way ANOVA test , one-way ANOVA test or Student's t-test and log-rank test for survival).

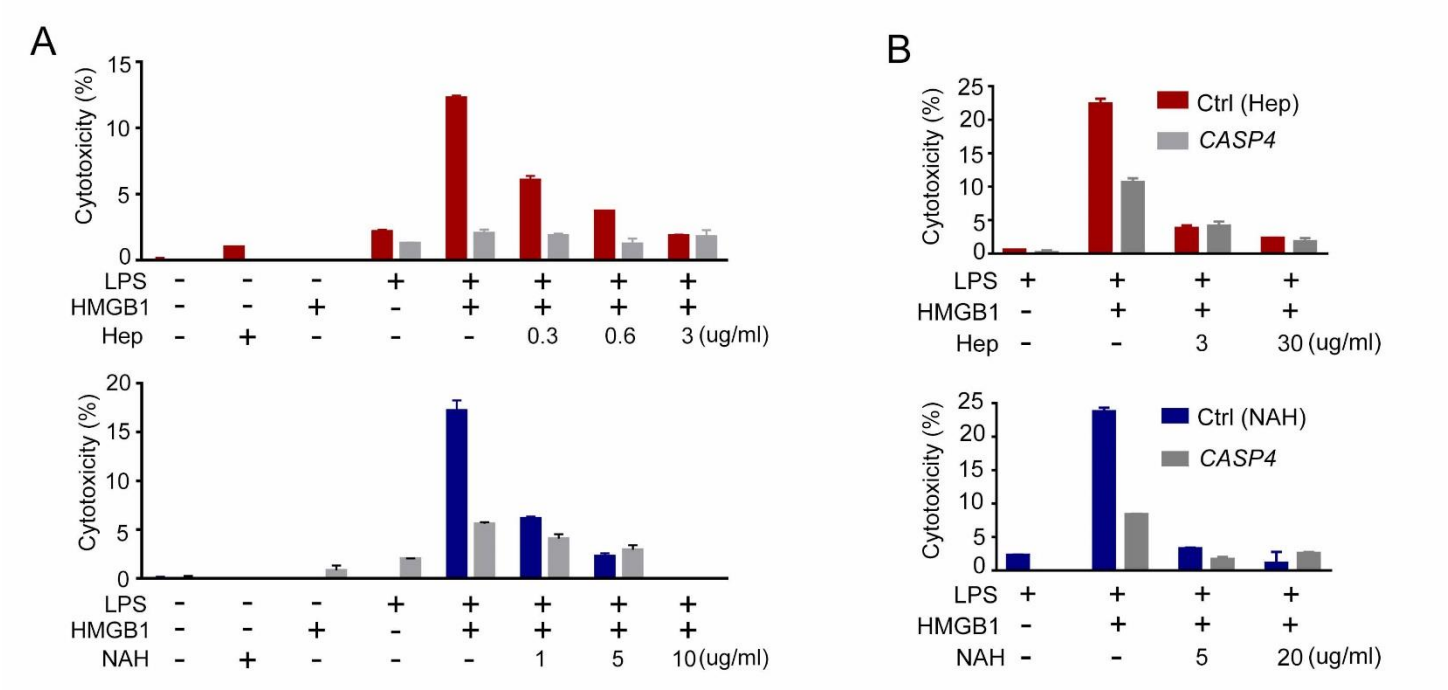

Figure S3. Heparin or NAH inhibits recombinant HMGB1- and caspase-11-dependent immune responses in vitro. Related to Figure 3.

(A) LDH assay in the supernatants of WT or *Casp11*$^{-/-}$ peritoneal macrophages stimulated with LPS alone (1μg/ml) or LPS (1μg/ml)+HMGB1 (400ng/ml) in the presence or the absence of indicated doses of heparin (Hep) or NAH for 16h.

(B) LDH assay in the supernatants of human monocytic THP-1 cells primed by PMA (100ng/ml) for 12h and then transfected with scrambled siRNA or CASP4-specific siRNA upon HMGB1 (400ng/ml) and LPS (1μg/ml) stimulation in the presence or the absence of indicated doses of heparin (Hep) or NAH for 16h.

Graphs show the mean ± SD of technical replicates and are representative of at least three independent experiments.

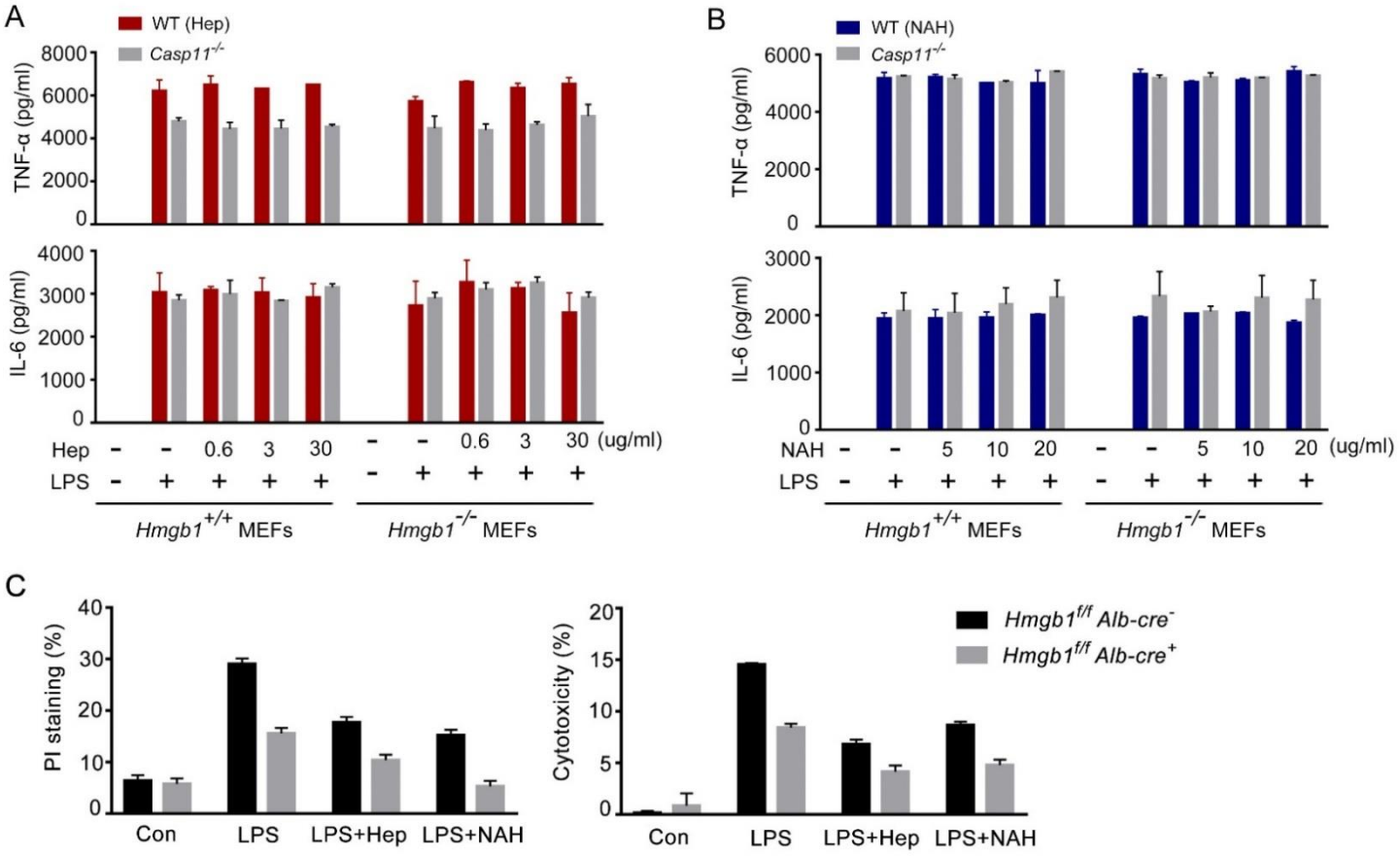

Figure S4. Heparin or NAH selectively inhibits endogenous HMGB1- and caspase-11-dependent immune responses in vitro. Related to Figure 4.

(A-B) ELISA for TNF-α and IL-6 in the supernatants of WT or *Casp11*$^{-/-}$ peritoneal macrophages stimulated with LPS alone (1μg/ml), LPS (1μg/ml)+*Hmgb1*$^{+/+}$ or *Hmgb1*$^{-/-}$ MEF cells in the presence or the absence of indicated doses of heparin (Hep, A) or NAH*(B)* for 16h.

(C) Macrophages PI staining and LDH assay in the supernatants from the macrophage-hepatocyte co-culture system (As shown in figure 4C).

Graphs show the mean ± SD of technical replicates and are representative of at least three independent experiments.

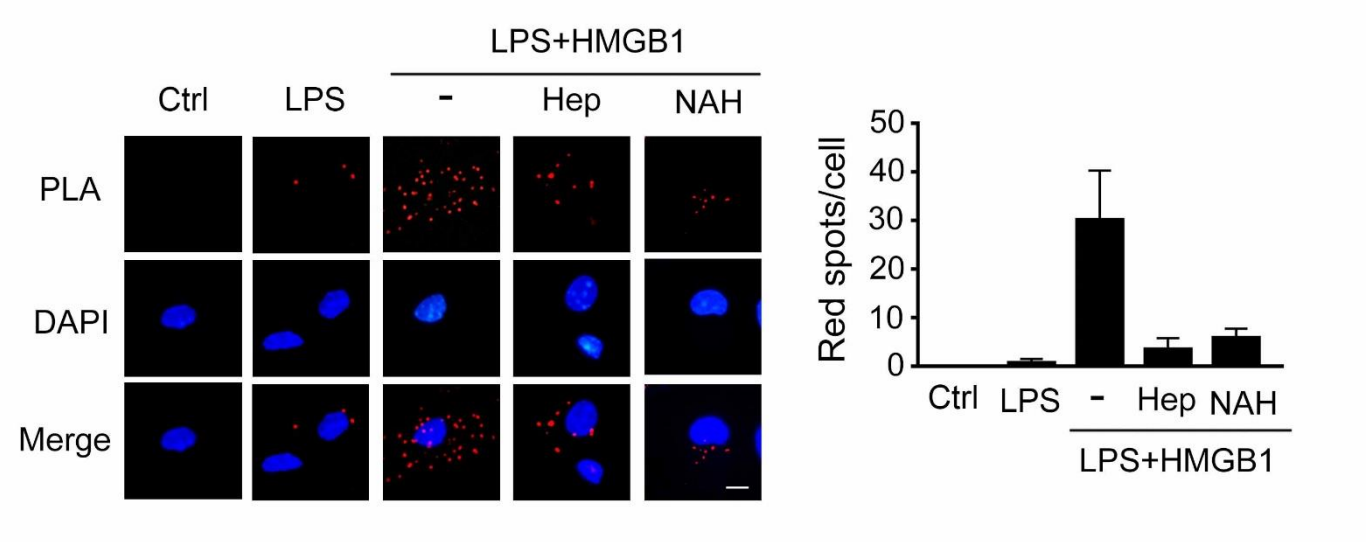

Figure S5. Heparin and NAH decreases the number of HMGB1-LPS complexes detected on the cell surface or inside cells. Related to Figure 6.

The physical interaction between HMGB1 and LPS were visualized as the red spots by PLA in mouse peritoneal macrophages stimulated with LPS alone (L, 5μg/ml) or LPS (5μg/ml)+HMGB1 (10μg/ml) (LH) in the presence or the absence of heparin (Hep, 3μg/ml) or NAH (10μg/ml) for 2h. Scale bar represents 10μm.

Graphs show the mean ± SD of technical replicates and are representative of at least three independent experiments.

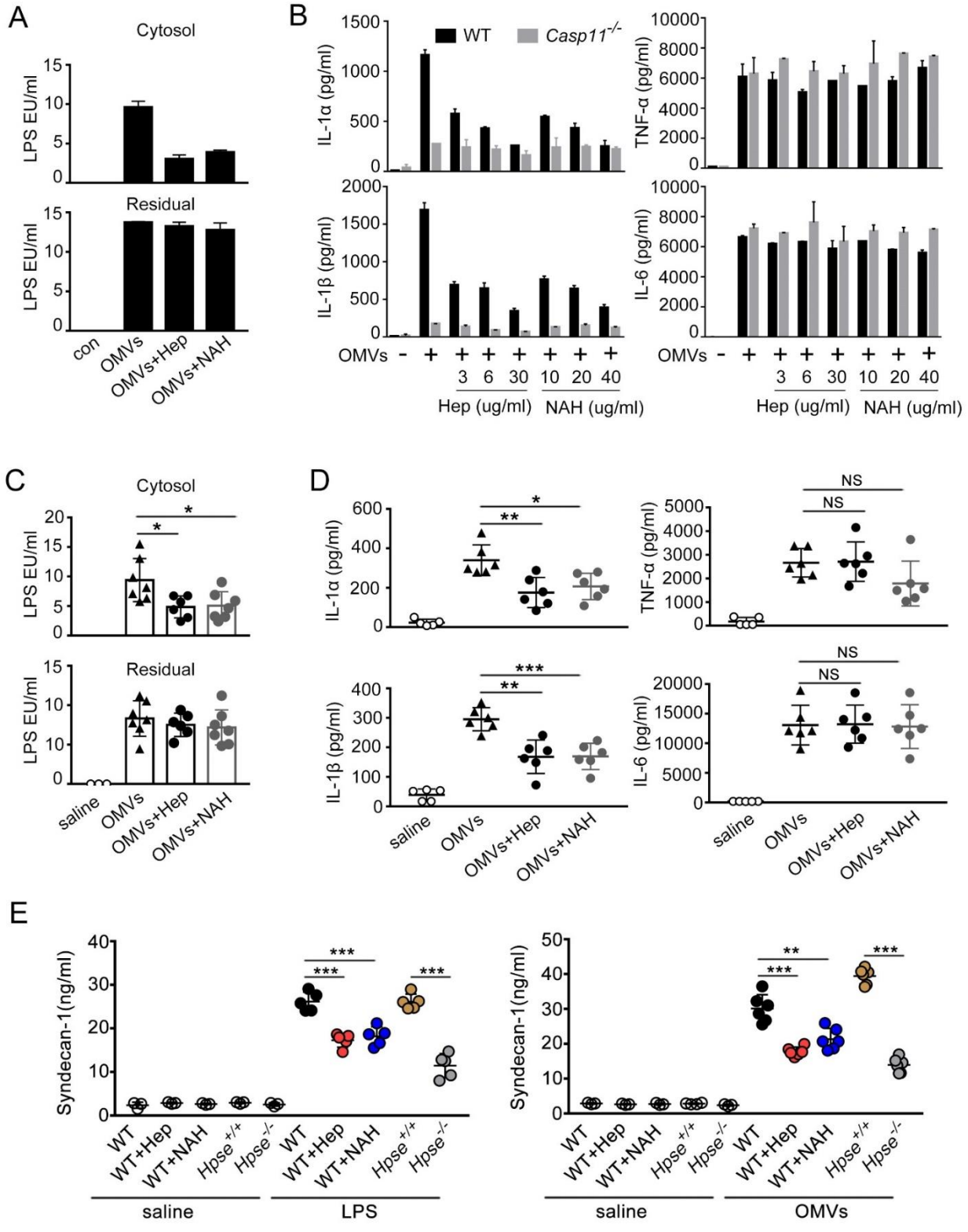

Figure S6. Heparin or NAH inhibits the cytosolic delivery of LPS and prevents heparanase-mediated glycocalyx degradation. Related to Figure 7.

(A) LPS activity assay in the cytosolic and residual fraction (including cytoplasmic

membranes, endosomes, lysosomes, nuclei, etc) from mouse peritoneal macrophages stimulated with OMVs (10µg /ml), in the presence or not of heparin (Hep, 12µg/ml) or NAH (40µg/ml) for 2h.

(B) ELISA for IL-1α, IL-1β, TNF-α and IL-6 in the supernatants of WT or

Casp11$^{-/-}$ mice peritoneal macrophages stimulated with OMVs (10µg/ml), in the presence or the absence of indicated doses of heparin (Hep) or NAH for 16h.

(C) LPS activity assay in the cytosolic and residual fraction of spleen cells from mice injected intraperitoneally with OMVs (200µg /mouse). Mice were first primed with 200µg Poly(I:C) for 6h. Heparin (Hep, 50units/mouse) or NAH (300µg/mouse) or was administered subcutaneously 30min after OMVs injection.

(D) Serum IL-1α, IL-1β, TNF-α and IL-6 concentrations of mice injected intraperitoneally with OMVs (200µg/mouse). Mice were first primed with 200µg Poly(I:C) for 6h.Heparin (Hep, 50units/mouse) or NAH (300µg/mouse) or was administered subcutaneously 30min after OMVs injection.

(E) Serum syndecan-1 concentrations from mice of indicated genotypes injected intraperitoneally with LPS (25mg/kg) or OMVs (200µg/mouse) with Poly(I:C) priming. Heparin or NAH was administered subcutaneously 30min after LPS or OMVs injection.

Graphs show the mean ± SD of technical replicates and are representative of at least three independent experiments. Circles represent individual mice or patient. *P < 0.05; **P < 0.01; ***P < 0.001; NS: not significant (Two-way ANOVA test, one-way ANOVA test or Student's t-test)

Supplementary Table S1 Patients Characteristics

Demographics	Sepsis			Non sepsis		
	All patients (41)	**With heparin (20)**	**Without heparin (21)**	**All patients (30)**	**With heparin (10)**	**Without heparin (20)**
ages (yrs, median(IQR)	60 (36,70)	64 (45,70)	54 (36,67)	52 (43,60)	50 (43,54)	55 (46,61)
male (n, %)	23 (56.10%)	13 (65.00%)	10 (47.62%)	10 (33.33%)	4 (40.00%)	6 (30.00%)
Source of Infection						
Abdominal (n, %)	12 (29.27%)	4 (20.00%)	8 (38.10%)	8 (26.67%)	1 (10.00%)	7 (35.00%)
Pulmonary (n, %)	11 (26.83%)	8 (40.00%)	3 (14.29%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
Urinary (n, %)	9 (21.95%)	3 (15.00%)	6 (28.57%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
Soft tissue (n, %)	8 (19.51%)	4 (20.00%)	4 (19.05%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
Others (n, %)	1 (2.44%)	1 (5.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
comorbidities						
≥2 (n, %)	14 (34.15%)	7 (35.00%)	7 (33.33%)	1 (3.33%)	1 (10.00%)	0 (0.00%)
None (n, %)	11 (26.83%)	5 (25.00%)	6 (28.57%)	20 (66.67%)	3 (30.00%)	17 (85.00%)
Diabetes (n, %)	8 (19.51%)	4 (20.00%)	4 (19.05%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
Trauma/Surgery (n,%)	6 (14.63%)	4 (20.00%)	2 (9.52%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
Cancer (n, %)	6 (14.63%)	2 (10.00%)	4 (19.05%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
Heart Failure (n, %)	8 (19.51%)	4 (20.00%)	4 (19.05%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
Kidney Disease (n, %)	12 (29.27%)	5 (25.00%)	7 (33.33%)	4 (13.33%)	4 (40.00%)	0 (0.00%)
Others (n, %)	1 (2.44%)	1 (5.00%)	0 (0.00%)	3 (10.00%)	1 (10.00%)	2 (10.00%)

Related to Figure 1