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

Highlights

- Heparin prevents caspase-11-dependent immune responses and lethality in sepsis
- Non-anticoagulant heparin prevents caspase-11-dependent coagulation and lethality
- Heparin inhibits caspase-11 activation by blocking cytosolic delivery of LPS
- Glycocalyx degradation, prevented by heparin, promotes cytosolic delivery of LPS

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In Brief

Caspase-11, a cytosolic receptor of LPS, triggers lethal immune responses in sepsis. Tang et al. reveal that heparin prevents cytosolic delivery of LPS and caspase-11 activation in sepsis through inhibiting the heparanase-mediated glycocalyx degradation and the HMGB1-LPS interaction.

Tang et al., 2021, Immunity *54*, 1–14
March 9, 2021
https://doi.org/10.1016/j.immuni.2021.01.007

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

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https://doi.org/10.1016/j.immuni.2021.01.007

SUMMARY

Heparin, a mammalian polysaccharide, is a widely used anticoagulant medicine to treat thrombotic disorders. It is also known to improve outcomes in sepsis, a leading cause of mortality resulted from infection-induced immune dysfunction. Whereas it is relatively clear how heparin exerts its anticoagulant effect, the immunomodulatory mechanisms enabled by heparin remain enigmatic. Here, we show that heparin prevented caspase-11-dependent immune responses and lethality in sepsis independent of its anticoagulant properties. Heparin or a chemically modified form of heparin without anticoagulant function inhibited the alarmin HMGB1-lipopolysaccharide (LPS) interaction and prevented the macrophage glycocalyx degradation by heparanase. These events blocked the cytosolic delivery of LPS in macrophages and the activation of caspase-11, a cytosolic LPS receptor that mediates lethality in sepsis. Survival was higher in septic patients treated with heparin than those without heparin treatment. The identification of this previously unrecognized heparin function establishes a link between innate immune responses and coagulation.

INTRODUCTION

Heparin is a mammalian polysaccharide with anticoagulant properties (Hirsh and Levine, 1992). Since its discovery in 1916 and initial introduction into clinical practice in the late 1930's, heparin has been extensively used to treat various thrombotic disorders, such as venous thromboembolism (Hirsh and Levine, 1992; Li and Ma, 2017). Heparin exerts its anticoagulant effect by binding to the lysine residue in antithrombin, thereby inducing a non-reversible conformational change at the arginine-reactive site (Rezaie et al., 2004; Yang et al., 2004). This leads to over a hundred-fold increase in antithrombin activity (Chuang et al., 2001; Rezaie et al., 2004). In addition to thrombotic diseases, accumulated evidence from basic research and clinical practice implicate that heparin treatment improves the outcome of sepsis (Cornet et al., 2007; Fan et al., 2016; Li and Ma, 2017; Li et al., 2011; Liu et al., 2014; Wang et al., 2014; Zarychanski et al., 2015), a leading cause of mortality defined as infection-induced critical illness with organ dysfunction (Angus and van der Poll, 2013). Though it is assumed that heparin exerts beneficial effects in sepsis through its anticoagulant properties, there is no unanimous evidence to support this notion.

Recent advances reveal that the caspase-11 signaling plays a key role in septic lethality (Cheng et al., 2017; Deng et al., 2018; Kayagaki et al., 2015; Kayagaki et al., 2011). Caspase-11 is expressed in various types of cells, such as macrophages and endothelial cells (Cheng et al., 2017; Deng et al., 2018). Upon activation by intracellular bacterial endotoxin (lipopolysaccharide: LPS), the major cell-wall component of Gram-negative bacteria, caspase-11 enzymatically cleaves gasdermin D (GSDMD) into peptides that form nano-pores in the cytoplasmic membrane (Ding et al., 2016; He et al., 2015; Kayagaki et al., 2015; Shi et al., 2015). This event either leads to pyroptosis, a lytic form of programmed cell death or renders viable cells hyperactive for the release of interleukin-1 (IL-1) (Ding et al., 2016; Evavold et al., 2018; He et al., 2015; Zanoni et al., 2016). Formation of GSDMD pores also triggers systemic activation of coagulation cascades, which contribute to the septic lethality (Wu et al., 2019; Yang et al., 2019). Mechanistically, GSDMD pore-mediated calcium influx activates the Transmembrane protein 16F (TMEMF16), a transmembrane phospholipid scramblase that induces phosphatidylserine (PS) exposure. This, in turn, markedly enhances the pro-coagulant activity of tissue factor (TF) and activation of coagulation cascades (Wu et al., 2019; Yang et al., 2019). Activation of

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caspase-11 and GSDMD in endotoxemia or sepsis is promoted by high mobility group box 1 protein (HMGB1). LPS triggers the release of HMGB1 from hepatocytes, the major source of plasma HMGB1 in sepsis (Deng et al., 2018). Circulating HMGB1 binds extracellular LPS and mediates the cytosolic delivery of LPS into the cytoplasm of myeloid or endothelial cells through receptor for advanced glycation end-products (RAGE)-dependent internalization followed by HMGB1-induced lysosomal rupture. This culminates in the activation of caspase-11 and GSDMD cleavage (Deng et al., 2018). Deletion of HMGB1 in hepatocytes, neutralizing extracellular HMGB1, loss of caspase-11, or deficiency in GSDMD uniformly leads to improved survival in experimental sepsis (Cheng et al., 2017; Deng et al., 2018).

In the course of studying how HMGB1 binds LPS, we found that the polysaccharide motif of LPS is important for HMGB1-LPS interaction. As a mammalian polysaccharide, heparin was also able to physically interact with HMGB1. Together with our recent findings that HMGB1 mediates caspase-11-dependent septic lethality (Deng et al., 2018), we postulated that heparin might prevent HMGB1- and caspase-11-dependent lethality in sepsis. Here, we found that heparin treatment improves the outcome of sepsis through inhibition of the caspase-11 signaling. The minimum dose of heparin that efficiently inhibits caspase-11-dependent immune response is much lower than that required to inhibit coagulation. By using chemically modified non-anticoagulant heparin (NAH), we show that NAH treatment could prevent caspase-11-dependent disseminated intravascular coagulation (DIC) in sepsis. Mechanistically, both heparin and NAH bind to HMGB1, inhibit the HMGB1-LPS interaction, and prevent the macrophage glycocalyx degradation by heparanase. These events blocked the cytosolic delivery of LPS, resulting in diminished activation of caspase-11. Thus, this study identifies a biological function of heparin and provides proof-of-concept that heparin prevents caspase-11-dependent coagulation activation and lethality in sepsis independent of its direct anticoagulant properties.

RESULT

Heparin prevents caspase-11-dependent immune responses and lethality in sepsis

To test whether heparin could prevent HMGB1- and caspase-11-dependent immune responses and lethality in sepsis, wild-type (WT) and caspase-11 deficient (*Casp11*$^{-/-}$) mice were subjected to lethal endotoxemia and treated with control vehicle or heparin in parallel. We observed that heparin treatment almost completely blocked the release of IL-1α and IL-1β as well as GSDMD cleavage in the lung after LPS challenge to similar degree seen with caspase-11 deficiency or depletion of HMGB1 in hepatocytes (Figures 1A and 1B). Heparin did not affect toll-like receptor 4 (TLR4)-dependent caspase-11 expression (Figure S1A). Further, heparin treatment or caspase-11 deficiency did not alter the release of tumor necrosis factor (TNF) or interleukin-6 (IL-6) during lethal endotoxemia (Figure 1A; Figure S1B). Administration of heparin or deletion of caspase-11 attenuated lung injury and markedly promoted survival in lethal endotoxemia (Figures 1C and 1D). The optimum dose of heparin for the protection against LPS lethality was 2 to 5 unit/mouse (Figure S1C). Similar observations were made in a clinically relevant murine model of bacterial sepsis induced by the surgical procedure called cecum ligation and puncture (CLP) (Figures 1E and 1F; Figures S1D–S1F). We previously show that hepatocyte-released HMGB1 is critical for caspase-11-dependent immune responses and lethality after CLP (Deng et al., 2018). Consistently, depletion of hepatocyte HMGB1 conferred protection against CLP-induced lethal sepsis (Figure S1G). Heparin treatment significantly promoted survival in WT but not hepatocyte HMGB1-deficient mice (Figure S1G). However, caspase-11 deficiency or heparin treatment failed to confer protection against sepsis caused by *Staphylococcus aureus*, a type of Gram-positive bacterium (Figure S1H). These findings suggest heparin prevents caspase-11-dependent immune responses and lethality in Gram-negative sepsis.

We then tested whether heparin treatment is associated with attenuated caspase-4 (the human ortholog of caspase-11 (He et al., 2015) activation in sepsis, and measured plasma concentrations of IL-1α and IL-1β, markers of caspase-4 activation, in 20 septic patients that received heparin treatment and 21 septic patients without heparin treatment (patient characteristics are shown in Table S1). Notably, plasma concentrations of IL-1α and IL-1β in patients treated with heparin were significantly lower than those in patients without heparin treatment (Figure 1G). In agreement with previous studies (Cornet et al., 2007; Li and Ma, 2017; Li et al., 2011), heparin treatment in septic patients was associated with an improved survival (Figure 1G). Taken together, these findings demonstrate that heparin prevents caspase-11-dependent immune responses and lethality in endotoxemia and bacterial sepsis.

Heparin inhibits caspase-11 activation independent of its anticoagulant properties

We observed that heparin at a relatively low dose (5 U/mouse or 28 μg/mouse) markedly reduced caspase-11-dependent immune responses without affecting the activated partial thromboplastin time (APTT) and the prothrombin time (PT) (Figure 2A), which are the indicators of systemic coagulant activity. These observations suggest that heparin might prevent caspase-11-dependent immune responses and lethality in a mechanism independent of its anticoagulant properties. To test this possibility, experiments were performed using sulfated heparin, a chemically modified heparin that does not exert anticoagulant properties (Yang et al., 2019). Sulfated non-anticoagulant heparin (NAH) dose-dependently blocked caspase-11-mediated immune responses and animal lethality in endotoxemia without affecting the plasma concentrations of TNF or IL-6 (Figure 2B and 2E; Figure S2A). Similar observations were made in cecal ligation puncture (CLP)-induced bacterial sepsis (Figures S2B–S2E). Further, administration of hirudin, a well-known anticoagulant that markedly increased the APTT and PT (Figure S2F), failed to reduce the release of IL-1α and IL-1β during endotoxemia or bacterial sepsis (Figure 2B; Figure S2B). These observations indicate that heparin inhibits caspase-11-dependent immune responses and lethality in sepsis independent of its anticoagulant properties.

We and others recently have shown that caspase-11 activates the lethal coagulation cascades through GSDMD during endotoxemia and bacterial sepsis (Wu et al., 2019; Yang et al., 2019). In line with these observations, deletion of Caspase-11

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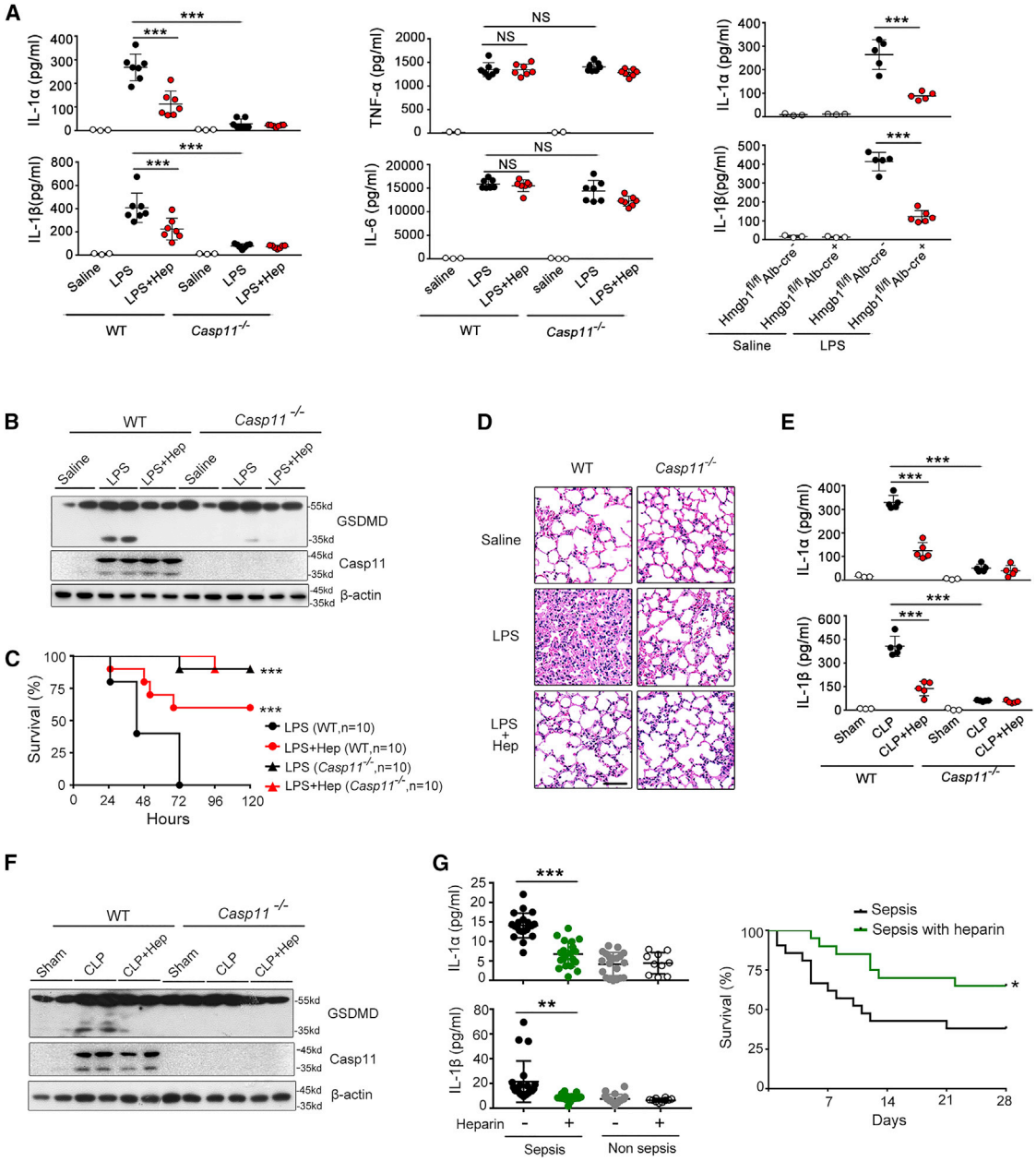

Figure 1. Heparin prevents caspase-11-dependent immune responses and lethality in sepsis

(A–D) Serum IL-1α, IL-1β, TNF-α, and IL-6 concentrations (A), immuno-blot to detect the GSDMD cleavage and caspase11 (Casp11) expression in lung (B), Kaplan Meier survival curves (C), and histopathological images of lung tissues (D) from mice of indicated genotypes injected intraperitoneally with LPS (25mg/kg) or saline. Heparin 5units/mouse (Hep) was administered subcutaneously 30min after LPS injection. Scale bar represents 50 μm.

(E and F) Serum IL-1α and IL-1β concentrations (E), immuno-blot to detect the GSDMD cleavage and caspase11 (Casp11) expression in lung (F) from WT or Casp11$^{-/-}$ mice subjected to either cecum ligation and puncture (CLP) or sham operation. Heparin 5units/mouse (Hep) was administered subcutaneously 2 h and 16 h after CLP.

(G) Serum IL-1α and IL-1β concentrations, Kaplan Meier survival curves from septic patient with or without heparin treatment.

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).

See also Figure S1 and Table S1.

or prevention of coagulation by hirudin administration reduced organ injury and lethality in lethal endotoxemia (Figure S2G). To confirm that heparin prevents caspase-11-mediated lethality independent of its direct anticoagulant properties, we tested whether non-anticoagulant heparin (NAH) treatment could prevent LPS-induced coagulation. The activation of the coagulation

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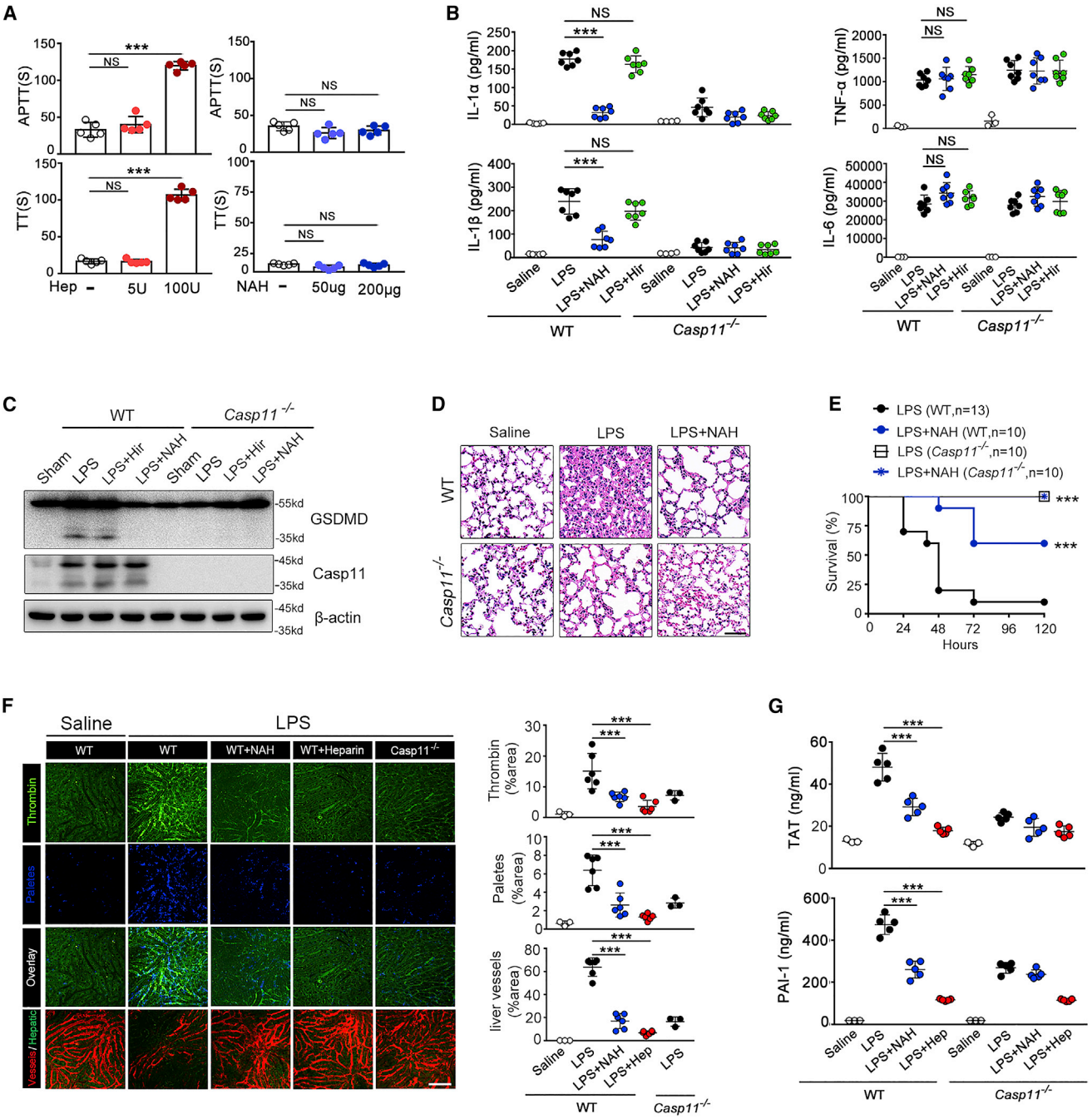

Figure 2. Heparin inhibits caspase-11 activation independent of its anticoagulant properties

(A) Plasma activated partial thromboplastin time (APTT) and thromboplastin time (TT) from WT mice administered subcutaneously with Heparin (Hep, 5units/mouse or 100 units/mouse) or non-anticoagulant heparin (NAH, 50 μg/mouse or 200 μg/mouse).

(B) Serum IL-1α and IL-1β concentrations (B) and histopathological images of lung tissues (D) from WT or *Casp11*$^{-/-}$ mice injected intraperitoneally with LPS (25mg/kg) or saline. NAH (200 μg/mouse) or Hirudin (Hir, 300units/mouse) was administered subcutaneously 30min after LPS injection.

(C) Immuno-blot to detect the GSDMD cleavage and Casp11 expression in lung from WT or *Casp11*$^{-/-}$ mice injected intraperitoneally with LPS (25mg/kg) or saline. NAH or Hirudin was administered subcutaneously 30 min after LPS injection.

(D and E) Histopathological images of lung tissues (D) and Kaplan Meier survival curves (E) from WT or *Casp11*$^{-/-}$ mice injected intraperitoneally with LPS (25mg/kg). NAH (200 μg/mouse) was administered subcutaneously 30min after LPS injection. Scale bar represents 50 μm.

(F) WT or *Casp11*$^{-/-}$ mice were injected intraperitoneally with LPS (10mg/kg) or saline. NAH (200 μg/mouse) or low molecular Heparin (200units/kg) was administered subcutaneously 30 min before LPS injection. Representative SD-IVM images of thrombin (green), platelet adhesion (blue, AF647 anti-CD49b), and Dextran (red, AF594-albumin) within the liver microvasculature at 6 h after LPS challenge of two independent experiments. Texas Red labeled Dextran (red) was regarded as a contrast material to identify perfused versus occluded vessels. Quantitative analysis of thrombin, platelets, and occluded vessels fluorescence

(legend continued on next page)

cascades within the liver vasculatures was visualized through the systemic introduction of the internally quenched 5-FAM/QXL-520 fluorescence resonance energy transfer (FRET) substrate of thrombin under an intravital microscope. Cleavage of the substrate by thrombin leads to the generation of a green fluorescence signal within the liver vasculature sinusoids (Figure 2F). We observed that NAH treatment markedly reduced thrombin activation after LPS challenge in a manner similar to that of caspase-11 deficiency (Figure 2F). Intravital microscopy revealed that endotoxemia also induced a profound aggregation of platelets, deposition of fibrin and occlusion of microcirculation in the liver, which were all markedly attenuated by NAH treatment (Figure 2F).

Systemic activation of the coagulation cascades in sepsis leads to the consumption of fibrinogen and the increase of plasma D-dimer, which is formed by plasmin degradation of fibrin (Koyama et al., 2014). Circulating markers of systemic activation of coagulation include the increases thrombin-antithrombin (TAT) complexes formed during coagulation and increases in concentrations of plasminogen activator inhibitor type-1 (PAI-1), an inhibitor of fibrinolysis (Gando et al., 2013). We observed that NAH treatment prevented LPS-induced increase in plasma TAT complexes, D-dimer and PAI-1 in a manner similar to that of caspase-11 deficiency (Figure 2F). Taken together, these data demonstrate that NAH treatment prevents LPS-induced coagulation in a caspase-11-dependent manner.

Heparin selectively inhibits HMGB1- and caspase-11-dependent immune responses *in vitro*

We previously have shown that HMGB1 enables extracellular LPS to activate caspase-11 in macrophages, which are critical for the activation of coagulation cascades during endotoxemia (Deng et al., 2018; Yang et al., 2020). To further confirm that heparin inhibits caspase-11 activation independent of its anticoagulant properties, we cultured mouse peritoneal macrophages in medium without pro-coagulant factors (including calcium, factor IV). Consistent with our previous findings, recombinant HMGB1 protein with LPS induced a robust release of IL-1α, IL-1β, and lactate dehydrogenase (LDH) as well as GSDMD cleavage in WT but not caspase-11-deficient peritoneal mouse macrophages. Caspase-11-dependent immune responses and GSDMD cleavage were dose-dependently inhibited by heparin (Figure 3A; Figure S3A). Similar observations were made using NAH (Figure 3A; Figure S3A). By contrast, neither heparin nor NAH affected the HMGB1- and LPS-induced release of TNF and IL-6 (Figure 3B). Immunoblot analysis revealed that neither heparin nor NAH altered LPS + HMGB1-induced expression of IL-1α or pro-IL-1β (Figure 3A). Further, neither heparin nor NAH affected the release of IL-1α and IL-1β induced by ATP, nigericin, Monosodium Urate Crystals (MSU), or silicon crystals (SiO_2) (Figure 3C), all of which are known activators of the nucleotide-Binding Oligomerization Domain or Leucine Rich Repeat and Pyrin Domain Containing 3 (NLRP3) inflammasomes (Lu et al., 2012). We next examined whether heparin could inhibit HMGB1- and caspase-4-dependent immune responses in human cells. Consistent with our previous findings (Deng et al., 2018), silencing of caspase-4 by shRNA in human monocytic THP-1 cells blocked LPS-induced release of IL-1α, IL-1β, and LDH in the presence of recombinant HMGB1 (Figure 3D; Figure S3B). The recombinant HMGB1- and caspase-4-dependent immune responses were abrogated by addition of heparin or NAH to cell-culture medium (Figure 3D; Figure S3B).

To confirm that heparin inhibits caspase-11 activation induced by HMGB1 and LPS *in vitro*, we stimulated mouse peritoneal macrophages with LPS and necrotic cell lysate from *Hmgb1*$^{+/+}$ or *Hmgb1*$^{-/-}$ mouse embryonic fibroblasts (MEFs). Consistent with our previous finding (Deng et al., 2018), necrotic cell lysate derived from *Hmgb1*$^{+/+}$ MEFs but not *Hmgb1*$^{-/-}$ MEFs enabled LPS to induce robust release of IL-1α and IL-1β as well as GSDMD cleavage in a caspase-11-dependent manner (Figure 4A). In this context, addition of heparin dose-dependently inhibited the release of IL-1α and IL-1β and blocked the cleavage of GSDMD (Figure 4A). Heparin addition did not alter the expression of IL-1α, IL-1β, caspase-11, and GSDMD, nor did it inhibit the release of TNF and IL-6 (Figure 4A; Figures S4A and S4B), further supporting the notion that heparin treatment does not affect inflammatory responses independent of caspase-11 or the priming step of inflammasome activation. Similar observations were made when we added NAH to the macrophages culture medium (Figure 4B; Figures S4A and S4B). Hepatocytes are the major source of circulating HMGB1 that drives the activation of caspase-11 in endotoxemia and bacterial sepsis (Deng et al., 2018). To determine whether heparin inhibits caspase-11 activation driven by hepatocyte-released HMGB1 *in vitro*, we co-cultured *Hmgb1*$^{+/+}$ or *Hmgb1*$^{-/-}$ mouse hepatocytes with mouse peritoneal macrophages. Consistent with our previous study (Deng et al., 2018), hepatocytes-derived HMGB1 was critical for LPS-induced the release of IL-1α and IL-1β from macrophages (Figure 4C). These caspase-11-dependent immune responses and GSDMD pore formation were blocked by addition of heparin or NAH to the cell-culture medium (Figure 4C; Figure S4C). As dead macrophages were fewer than propidium iodide (PI)-positive cells (Figure S4C), it is likely that heparin or NAH inhibited HMGB1-dependent macrophage pyroptosis and hyperactivation. Taken together, these data demonstrate that heparin selectively inhibited HMGB1 and caspase-11-dependent immune responses independent of its anticoagulant properties.

Heparin inhibits caspase-11 activation by inhibiting HMGB1-mediated cytosolic delivery of LPS

Next, we investigated the mechanisms by which heparin inhibits HMGB1- and caspase-11-dependent immune responses. Using

within the liver microcirculation by ImageJ software. The proportion of occluded vessels in liver was quantified per field of view in endotoxemic WT versus *Casp11*$^{-/-}$ mice. Scale bar represents 100 μm.

(G) Plasma concentrations of TAT complexes and PAI-1 were measured at 8 h (the peak time of thrombin generation, data not show) in mice injected with LPS (25 mg/kg) with or without NAH (200 μg /mouse) or low molecular Heparin (200 units/kg) administration.

Circles represent individual mice. *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). See also Figure S2.

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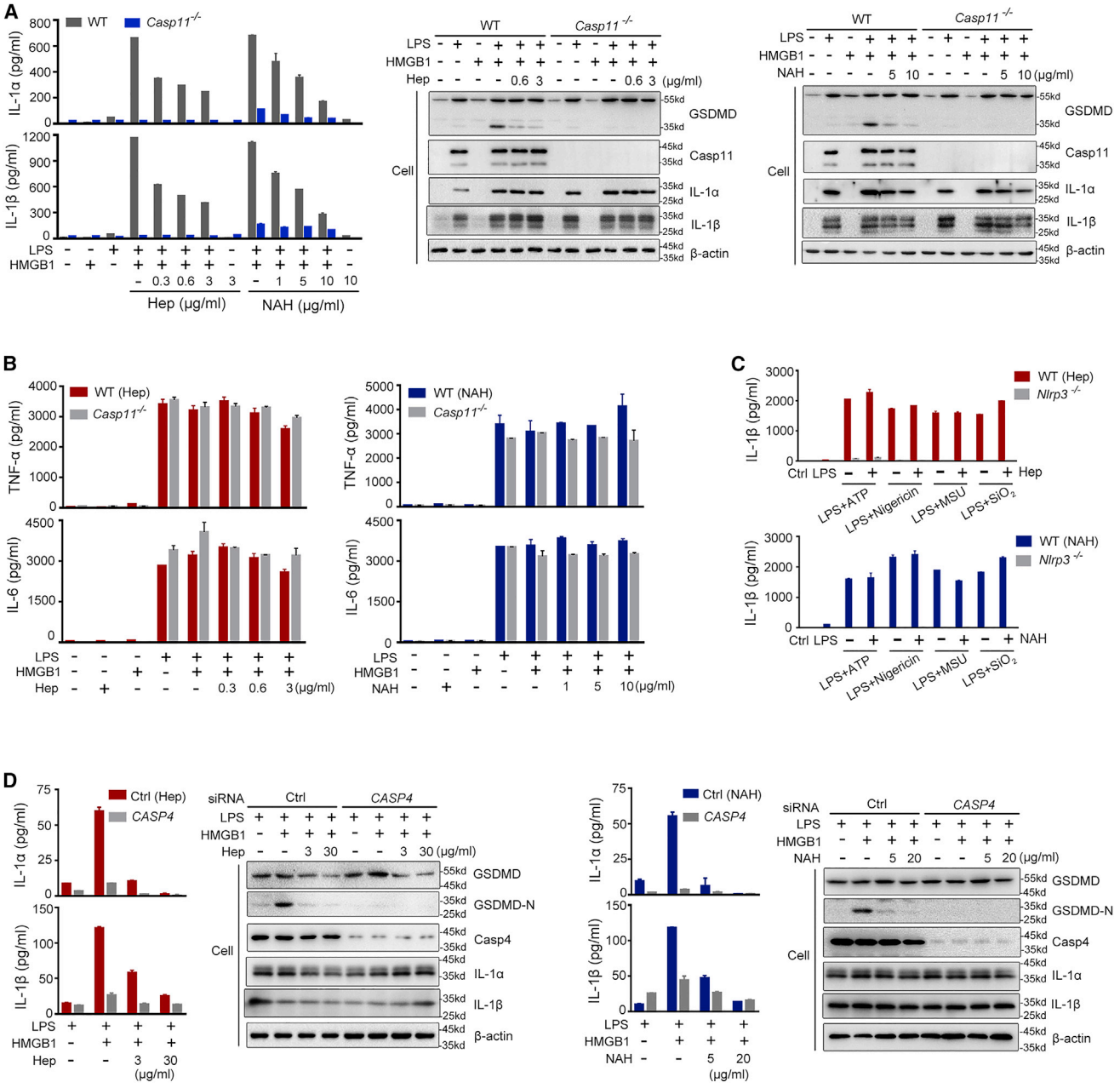

Figure 3. Heparin selectively inhibits recombinant HMGB1- and caspase-11-dependent immune responses *in vitro*

(A) ELISA for IL-1α and IL-1β in the culture supernatants, immuno-blots for GSDMD cleavage and caspase11 (Casp11), IL-1α and IL-1β expression in the cell lysates of WT or *Casp11*$^{-/-}$ mouse peritoneal macrophages stimulated with LPS alone (1 μg/mL) or LPS (1 μg/mL)+HMGB1 (400ng/mL) in the presence or the absence of indicated doses of heparin (Hep) or NAH for 16 h.

(B) ELISA for TNF-α and IL-6 in the supernatants of WT or *Casp11*$^{-/-}$ mouse peritoneal macrophages stimulated with LPS alone (1 μg/mL) or LPS (1 μg/mL)+HMGB1 (400ng/mL) in the presence or not of indicated doses of heparin or NAH for 16 h.

(C) ELISA for IL-1α and IL-1β in the supernatants of LPS-primed mouse peritoneal macrophages stimulated as indicated, in the presence or the absence of heparin (Hep, 3 μg/mL) or NAH (10 μg/mL) for 16 h.

(D) ELISA for IL-1α and IL-1β in the supernatants, immuno-blot to detect GSDMD cleavage (GSDMD-N) and caspase4 (Casp4), IL-1α, and IL-1β expression in cell lysates of human monocytic THP-1 cells primed by PMA(100ng/mL) for 12 h and then transfected with scrambled siRNA or CASP4-specific siRNA upon HMGB1 (400ng/mL) and LPS (1 μg/mL) stimulation in the presence or not of indicated doses of heparin (Hep) or NAH for 16 h.

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

See also Figure S3.

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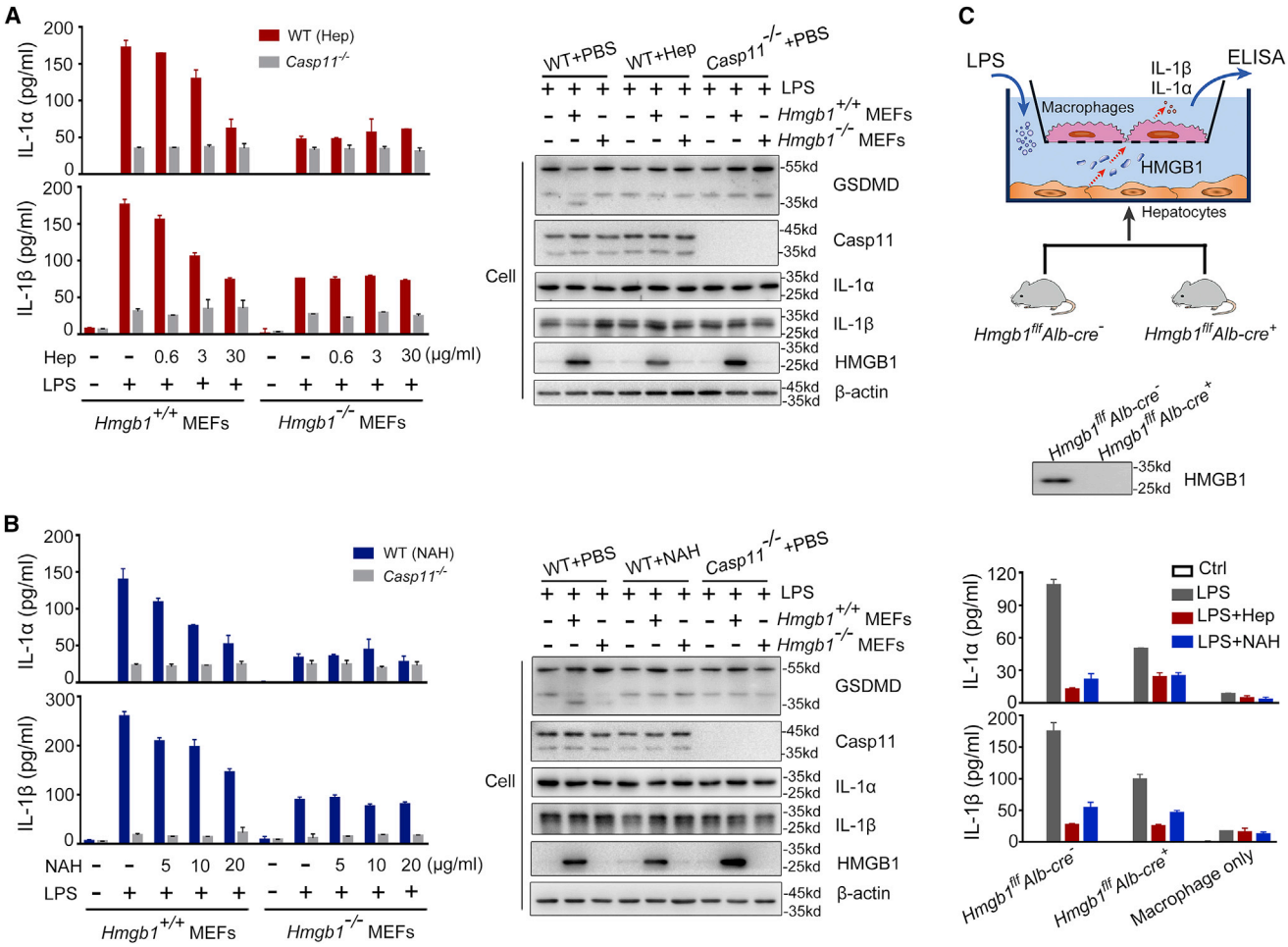

Figure 4. Heparin inhibits endogenous HMGB1- and caspase-11-dependent immune responses *in vitro*.

(A and B) ELISA for IL-1α and IL-1β in the supernatants, immuno-blots for GSDMD cleavage and caspase11 (Casp11), IL-1α and IL-1β expression in the cell lysates of WT or *Casp11*$^{-/-}$ mouse peritoneal macrophages stimulated with LPS alone (1 μg/mL), LPS (1 μg/mL)+*Hmgb1*$^{+/+}$, or *Hmgb1*$^{-/-}$ MEF cells in the presence or the absence of indicated doses of heparin (Hep, A) or NAH (B) for 16 h.

(C) Primary hepatocytes isolated from mice of indicated genotypes co-cultured with WT mouse peritoneal macrophages are shown in the upper panel. IL-1α released from macrophages after stimulation of LPS (0.1 μg/mL) in the presence or the absence of heparin (Hep) or NAH is shown in the bottom panel.

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

See also Figure S4.

a cell-free system (Shi et al., 2014), we found that addition of heparin or NAH failed to directly inhibit LPS-induced caspase-11 activation (Figure 5A). By contrast, addition of Z-VAD-FMK, a pan-caspase inhibitor, markedly reduced caspase-11 activity (Figure 5A). When LPS was artificially delivered into the cytosol of macrophages during electroporation, heparin or NAH failed to inhibit the activation of caspase-11 (Figure 5B), suggesting that heparin is not a direct inhibitor of caspase-11. To determine whether heparin inhibits HMGB1-mediated cytosolic delivery of LPS, we used low concentrations of digitonin to isolate the cytosol fraction devoid of cytoplasmic membranes, endosomes or lysosomes from macrophages that were stimulated with LPS and HMGB1 in the presence or the absence of heparin (Figure 5C). Addition of heparin markedly reduced the concentrations of LPS in the cytosol of macrophages stimulated with LPS and HMGB1 (Figure 5C). To confirm that heparin inhibits HMGB1-mediated cytosolic delivery of LPS, we assessed the physical interaction between LPS and caspase-11 by using a proximity-ligation assay (PLA). In agreement with our previous findings (Deng et al., 2018), HMGB1 markedly promoted the LPS-caspase-11 interaction (Figure 5C), manifested by the cytosolic co-localization of LPS with caspase-11. Addition of heparin or NAH to the cell culture medium diminished the detection of LPS-caspase-11 interaction in the cytosol (Figure 5D).

To examine whether heparin inhibits HMGB1-dependent cytosolic delivery of LPS *in vivo*, hepatocyte HMGB1-deficient mice (*Hmgb1*$^{fl/fl}$*Alb-Cre*$^{+}$) and their controls (*Hmgb1*$^{fl/fl}$*Alb-Cre*$^{-}$) were subjected to lethal endotoxemia with or without heparin or NAH treatment. The splenocytes were isolated and fractionated to obtain the cytosolic fraction. In line with our previous findings that deletion of HMGB1 in hepatocytes markedly reduces the concentrations of serum HMGB1 (Deng et al., 2018), heparin or NAH

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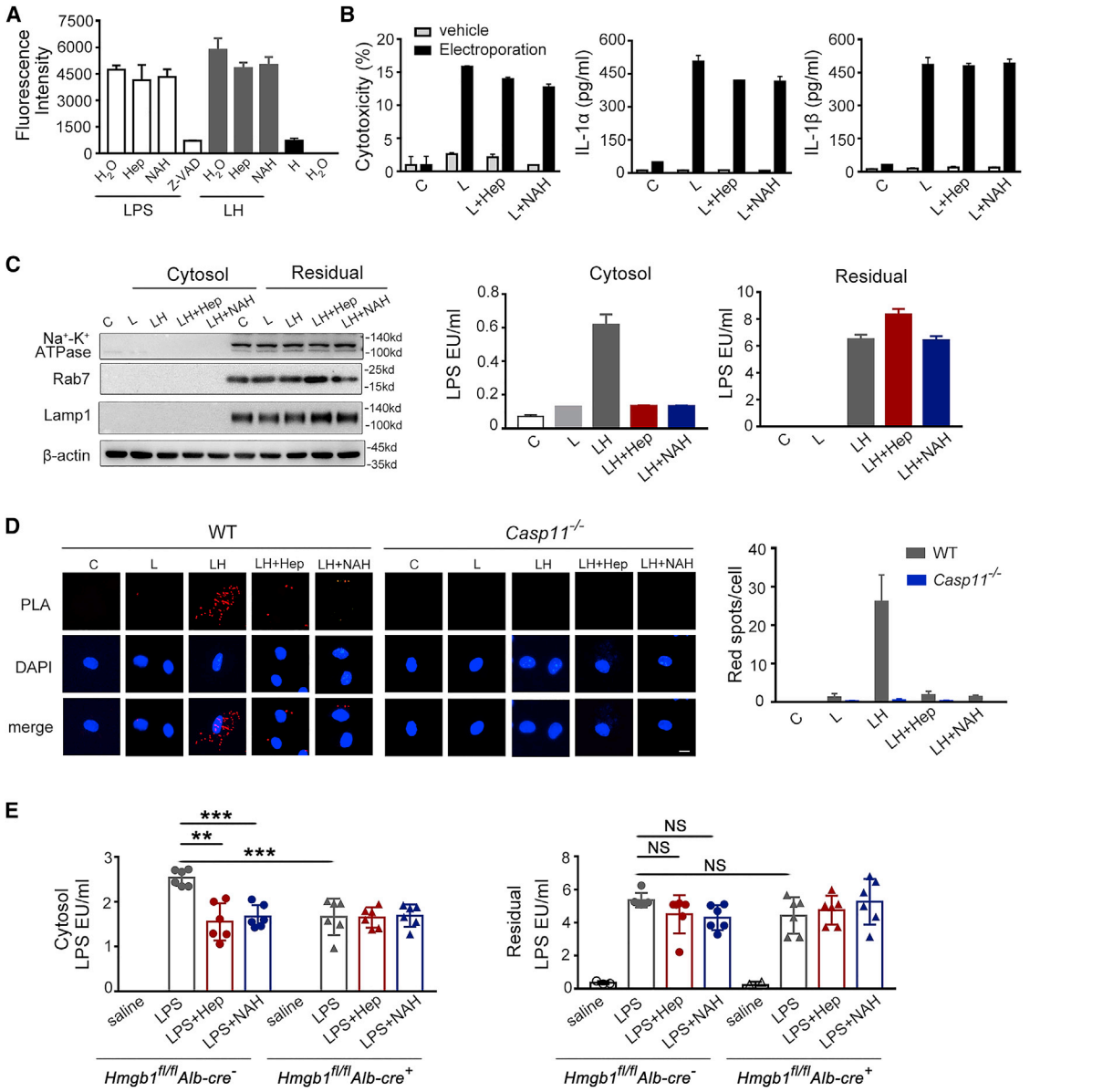

Figure 5. Heparin inhibits caspase-11 activation by inhibiting HMGB1-mediated cytosolic delivery of LPS.

(A) LPS or LPS+HMGB1 (LH) induced activation of insect cell-derived caspase-11 in the presence or the absence of heparin (Hep), NAH, or pan-caspase inhibitor Z-VAD-FMK (Z-VAD). HMGB1 alone (H) or H_2O was added as control. The caspase activity was determined by measuring the fluorescence intensity of free AMC hydrolyzed from zVAD-AMC.

(B) LDH assay and ELISA for IL-1α and IL-1β in the supernatants of mouse peritoneal macrophages electroporated with LPS (L) in the presence or the absence of heparin (Hep, 3 μg/mL) or NAH (10 μg/mL).

(C) Immuno-blot for Na^+-K+ ATPase, Rab7, and Lamp1 (left panel) and LPS activity assay (right panel), in the cytosolic and residual fraction (including cytoplasmic membranes, endosomes, lysosomes, nuclei, etc.) from mouse peritoneal macrophages stimulated with LPS (L, 1 μg/mL) or LPS (1 μg/mL) + HMGB1 (400ng/mL) (LH), in the presence or not of heparin (Hep, 3 μg/mL) or NAH (10 μg/mL) for 2 h.

(D) The physical interaction between caspase-11 and LPS were visualized as the red spots by PLA in mouse peritoneal macrophages stimulated with LPS alone (L, 5 μg/mL), HMGB1 alone (10 μg/mL) (H), or LPS (5 μg/mL)+HMGB1 (10 μg/mL) (LH) in the presence or the absence of heparin (Hep,3 μg/mL) or NAH (10 μg/mL) for 2 h. Scale bar represents 10 μm.

(E) LPS activity assay in the cytosolic and residual fraction of spleen cells from indicated mice genotypes injected intraperitoneally with LPS (25mg/kg) or saline. Heparin 5units/mouse (Hep) or NAH (200 μg/mouse) was administered subcutaneously 30min after LPS injection.

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

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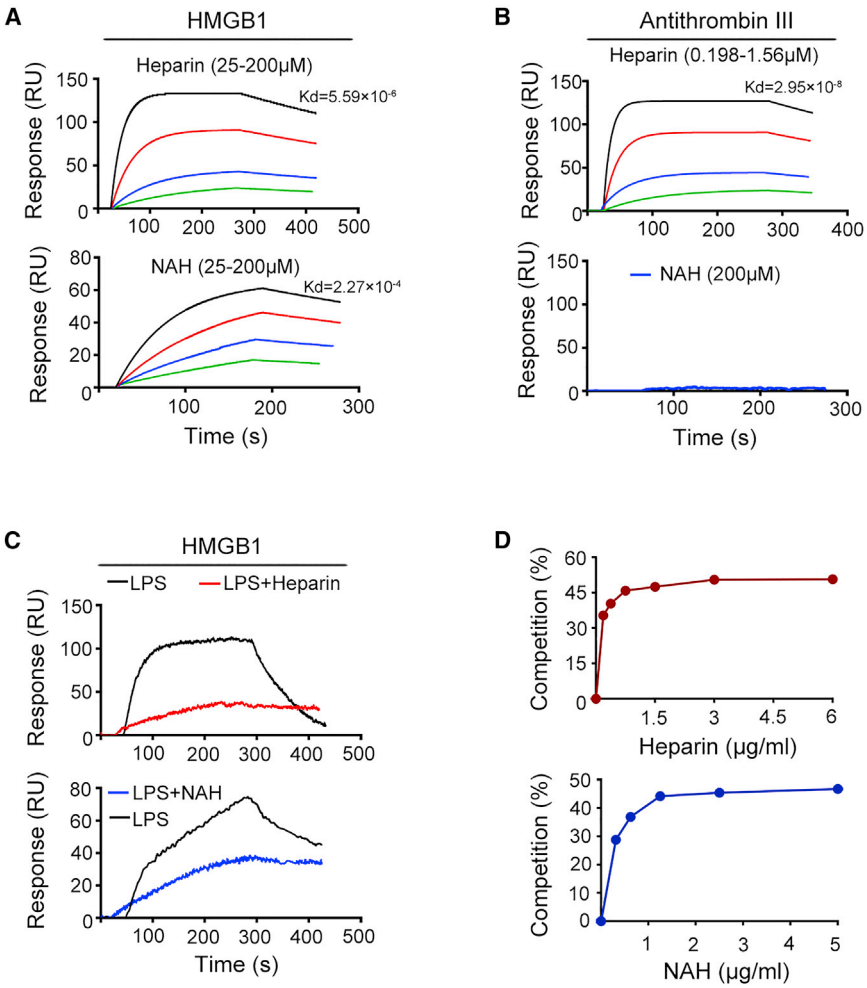

Figure 6. Heparin inhibits HMGB1-LPS binding

(A) Recombinant HMGB1 proteins were immobilized to the chips. The binding between HMGB1 and heparin (25, 50, 100, 200 μM, from the bottom to top) or NAH (25, 50, 100, 200 μM, form the bottom to top) was assessed by surface plasmon resonance.

(B) Antithrombin III were immobilized to the chips. The binding between antithrombin III and heparin (25, 50, 100, 200 μM, form the bottom to top) or NAH (25, 50, 100, 200 μM, form the bottom to top) was assessed by surface plasmon resonance.

(C) Recombinant HMGB1 proteins were immobilized to the chips. The binding between LPS and HMGB1 in the presence or not of heparin (up panel) or NAH (bottom panel) was assessed by surface plasmon resonance.

(D) The LPS-binding capacity of HMGB1 incubated with different concentrations of heparin or NAH. Plates coated with recombinant HMGB1 (4 μg/mL) were incubated with biotin-labeled LPS (1 μg/mL) with indicated concentration of heparin or NAH. Binding between plate-coated HMGB1 and biotin-labeled LPS was measured by using streptavidin-HRP. The percentage of binding competition by heparin or NAH was determined. Graphs shown are representative of at least two independent experiments.

See also Figure S5.

treatment significantly reduced the LPS concentrations in the cytosol of splenocytes in *Hmgb1*$^{fl/fl}$*Alb-Cre*$^{-}$ but not *Hmgb1*$^{fl/fl}$*Alb-Cre*$^{+}$ mice (Figure 5E). Collectively, these findings indicate that heparin prevents caspase-11-dependent immune responses by inhibiting HMGB1-mediated cytosolic delivery of LPS.

Heparin directly inhibits HMGB1-LPS binding

We then investigated the mechanisms by which heparin inhibits HMGB1-mediated cytosolic delivery of LPS. The polysaccharide motif of LPS is important for HMGB1 interaction with LPS (Deng et al., 2018). As heparin is a mammalian polysaccharide, we first determined whether heparin could directly bind HMGB1. As shown by surface plasmon resonance (SPR), both heparin and NAH bound recombinant HMGB1 protein with high affinity (Figure 6A). However, only heparin, and not NAH, bound recombinant antithrombin III (Figure 6B). We previously showed that HMGB1-LPS binding is required for the cytosolic delivery of LPS (Deng et al., 2018); SPR revealed that both heparin and NAH were able to prevent the HMGB1-LPS binding (Figure 6C). To confirm that heparin could competitively inhibit the HMGB1-LPS interaction, plates coated with recombinant HMGB1 (16 μg/mL) were incubated with biotin-labeled LPS (0.2 μg/mL) in the presence of heparin or NAH. Binding between plate-coated HMGB1 and biotin-labeled LPS was measured by using streptavidin-horseradish peroxidase. Notably, heparin and NAH dose-dependently inhibited the interaction HMGB1 with LPS (Figure 6D). To further confirm that heparin is able to competitively inhibit HMGB1-LPS interaction, we quantitatively measured HMGB1-LPS complexes on the cell surface and inside the HMGB1 + LPS-stimulated macrophages by using PLA. Addition of heparin markedly decreased the number of HMGB1-LPS complexes detected on the cell surface or inside cells (Figure S5). Thus, heparin suppresses HMGB1-mediated cytosolic delivery of LPS, at least in part, through direct inhibition of the HMGB1-LPS binding.

Heparin inhibits the cytosolic delivery of LPS through preventing heparanase-mediated glycocalyx degradation

In addition to HMGB1, bacteria outer membrane vesicles (OMVs) and cholera toxin B are capable of delivering LPS into the cytosol of macrophages (Kayagaki et al., 2015; Vanaja et al., 2016). Next, we determined whether heparin specifically inhibits HMGB1-mediated cytosolic delivery of LPS in macrophages. Addition of heparin or NAH markedly reduced OMVs-mediated cytosolic delivery of LPS in mouse peritoneal macrophages (Figure S6A) and dose-dependently inhibited the release of IL-1α and IL-1β from OMVs-stimulated mouse macrophages (Figure S6B) without affecting the secretion of TNF or IL-6 (Figure S6B). Further, heparin or NAH treatment significantly reduced the LPS concentrations in the cytosol of splenocytes and the serum

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concentrations of IL-1α and IL-1β in mice injected with OMVs (Figure S6C). These observations suggest that heparin or NAH inhibited OMVs-mediated cytosolic delivery of LPS through a mechanism independent of HMGB1. Heparin and NAH are known inhibitors of heparanase, which degrades the glycocalyx, the extracellular layer of glycoprotein, proteoglycan, and glycosaminoglycans that coat the exterior cell surface (Paszek et al., 2014; Schmidt et al., 2012). The glycocalyx is involved in endothelial protection, tissue generation, and tumor metastasis (Kiyan et al., 2019; Paszek et al., 2014; Schmidt et al., 2012). However, the roles of the glycocalyx in innate immune responses remain largely unknown. Together with the fact that inflammation upregulates the expression of heparanase and induces the degradation of the glycocalyx, we postulated that heparin or NAH might inhibit the cytosolic delivery of LPS by preventing heparanase-induced glycocalyx degradation in macrophages.

As expected, HMGB1+LPS or OMVs stimulation markedly decreased the density of glycocalyx, which were prevented by genetic deletion of Heparanase or addition of heparin or NAH (Figure 7A). Heparanase deficiency inhibited HMGB1- or OMVs-mediated cytosolic delivery of LPS in a manner similar to that of addition of heparin or NAH to the cell-culture medium (Figure 7B). Accordingly, loss of Heparanase markedly reduced the release of LDH, IL-1α and IL-1β from mouse macrophages stimulated with HMGB1+LPS or OMVs (Figure 7B). Addition of heparin or NAH inhibited the release of IL-1α and IL-1β from heparanase-deficient macrophages stimulated with HMGB1+LPS but not OMVs (Figure S6D), further supporting the notion that heparin inhibits HMGB1-mediated cytosolic delivery through both the disruption of HMGB1-LPS interaction and suppression of heparanase-mediated glycocalyx degradation. Loss of Heparanase did not affect the secretion of TNF or IL-6 (Figure 7C). To provide further evidence that glycocalyx degradation promotes the cytosolic delivery of LPS, we artificially degraded the glycocalyx of macrophages by using recombinant heparanase (Figure 7A). This culminating in the increase of LPS concentrations in the cytosol and the release of LDH, IL-1α and IL-1β from macrophages stimulated with HMGB1+LPS or OMVs (Figure 7D). Taken together, our findings establish that heparin inhibits the cytosolic delivery of LPS in macrophages and the activation of caspase-11 through the disruption of HMGB1-LPS interaction and the prevention of glycocalyx degradation by heparanase.

Finally, we sought to determine whether glycocalyx degradation promotes the cytosolic delivery of LPS in splenocytes and the caspase-11-dependent immune responses *in vivo*. Heparanase-deficient mice and their WT littermate controls were subjected to lethal endotoxemia. Heparanase deficiency prevented glycocalyx degradation in a manner similar to that of heparin or NAH treatment (Figure S6E). Deletion of Heparanase significantly attenuated the caspase-11-dependent release of IL-1α and IL-1β but did not affect the secretion of IL-6 (Figure 7E). The cytosolic fraction of splenocytes isolated from heparanase-deficient mice contained significant less LPS as compared to that of from their WT littermate controls (Figure 7E). Accordingly, heparanase deficiency significantly promoted survival during lethal endotoxemia (Figure 7F). Collectively, these findings indicate that heparin inhibits the cytosolic delivery of LPS in splenocytes and the caspase-11-dependent immune responses, at least in part, through preventing heparanase-induced glycocalyx degradation during endotoxemia.

DISCUSSION

In this study, we discovered that heparin, a mammalian polysaccharide expressed in a variety of tissue types, is a potent inhibitor of the caspase-11 pathway in sepsis. Caspase-11 plays an adaptive role in innate immunity by destroying the intracellular niche for certain invading microbes that evade intracellular killing mechanisms, such as *Burkholderia pseudomallei* (Aachoui et al., 2013). However, caspase-11 can also become activated when hepatocyte-secreted HMGB1 or bacteria-released OMVs deliver LPS into the cytosol of target cells such as macrophages or endothelial cells (Deng et al., 2018; Vanaja et al., 2016). This causes immune cell death and endothelial dysfunction (Cheng et al., 2017; Deng et al., 2018; Vanaja et al., 2016). By disrupting the HMGB1-LPS interaction and inhibiting the heparanase-induced glycocalyx degradation in macrophages, heparin prevented the cytosolic delivery of LPS, thereby attenuating the over-activation of this detrimental cascade. Heparin also directly binds to antithrombin and other pro-coagulant factors, through which it exerts anticoagulant properties (Chuang et al., 2001; Rezaie et al., 2004; Yang et al., 2004). A chemically modified form of heparin without anticoagulant function inhibits the caspase-11 activation in a manner similar to that of heparin. These findings dissect the roles of heparin in regulating inflammation and coagulation.

Previous studies reported that heparin could inhibit the LPS-induced expression of tumor necrosis factor (TNF) and interleukin-6 (IL-6) *in vivo* or *in vitro* (Li and Ma, 2017; Li et al., 2018). However, we discovered that heparin treatment prevented caspase-11-dependent immune responses but did not affect the release of TNF or IL-6 in animal models of lethal endotoxemia or sepsis *in vivo*, as well as in primary mouse macrophages *in vitro*. The discrepancy is likely due to the different doses of LPS used in the experiments. That is, heparin partially reduced the release of TNF and IL-6 if mice were challenged with a low dose of LPS (0.1mg/kg), but failed to inhibit the production of TNF and IL-6 when mice were subjected to lethal endotoxemia or sepsis. Though the underlying mechanisms require further investigation, our data clearly suggest that heparin selectively inhibits the detrimental cascade mediated by HMGB1 and caspase-11 in sepsis.

Sepsis is a major public health concern, accounting for more than $20 billion (5.2%) of total US hospital costs in 2011 (Angus and van der Poll, 2013; Li and Ma, 2017; Seymour et al., 2016). Although the true incidence of sepsis is unknown, epidemiological studies have established that sepsis is the leading cause of death in intensive care units (Seymour et al., 2016). Heparin has previously, and confirmed by us now, been demonstrated to prevent organ damage and lethality in experimental sepsis models (Li and Ma, 2017; Li et al., 2011). In agreement with these findings, clinical evidence suggests that heparin treatment improves the outcome of patients with sepsis or septic shock (Fan et al., 2016; Liu et al., 2014; Wang et al., 2014; Zarychanski et al., 2015). However, one obstacle for wider use of heparin to treat sepsis is the bleeding risk (Zarychanski et al., 2015). Therapeutic administration of modified heparin without anticoagulant activity

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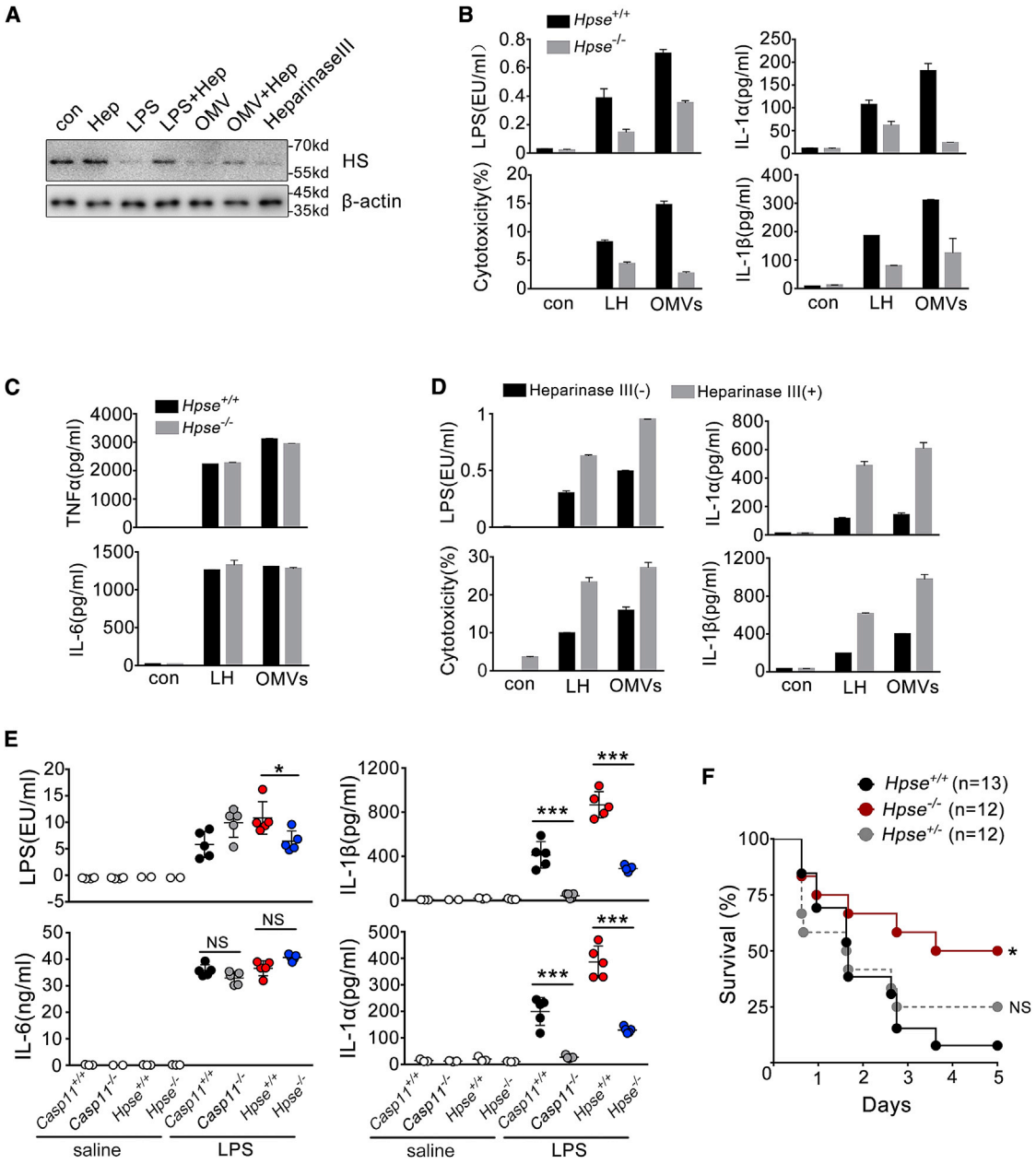

Figure 7. Heparin inhibits the cytosolic delivery of LPS through preventing heparanase-mediated glycocalyx degradation

(A) Immuno-blot to detect the heparan sulfate (HS) expression in the cell lysates of mouse peritoneal macrophages stimulated with LPS alone (1 μg/mL) or OMVs (10 μg/mL) in the presence or the absence of indicated doses of heparin (Hep).

(B) LPS activity assay in the cytosolic, LDH assay, and ELISA for IL-1α and IL-1β in the supernatants of heparanase deficiency (*Hpse*$^{-/-}$) or wild-type (*Hpse*$^{+/+}$) mouse peritoneal macrophages stimulated with LPS (1 μg/mL) + HMGB1 (400ng/mL) (LH) or OMVs (10 μg/mL).

(C) ELISA for TNF-α and IL-6 in the supernatants of heparanase deficiency (*Hpse*$^{-/-}$) or wild-type (*Hpse*$^{+/+}$) mouse peritoneal macrophages stimulated with LPS (1 μg/mL) + HMGB1 (400ng/mL) (LH) or OMVs (10 μg/mL).

(D) LPS activity assay in the cytosolic, LDH assay, and ELISA for IL-1α and IL-1β in the supernatants of mouse peritoneal macrophages pre-treated with heparinaseIII (1U/mL for 6 h) and then stimulated with LPS (1 μg/mL) + HMGB1 (400ng/mL) (LH) or OMVs (10 μg/mL).

(E) LPS activity assay in the cytosolic of spleen cells, serum IL-1α, IL-1β, and IL-6 concentrations from mice of indicated genotypes injected intraperitoneally with LPS (25mg/kg) or saline.

(F) Kaplan Meier survival curves from heparanase deficiency homozygotes (*Hpse*$^{-/-}$), heterozygotes (*Hpse*$^{+/-}$), or wild-type (*Hpse*$^{+/+}$) mice injected intraperitoneally with LPS (25mg/kg) or saline.

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

See also Figure S6.

or heparin in lower doses without effects on coagulation but capable of preventing HMGB1-mediated intracellular LPS delivery and heparanase-mediated glycocalyx degradation, might offer strategies to minimize the risk for bleeding.

In current study, we found that NAH, a chemically modified heparin without anticoagulant activity, was able to rescue mice from lethal sepsis. Similar observations were made using non-anticoagulant heparin purified from unfractionated heparin using antithrombin columns (Wildhagen et al., 2014). We also noted that NAH administration prevented caspase-11-dependent DIC in endotoxemia and bacterial sepsis, further supporting the notion that heparin could attenuate coagulopathy independent of its anticoagulant property. Non-anticoagulant heparin can also bind histones and prevent histone-mediated cytotoxicity *in vitro* and *in vivo* (Wildhagen et al., 2014). As histones are released from damaged or dead cells, these findings suggest that heparin might exert its beneficial effects in sepsis in two ways: i) preventing caspase-11-dependent DIC and pyroptosis of macrophages and endothelial cells, culminating in the reduced concentration of histones in the circulation; ii) neutralizing circulating histones and thereby reducing histone-mediated cytotoxicity. Taken together, we provide a mechanistic basis in support of the development of safe heparin-based therapies for sepsis.

LIMITATIONS OF STUDY

This study shows that heparin treatment could prevent lethal responses in experimental sepsis and was associated with an improved survival in human sepsis. However, we cannot draw the conclusion that heparin treatment is protective for all septic patients. Gram-positive sepsis causes death through mechanisms distinct from that of Gram-negative sepsis (Popescu et al., 2018). Caspase-11 deficiency or heparin treatment prevented lethality in Gram-negative sepsis but failed to confer any protection against sepsis caused by *Staphylococcus aureus*, a type of Gram-positive bacterium. Furthermore, some clinical studies demonstrate that heparin treatment improves the outcome of patients with sepsis (Fan et al., 2016; Liu et al., 2014; Wang et al., 2014; Zarychanski et al., 2015), whereas other studies show that heparin treatment does not significantly reduce the 28-day mortality of septic patients (Jaimes et al., 2009; Li and Ma, 2017). The discrepancy between these studies might be due to the difference in infected pathogens. Thus, stratification of patients based on the type of invading pathogens might improve the therapeutic efficiency of heparin in sepsis, and this merits future investigations.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.immuni.2021.01.007.

ACKNOWLEDGMENTS

The authors thank Professor Rongbin Zhou for sharing mouse strains (*Nlrp3*$^{-/-}$ mice), Dr. Kevin J. Tracey for providing recombinant HMGB1 protein, and Professor Feng Shao for providing recombinant caspase-11. We also thank Dr. Jianfeng Wu for *in vitro* fertilization of mice and Dr.Yanjun Zhong for sharing microbe strains. This work was supported by National Natural Science Foundation of China (No. 82025021 and No. 81930059 to B.L.; No. 81971893 to Y.T.), and was an Innovation-driven scientific project of CSU (No.2019CX013).

AUTHOR CONTRIBUTIONS

B.L. conceived the project, designed experiments, and wrote the paper; Y.T. supervised the study, designed experiments, performed the experiments, analyzed the data, and made the figures; X.W. and R.Z. performed the experiments and analyzed the data; Z.L. and Q.X. performed the mouse experiments; X.Y. and X.C performed Spinning Disk Confocal Intravital Microscopy analysis; Z.L., Y.P., and, Y.B. performed human serum samples. Y.T. and B.L. interpreted the data and wrote the manuscript; and K.Z., Z.H., L.F., X.X., U.A., H.W., and T.R.B. assisted in data interpretation and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: December 4, 2019
Revised: November 9, 2020
Accepted: January 13, 2021
Published: February 8, 2021

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-Heparin/Heparan Sulfate Antibody	Sigma	Cat#:T320.11;RRID:AB_94518
Anti-caspase-11 antibody	Sigma	Cat#:C1354; RRID: AB_258736
Anti-IL-1β antibody	R&D system	Cat#:AF-401-NA; RRID: AB_416684
Anti-IL-1a antibody	Abcam	Cat#:ab7632;RRID:AB_306001
Anti-Gasdermin D-antibody	Abcam	Cat#:ab209845;RRID:AB_2783550
Anti-caspase-1 antibody	Abcam	Cat#:ab179515
Anti-HMGB1 antibody	Abcam	Cat#:ab79823; RRID: AB_1603373
Anti-E.coli LPS antibodies	Abcam	Cat#:Ab35654; RRID: AB_732222
Anti-human Gasdermin D-antibody	Abcam	Cat#:ab210070
Anti-human cleaved N-terminal GSDMD-antibody	Abcam	Cat#:ab215203
Anti-human IL-1a antibody	Abcam	Cat#:ab206410
Anti-human IL-1β antibody	Abcam	Cat#:ab9722;RRID:AB_308765
Anti-LAMP1 antibody (clone 1D4B)	eBioscience	Cat#:14-1071-85; RRID: AB_65753
Sodium Potassium ATPase Alpha 1 Antibody	Novus Biological	Cat#:NB300-146; RRID: AB_2060981
Anti-Rab7 antibody	Cell Signaling Technologies	Cat#:9367S; RRID: AB_1904103
β-actin antibody (clone 8H10D10)	Cell Signaling Technologies	Cat#: 3700S; RRID: AB_2242334
anti-caspase-4 antibody	MBL International	Cat#:M029-3;RRID:AB_590743
AF647-labeled anti-mouse CD49b	Biolegend	Cat#: 103511; RRID: AB_528830
5-FAM/QXL-520 FRET thrombin substrate	Anaspec	Cat#: AS-72129
Chemicals, Peptides, and Recombinant Proteins		
Unfractionated Heparin	Sigma-Aldrich	H3149
N-Acetyl heparin sodium salt	Sigma-Aldrich	A8036
Heparinase III	Sigma-Aldrich	H88991
Antithrombin III	Sigma-Aldrich	SRP6316
Ultrapure LPS (*E. coli* 0111:B4)	InvivoGen	tlrl-3pelps
LPS-EB Biotin	InvivoGen	tlrl-3blps
ATP	InvivoGen	tlrl-atp
Nigericin	InvivoGen	tlrl-nig
MSU Crystals	InvivoGen	tlrl-msu
Nano-SiO2	InvivoGen	tlrl-sio
Cholera Toxin B Subunit (Choleragenoid) from Vibrio cholerae	List Biological Laboratories, INC.	103B
Lipofectamine 3000 Transfection Reagent	Thermo Fisher Scientific	L3000015
Z-VAD-AMC	BACHEM	I-1710
Recombinant mouse LPS-binding protein	R&D Systems Inc	6635-LP-025/CF
Recombinant HMGB1 protein	Prepared in K.J.T. Lab	(Wang et al., 1999)
Recombinant caspase-11 protein	Prepared in Dr.Feng shao Lab	(Shi et al., 2014)
Critical Commercial Assays		
LDH Cytotoxicity Assay kit	Beyotime Biotechnology	C0016
IL-1 alpha Mouse Uncoated ELISA Kit	eBioscience	88-5019-77
IL-1 beta Mouse Uncoated ELISA Kit	eBioscience	88-7013-77
IL-6 Mouse Uncoated ELISA Kit	eBioscience	88-7064-77
TNF alpha Mouse Uncoated ELISA Kit	eBioscience	88-7324-77

(*Continued on next page*)

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Human IL-1α ELISA MAX™ Deluxe	Biolegend	445804
Human IL-1β ELISA MAX™ Deluxe	Biolegend	437004
PAI-1Mouse Simple Step ELISA Kit,	Abcam	ab197752
TAT Complexes Mouse ELISA Kit	Abcam	ab137994
Murine CD138 ELISA kit	Diaclone	860.090.192
HMGB1 ELISA	TECAN	ST51011
Duolink® *In Situ* Detection Reagents Red	Sigma-Aldrich	DUO92008
Duolink® *In Situ* Probemaker MINUS	Sigma-Aldrich	DUO92010
Duolink® *In Situ* PLA® Probe Anti-Mouse PLUS	Sigma-Aldrich	DUO92001
Duolink® *In Situ* PLA® Probe Anti-Mouse MINUS	Sigma-Aldrich	DUO92004
Duolink™ *In Situ* PLA® Probe Anti-Rabbit PLUS	Sigma-Aldrich	DUO92002
Experimental Models: Cell Lines		
MEF cells	HMGBiotech, Inc	N/A
Mouse Hepatocytes	Prepared in T.R.B. Lab	Described in current manuscrip
Mouse Macrophages	Prepared in B.L. Lab	Described in current manuscript
Experimental Models: Organisms/Strains		
C57BL/6 mice	Jackson Laboratories	664
Casp11$^{-/-}$ mice	University of Pittsburgh Animal Housing	Described in current manuscrip
Hpse$^{-/-}$ mice	GemPharmatech Co. Ltd	Described in current manuscrip
Hmgb1 $^{fl/fl}$ mice	University of Pittsburgh Animal Housing	Described in current manuscript
Nlrp3$^{-/-}$ mice	Genentech Inc	(Mariathasan et al., 2006)
Oligonucleotides		
5¢ -ACT TTC TCT CTT CTC ACT-3¢,	Invitrogen	*Casp11*-specific primers
5¢ -TGT CTA ACT ATA TTG AAA TGT G-3¢		
5¢-AGTCATACCTAGAGGTGTGTTGCC-3¢	Sangon Biotech	*Hpse*-specific primers
5¢-TCGCTACCGTAGCCAGAATG-3¢		
5¢-TTTCTTGCCTGTCTTGTAACAGC-3¢	Sangon Biotech	*Hpse*-specific primers
5¢-TTGGGTTCTCTACTTGTCTAAGGG-3¢		
TCTACACTATAGTCCAGACCC	(Shi et al., 2014)	*CASP4*-specific siRNA
GTCTGGACTATAGTGTAGATG	(Shi et al., 2014)	*CASP4*-specific siRNA
CGTACGCGGAATACTTCGA	(Shi et al., 2014)	Control-specific siRNA
Software and Algorithms		
Graphpad Prism 7 software	https://www.graphpad.com	N/A
Adobe Photoshop CS6	https://www.photoshop.com/en	N/A
NIS Elements software	https://www.microscope.healthcare.nikon.com/	N/A
ImageJ	https://imagej.nih.gov/	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Ben Lu (xybenlu@csu.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate any unique datasets or code.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice

Casp11$^{-/-}$ mice were generated as previously described (Deng et al., 2018). The mutated alleles were monitored and tracked in the progenies with a set of PCR genotyping primers (forward, 5¢-ACT TTC TCT CTT CTC ACT −3¢, reverse, 5¢-TGT CTA ACT ATA TTG AAA TGT G-3¢). WT littermates (C57BL/6J background) were used as the controls for the *Casp11*$^{-/-}$ mice. *Nlpr3*$^{-/-}$ mice were provided by Professor Rongbin Zhou.

Hmgb1 $^{fl/fl}$ mice (*Hmgb1* $^{fl/fl}$ allele) were generated by inserting loxP sites within intron 1 and intron 2, flanking exon 2 of Hmgb1. *Hmgb1* $^{fl/fl}$ mice were interbred with heterozygous stud males (*Alb-cre*$^{+}$) to generate *Hmgb1* $^{fl/fl}$ *Alb-cre* mice. Transgenic mice used for experiments were confirmed to be desired genotype via standard genotyping techniques.

Hpse$^{-/-}$ mice were generated by the CRISPR/Cas9-mediated genome editing (GemPharmatech Co. Ltd). Briefly, the vectors encoding Cas9 and guide RNA were *in vitro* transcribed into mRNA and gRNA, and then injected into the fertilized eggs which were transplanted into pseudo-pregnant mice. The targeted genome of F0 mice was amplified with PCR and sequenced and the chimeras were crossed with wild-type C57BL/6 mice (GemPharmatech Co. Ltd) to obtain the *Hpse*$^{+/-}$ mice. The F1 *Hpse*$^{+/-}$ mice were further crossed with wild-type C57BL/6 mice for at least three generations. Mice were genotyped by PCR analysis (F1: AGTCATACCTAGAGGTGTGTTGCC, R1: TCGCTACCGTAGCCAGAATG and F2: TTTCTTGCCTGTCTTGTAACAGC, R2: TTGGGTTCTCTACTTGTCTAAGGG) of tail DNA followed by sequencing and the resulted *Hpse*$^{+/-}$ mice were crossed to generate *Hpse* $^{+/+}$ and *Hpse* $^{-/-}$ mice.

Experimental protocols were approved by the Institutional Animal Care and Use Committees of the University of Pittsburgh and Central South University. Mice 25 to 30 g in weight were used in current study, the skin was disinfected with a 2% iodine tincture and laparotomy was performed under 2% isoflurance (Piramal Critical Care) with oxygen. We used moribundity as the endpoint for our survival study following the Animal Research Advisory Committee Guidelines from National Institutes of Health. Briefly, mice were monitor 2-4 times daily by personnel experienced in recognizing signs of moribundity. Mice were euthanized with CO2, when they became moribund or at observation endpoint. Male mice with weight ranged from 25 to 30 g (8-12 weeks old) were used in *in vivo* experiments to generate data presented in Figure 1A-F, Figure 2, Figure 5E, Figure 7E-F and Figure S1, S2, S6C-E. Male or Female mice with weight ranged from 25 to 30 g(8-12 weeks old)were used in primary macrophages isolation to generate data presented in Figures 3, Figure 4, Figure 5B-D, Figure 7A-D and Figure S3,S4,S5,S6A-B.

Human samples

Patients were diagnosed with sepsis according to the 2016 International Sepsis Definitions (Seymour et al., Jama 315, 762-774.) Exclusion criteria were: age < 18 years, preexisting immunosuppression; transplant recipient; presence of decompensated cirrhosis (Child-Pugh class B or C), hematological diseases, any disease involving hemodialysis such as chronic renal failure, and history of anticoagulant therapy during the preceding four weeks. A total of 41 patients from the intensive care unit (ICU) at the Third Xiangya Hospital of Central South University from January 2018 to April 2019 met the above conditions. Patients without heparin treatment (21-75 years, 10 males, 11 females, n = 21) and patients with heparin treatment (23-85 years, 13 males, 7 females, n = 20) were included. Blood samples were collected on days 1 to 2 when these patients were recently diagnosed with sepsis after admission to ICU. Serum samples were performed as soon as possible (< 6 h) and stored at −80°C when all patients fulfilled septic shock criteria to avoid any therapeutic bias. We also collected blood samples from 30 patients with sepsis at the Third Xiangya Hospital of Central South University from June 2019 to August 2019. Patients without heparin treatment (17-79 years,6 males, 14 females, n = 20) and patients with heparin treatment (22-64 years,4 males,6 females, n = 10) were included. Serum concentrations of IL-1α and IL-1β were detected by ELISA according to manufacturer's instructions. Information about the patients was recorded, including 28-day mortality (Table S1). Informed consent was conferred by the patients or their families and this study was approved by the research ethics committee of the Third Xiangya Hospital of Central South University (2018-S178 and 2019-S093).

Primary cells

Mouse peritoneal macrophage preparation

Mouse peritoneal macrophages were isolated and cultured as described previously (Deng et al., 2018). Briefly, male or female mice (8–12 weeks old) were injected with 3 mL of sterile 3% thioglycollate broth intraperitoneally to elicit peritoneal macrophages. Cells were collected by lavage of the peritoneal cavity with 5 mL of RPMI medium 1640 72 h later. After washing, cells were resuspended in RPMI medium 1640 supplemented with 10% fetal bovine serum and antibiotics (GIBCO) and plated in 12-well plates (106 cells per well).

Mouse hepatocyte preparation

Mouse hepatocytes were isolated and cultured as described previously (Deng et al., 2018). Briefly, cells were isolated from male or female mice (8–12 weeks old) through an *in situ* collagenases (type VI, Sigma) perfusion technique. Hepatocyte purity exceeded 99% by flow cytometric assay. Cell viability was typically > 90% by trypan blue exclusion. Hepatocytes (150,000 cells/mL) were cultured on gelatin-coated culture plates or coverslips coated with collagen I (BD PharMingen) in Williams medium E with 10% calf serum, 15 mM HEPES, 1 μM insulin, 2 mM L-glutamine, penicillin (100 U/mL), and streptomycin (100 U/mL).

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Cell lines

Wild-type (*Hmgb1*$^{+/+}$) and *Hmgb1*$^{-/-}$ fibroblasts (MEF) were purchased from HMGBiotech, Inc. Cells were cultured in DMEM medium supplemented with 10% FBS, 2-mecaptoethanol (5 × 10−5 M), 100 U/mL penicillin, 100 μg/mL streptomycin, and 10 Mm HEPES.

Microbe strains

Staphylococcus aureus (ATCC25923) was a kind gift from Dr. Yanjun Zhong at ICU Center, The Second Xiangya Hospital, Central South University.

METHOD DETAILS

Endotoxemia model

Male mice with weight ranged from 25 to 30 g (8-12 weeks old) were used. *Casp11*$^{-/-}$ mice, their *Casp11*$^{+/+}$ littermates or *Hpse*$^{-/-}$ mice and their *Hpse*$^{+/+}$ littermates were injected intraperitoneally with a dose of LPS (25mg/kg). Heparin 5units/mouse (Hep5U), NAH 200 μg /mouse or hirudin 100units/mouse was administered subcutaneously 30min after LPS injection. Mice monitored 4 times daily for a total of 5 days or sacrificed at 16-18 h after LPS injection to collect serum or lung tissue.

CLP bacterial sepsis model

Sepsis was induced by cecum ligation and puncture (CLP) operation as described previously (Deng et al., 2018). Male mice with weight ranged from 25 to 30 g (8-12 weeks old) were used. *Casp11*$^{-/-}$ mice, their *Casp11*$^{+/+}$ littermates or *Hmgb1* $^{fl/fl}$ *Alb-cre*$^{-}$ and *Hmgb1* $^{fl/fl}$ *Alb-cre*$^{+}$ mice were subjected to CLP. Briefly, 75% of the cecum was ligated and punctured twice with an 18-gauge needle to generate the lethal model. Saline (1 mL) was given subcutaneously for resuscitation immediately after operation. Heparin 5units/mouse (Hep5U) or hirudin 100units/mouse was administered subcutaneously 2 h and 16 h after CLP. NAH 200ug /mouse was administered subcutaneously 1 h after CLP. Mice were monitored 4 times daily for a total of 7 days or sacrificed at 18-20 h after CLP to collect serum or lung tissue.

S. aureus systemic infection in mice

Staphylococcus aureus were grown in Luria-Bertani broth (LB) overnight at 37°C with 250 rpm shaking. After centrifugation at 5000 rpm for 10 min, the pellet was resuspended and washed with sterile phosphate-buffer saline (PBS) and then diluted with PBS to an appropriate concentration as determined by spectrophotometry at 600 nm. Male mice with weight ranged from 25 to 30 g (8-12 weeks old) were used. *Casp11*$^{-/-}$ mice and their *Casp11*$^{+/+}$ littermates were infected with S. aureus at 3 × 10^8 CFU/mouse intraperitoneally or 2 × 10^8 CFU/mouse intravenously. . Heparin or NAH at different dose were administered 30min before S. aureus challenged. The mortality was monitored for 7 days.

Spinning Disk Confocal Intravital Microscopy analysis

Livers were prepared for intravital microscopy as previously described (Yang et al., 2019). Briefly, mice were anesthetize and the exposed liver was visualized with a Nikon Ti2-E inverted microscope (Nikon Instruments) equipped with a Yokogawa CSU-W1 head (Yokogawa Electric Corporation). Thrombin activity was visualized using SensoLyte internally quenched 5-FAM/QXL-520 FRET thrombin substrate (2 μL/mouse). Platelets were visualized using AF647-anti-CD49b antibody (5 μL/mouse). Perfused vessels were visualized using Texas Red labeled Dextran (250ug/mouse). Reagents were administered via internal jugular vein catheterization 4 min before imaging. Image analysis methodology was performed as previously described (Yang et al., 2019). Briefly, images were saved in tiff format, exported, and analyzed in ImageJ (NIH). For the elimination of background autofluorescence, contrast was adjusted to minimize autofluorescent background staining, and a minimum brightness threshold was set to emerge only positive staining. Identical contrast and threshold values were applied to all images within our study. Thresholded images were converted to binary, and the area per field of view covered by positive fluorescence staining was through ImageJ software. Data are expressed as the percentage of area in each field of view covered by positive fluorescence staining.

Lung histology

After cardiac perfusion with PBS, the left upper of lung were fixed in 2% paraformaldehyde. Tissue was then placed in 2% paraformaldehyde for 2 h and then switched to 30% sucrose in distilled water solution for 12 h, then 4 μm thick sections were cut and stained with hematoxylin and eosin. Imaging was performed using a Nikon A1 confocal microscope (purchased with 1S10OD019973-01 awarded to Dr. Simon C. Watkins).

Peritoneal macrophage stimulation

Mouse peritoneal macrophages culture medium was changed with RPMI medium 1640 with or without indicated doses of heparin or NAH for 30mins, then macrophages were stimulated with ultra-pure LPS and HMGB1 which were pre-mixed at room temperature for 20 min. In some experiments, Cell lysates and supernatants were collected 16 h later for immune-blot, ELISA and LDH release.

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Mouse hepatocyte stimulation

Mouse hepatocytes were allowed to attach to plates overnight, and the culture media was replaced with fresh media before the cells were treated for experiments. After treatment, 1 mL of culture media was collected and centrifuged at 400 g for 5 min. The supernatant was removed to a new tube and stored at −80°C for further analysis.

Bacterial OMVs isolation and purification

OMVs were purified from E.coli BL21 as previously described with modification (Vanaja et al., 2016). Bacteria-free supernatant was collected and filtered through a 0.45 μm filter, then collected supernatant by centrifugation at 10,000x g for 15min at 4°C, further filtered through a 0.45 μm filter. OMVs were pelleted by ultracentrifugation at ~100,000 x g for 2 h at 4°C in a Beckman (optima L-100 XP) rotor. After removing the supernatant, OMVs were resuspended in 300 μL sterile PBS. The OMVs were filtered through a 0.22 μm filter. Purified OMVs were subjected to agar plating to ensure lack of bacterial contamination and Limulus Amebocyte Lysate (LAL) assay according to the manufacturer's instructions to quantify LPS. The protein content of OMV preparations was assessed by Pierce BCA protein assay kit according to the manufacturer's instructions.

Necrotic cell lysate preparation

Wild-type (*Hmgb1*$^{+/+}$) and *Hmgb1*$^{-/-}$ fibroblasts (MEF) were purchased from HMGBiotech, Inc. Cells were cultured in DMEM medium supplemented with 10% FBS, 2-mecaptoethanol (5 × 10^{-5} M), 100 U/mL penicillin, 100 μg/mL streptomycin, and 10 Mm HEPES. Necrotic cells were prepared by ten cycles of freezing and thawing. Necrosis was verified by microscopic evaluation showing cell fragments but no intact cells. The ratio of necrotic cells to macrophages in Figure 1F is 1:1. Briefly, 10^8 MEF cells were freezed and thawed 10 times in 1 mL serum-free RPMI 1640 medium, and then subjected to high-speed centrifugation. The supernatant was collected for macrophage stimulation. 10^6 macrophages were stimulated with 10 μL of such necrotic cell supernatant in the presence or the absence of LPS (1ug/mL).

LPS transfection

Macrophages were seeded into the six-well transfection plate (Bimake) in a total volume of 2ml (2 × 10^6 cells/well). Cells were priming with 1ug/mL of Pam3CSK4 for 4 h in RPMI medium 1640(GIBCO) and transfected with ultrapure LPS or ultrapure LPS+Heparin or LPS+NAH. The details for transfection was followed the rules of Auto Electroporator (Bimake).

Caspase activity assay

Caspase activity was measured as described previously (Shi et al., 2014). Hydrolysis of fluorogenic substrate z-VAD-AMC by recombinant caspase proteins was performed in a buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 3 mM EDTA and 0.005% (v/v) Tween-20 and 10 mM DTT. To measure ligand-induced caspase activation, each ligand was incubated with 0.25 mM caspase-11 in a 100 mL reaction at 37°C for 30 min. After incubation, zVAD-AMC was added into the reaction at a final concentration of 75 mM. The reaction mixture was transferred to a 96-well plate and incubated at 37°C for another 30 min. Substrate cleavage was monitored by measuring the emission at 450 nm on excitation at 365 nm on a fluorescent multi-well reader (Tecan Spark).

Isolation of cytosol fraction and LPS activity assay

Subcellular fractionation of mouse peritoneal macrophages or splenocytes from mice were conducted by a digitonin-based fractionation method as described previously with modifications (Vanaja et al., 2016). For macrophages, 5 × 10^6 cells were stimulated with or without LPS (1 μg/mL) alone or LPS (1 μg/mL) plus HMGB1 (400ng/mL) in the presence or not of heparin (3 μg/mL) or NAH (10 μg/mL) for 2 h. For splenocytes, cells were isolated from LPS challenged mice and resuspended in RPMI 1640 medium. Then cells were washed with sterile cold PBS 4 times, and subsequently treated with 300 μL of 0.005% digitonin extraction buffer for 10 min on ice and the supernatant containing cytosol was collected. The residual cell fraction containing cell membranes, organelles, and nuclei were collected in 300 μL of 0.1% CHAPS buffer. BCA assay was used for protein quantification and LPS activity assay for LPS quantification. Additionally the fractions were immunoblotted for Na^+-K+ ATPase, Rab7, LAMP1, and β-Actin to confirm the purity of cytosol fraction.

Proximity-ligation assay

A Proximity Ligation Assay kit (Sigma, DUO92101) was used to study the interaction between LPS and HMGB1 or caspase11 protein in mouse peritoneal macrophages which were cultured and stimulated on a six-well PTFE'' Printed Slides (Electron Microscopy Sciences, 63423-08) in RPMI medium 1640.

For LPS and HMGB1 interaction, cells were treated with or without LPS (5 μg/mL) alone or LPS (5 μg/mL) plus HMGB1 (10 μg/mL), in the presence or not of heparin (3 μg/mL) or NAH (10 μg/mL) for 2 h. To assess LPS and caspase11 interaction, cells were primed with 100ng/mL LPS for 4 h before this treatment. After fixation with 4% formaldehyde and permeabilization with 0.1%Triton, cells were incubated over night with primary antibody pair of different species directed to LPS (mouse monoclonal 2D7/1), HMGB1 (rabbit monoclonal EPR3507) or caspase11 (rat monoclonal 17D9).

In situ PLA was performed according to manufacturer's instructions. Briefly, after incubation with primary antibodies, the cells were incubated with a combination of corresponding PLA probes, secondary antibodies conjugated to oligonucleotides (mouse MINUS and rabbit PLUS for LPS and HMGB1 interaction, mouse PLUS and rat MINUS for LPS and caspase11 interaction). Subsequently,

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ligase was added forming circular DNA strands when PLA probes were bound in close proximity, along with polymerase and oligonucleotides to allow rolling circle amplification. Fluorescently labeled probes complementary to sequence to the rolling circle amplification product was hybridized to the rolling circle amplification product. Images were taken using a Nikon Ni-U microscope and quantified using Image-J software.

Surface plasmon resonance

The equilibrium-binding constant (KD) of heparin or NAH and HMGB1 was determined by Open SPR conducted using a BIAcore4000 instrument (BIAcore). In brief, HMGB1 protein (10 μg/mL in 200μl) was covalently immobilized on COOH-sensor chips by the EDC/NHS chemistry. Then, heparin or NAH was continuously diluted into several different concentrations using the running buffer and injected into the chip from low to high concentrations. In each cycle, a 200 μl sample was flowed through the chip for 4-6 min at a constant flow rate of 20 μl/min. After detection, 0.05% SDS was added to dissociate the peptides from target protein. Finally, the kinetic parameters of the binding reactions were calculated and analyzed by Trace Drawer software (Ridgeview Instruments AB, The Kingdom of Sweden). To evaluate if heparin or NAH can inhibit the binding of LPS and HMGB1, LPS was diluted in running buffer and passed over the sensor chip immobilized HMGB1 protein at a flow rate of 20 μl/min, then heparin (200μM) or NAH (200μM) was injected into the chip. An activated and blocked flow-cell without immobilized ligand was used to evaluate nonspecific binding.

RNAi Silencing

For siRNA Silencing of CASP4, THP1 were cultured in 12-well plates (3×10^5 cells/well), primed by PMA (100ng/mL) for 12 h before siRNA transfection was performed using the Lipofectamine RNAi MAX Transfection Reagent (Thermo Fisher Scientific, #13778150) by following the manufacturer's instructions. 72 h after transfection, cells were stimulated with LPS alone or LPS plus HMGB1 with or without indicated doses of heparin or NAH. The siRNA target sequences are TCTACACTATAGTCCAGACCC (CASP4-1), GTCTGGACTATAGTGTAGATG (CASP4-2) and CGTACGCGGAATACTTCGA (control), which were described previously (Shi et al., 2014). The silencing efficiency was examined by western-blot using the corresponding antibodies.

ELISA and LDH Assay

Mouse serum and macrophage cell culture supernatant samples were analyzed using eBioscience Ready-SET-Go ELISA kit: IL-1α (#88-5019-77), IL-1β (#88-7013-77), TNF-α (#88-7324-77) and IL-6 (#88-7064-77). Human serum and THP-1 cell culture supernatant were analyzed using human IL-1α ELISA MAX Deluxe (Biolegend, #445804), human IL-1β ELISA MAX Deluxe (Biolegend, #437004). PAI-1Mouse Simple Step ELISA Kit (ab197752) and TAT Complexes Mouse ELISA Kit (ab137994) were purchased from Abcam. Mouse serum samples were analyzed using Murine CD138 ELISA kit (Diaclone 860.090.192).

Cell death was assessed by LDH Cytotoxicity Assay kit (Beyotime Biotechnology, C0017).

Immuno-blot

Cells and tissue lysates were prepared and protein concentration was determined by the BCA method (Thermo Fisher Scientific, #23227). Samples were separated by 12% SDS-PAGE and transferred onto PVDF membranes (Millipore). Antibodies to mouse GSDMD was used at 1:1000 dilution; caspase-11 were used at 1:500 dilution; IL-1α was used at 1:1000 dilution; IL-1β was used at 1:1000 dilution and caspase-1 were used at 1:1000 dilution. Antibodies to Na-K-ATPase, Lamp1, and Rab7 were used at 1:1000 dilution. Antibodies to human GSDMD was used at 1:1000 dilution; caspase-11 were used at 1:500 dilution; IL-1α was used at 1:1000 dilution and IL-1β was used at 1:1000 dilution. Blots were normalized to β-actin expression.

QUANTIFICATION AND STATISTICAL ANALYSIS

All error bars corresponded to the mean ± SD (human samples: mean ± SEM). For all figures, the number of independent experiments (n) were described in the legend. For quantifications of SD-IVM images,we plotted data from mean fluorescent intensities. For comparison of two groups, two-sided Student's t test was used. For multiple comparisons, one-way ANOVA or two-way ANOVA tests were used followed by post hoc Bonferroni test in two comparison within groups. Survival data were analyzed using the log-rank test and Kaplan-Meier survival curves. A p value < 0.05 was considered statistically significant for all experiments. All data were analyzed using GraphPad Prism software (version 7.01), (GraphPad Software, Inc., La Jolla, CA, USA).

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Supplemental Information

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

Yiting Tang, Xiangyu Wang, Zhaozheng Li, Zhihui He, Xinyu Yang, Xiaoye Cheng, Yue Peng, Qianqian Xue, Yang Bai, Rui Zhang, Kai Zhao, Fang Liang, Xianzhong Xiao, Ulf Andersson, Haichao Wang, Timothy R. Billiar, and Ben Lu

Supplemental information

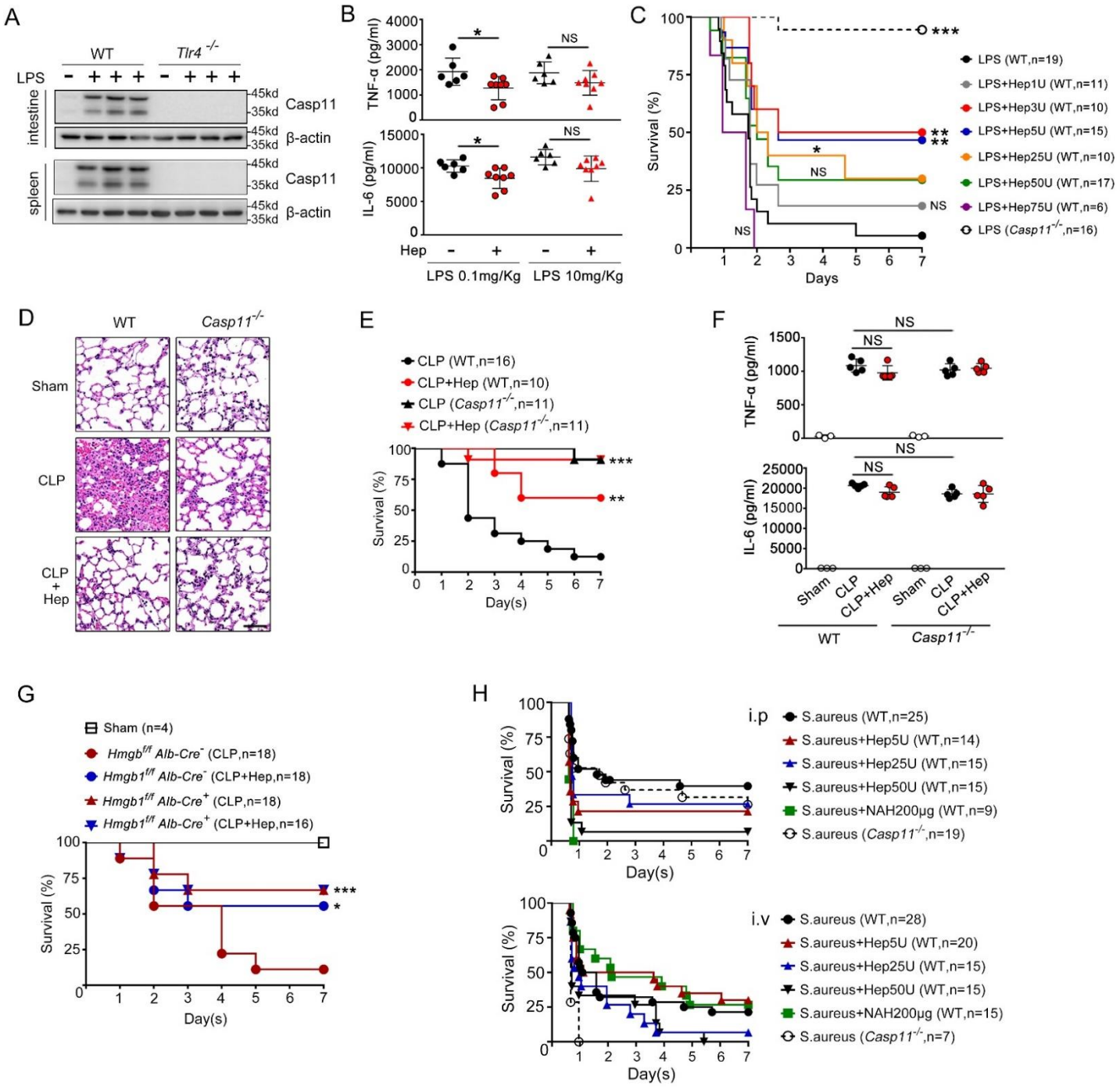

Figure S1. Heparin treatment prevents caspase-11-dependent immune responses and lethality in sepsis. Related to Figure 1.

(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).

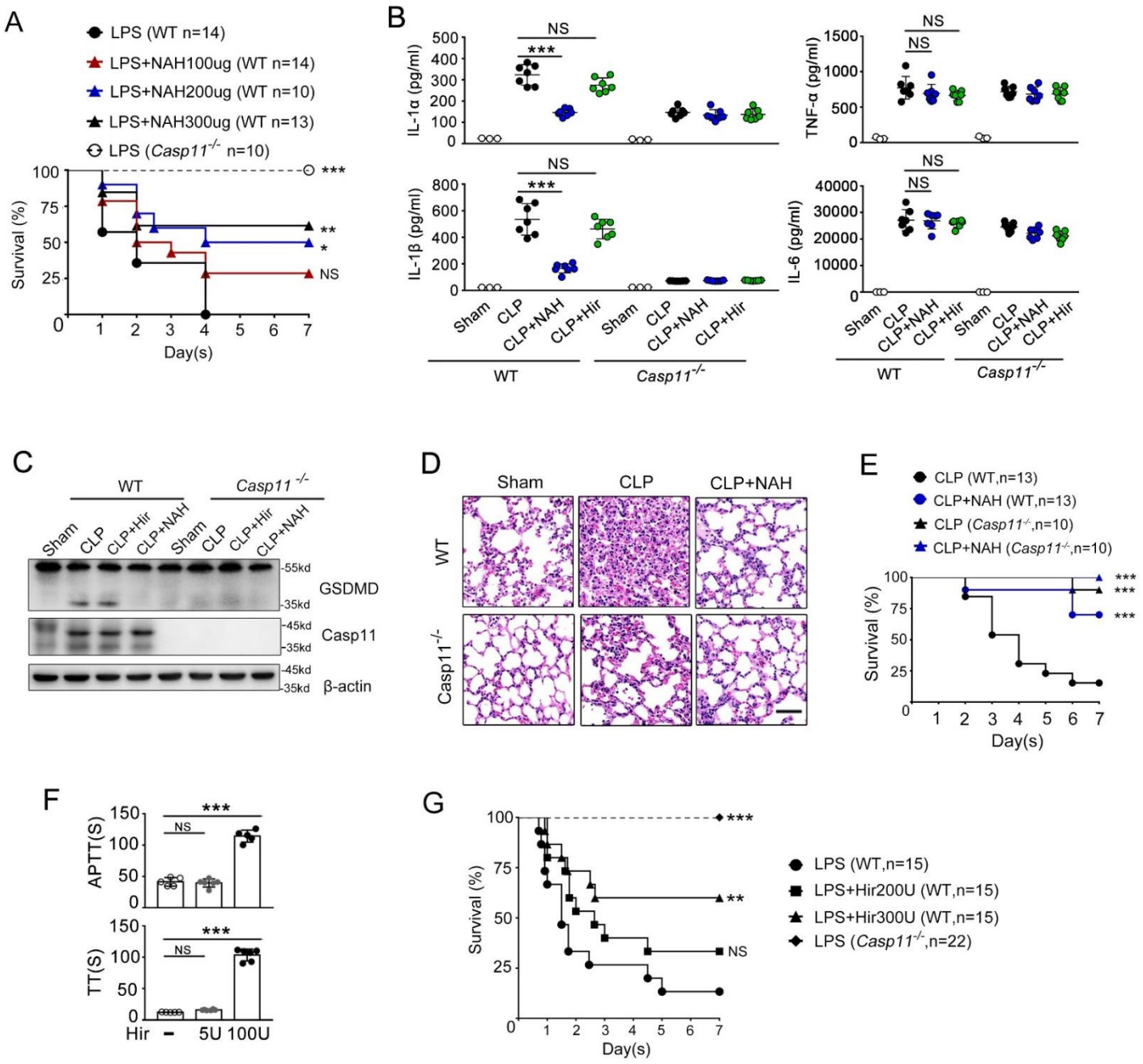

Figure S2. Heparin treatment inhibits caspase-11 activation in sepsis independent of its anticoagulant properties. Related to Figure 2.

(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).

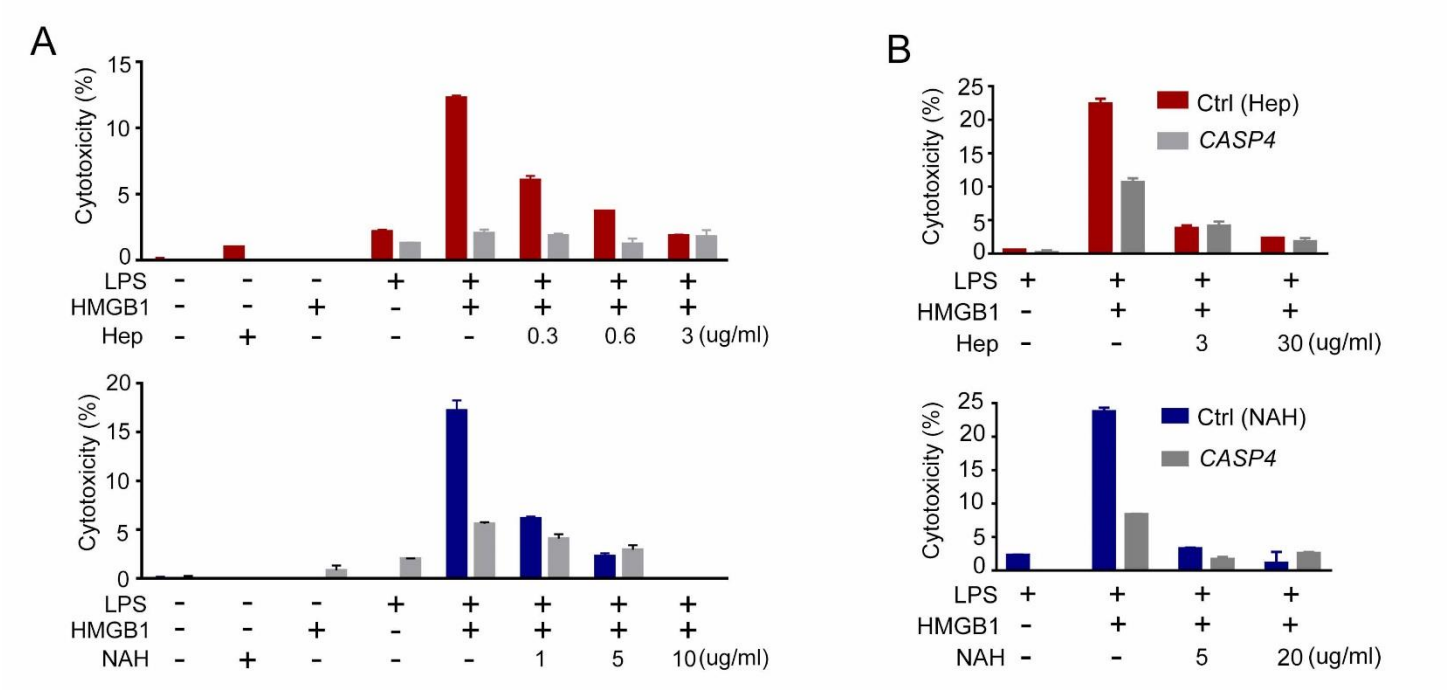

Figure S3. Heparin or NAH inhibits recombinant HMGB1- and caspase-11-dependent immune responses in vitro. Related to Figure 3.

(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.

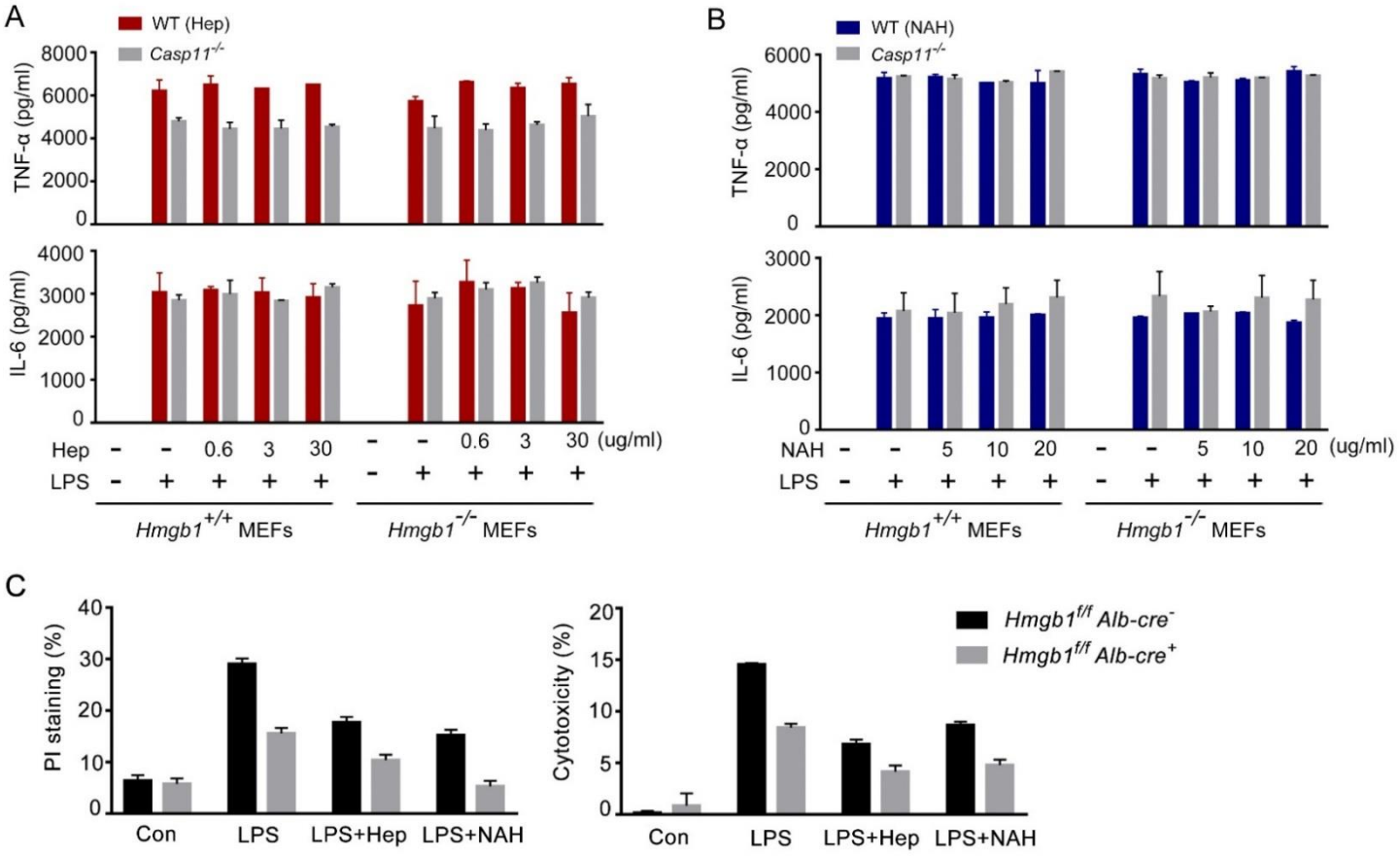

Figure S4. Heparin or NAH selectively inhibits endogenous HMGB1- and caspase-11-dependent immune responses in vitro. Related to Figure 4.

(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.

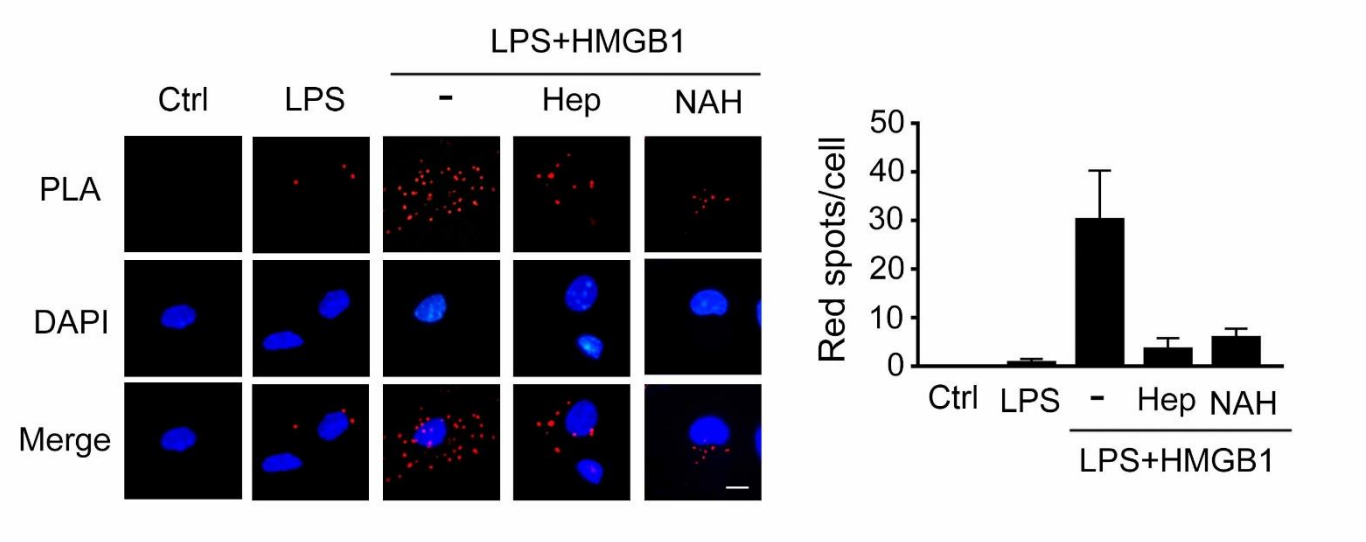

Figure S5. Heparin and NAH decreases the number of HMGB1-LPS complexes detected on the cell surface or inside cells. Related to Figure 6.

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.

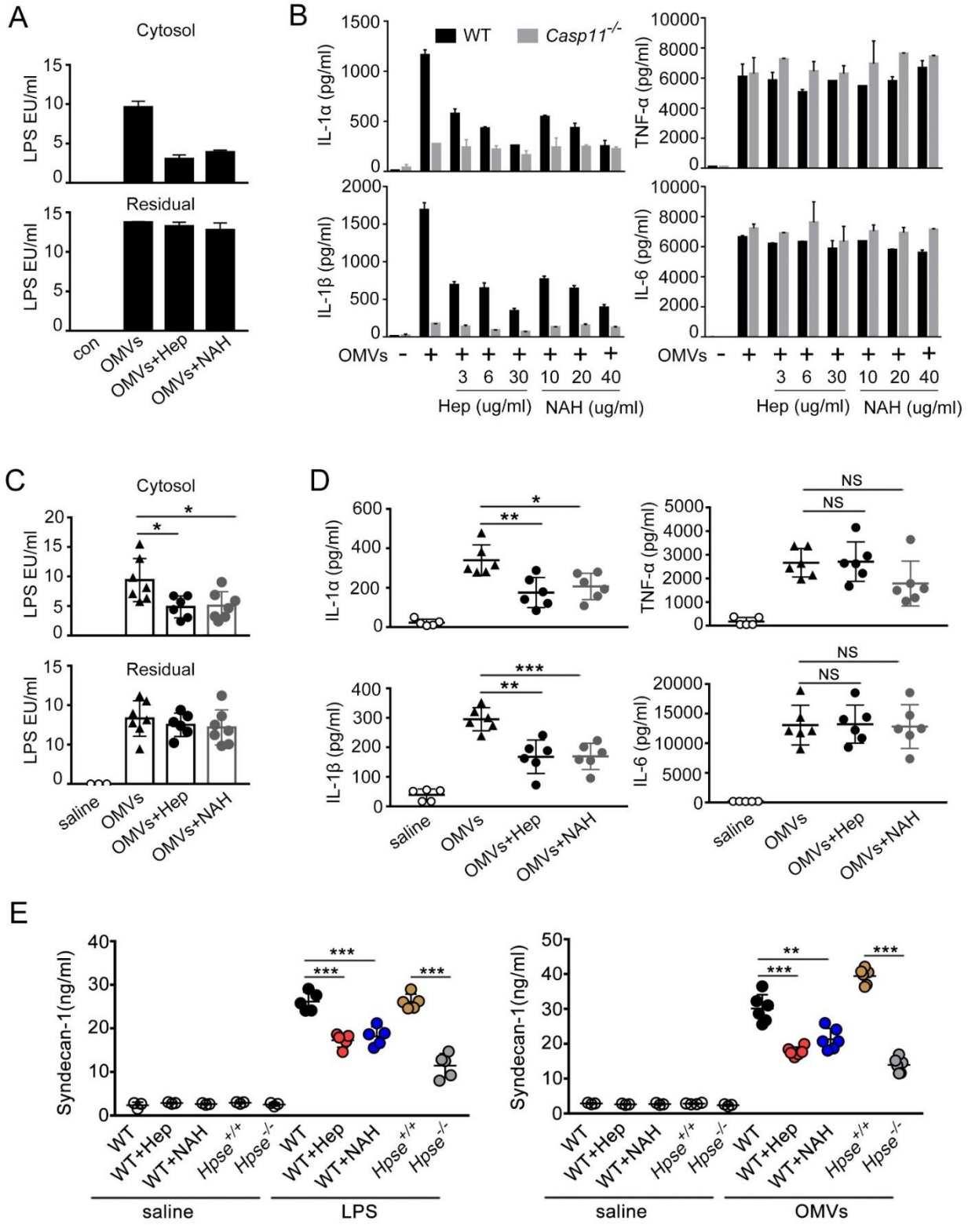

Figure S6. Heparin or NAH inhibits the cytosolic delivery of LPS and prevents heparanase-mediated glycocalyx degradation. Related to Figure 7.

(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