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Research Paper

NOX2 decoy peptides disrupt trauma-mediated neutrophil immunosuppression and protect against lethal peritonitis

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ABSTRACT

Trauma and sepsis are frequent causes of immunosuppression and risk of secondary bacterial infections and mortality among critically ill patients. Reduced activity of neutrophil NADPH oxidase 2 (NOX2) and impaired bacterial killing are among the major indices of immunosuppression. We hypothesize that NOX2-decoy peptides disrupt the inhibition of neutrophil NOX2 by plasma of patients with severe trauma and immunosuppression, thereby preserving the neutrophil respiratory burst that is a central antimicrobial mechanism. We demonstrate that plasma from trauma/hemorrhage (T/H) patients, but not healthy donors (HD), significantly reduced the activity of neutrophil NOX2 and impaired bacterial killing. The inhibitory action of plasma was associated with an increase in bacterial infections among trauma survivors. High Mobility Group Box 1 (HMGB1) is a mediator of lethality in trauma and sepsis and our mechanistic studies revealed that disulfide and oxidized forms of HMGB1 bind to the $gp91^{phox}$ subunit of NOX2, and thus decrease the neutrophil respiratory burst and bacterial killing. NOX2 decoy Anti-Immunosuppression (Ai) Peptides 1 and 3 effectively disrupted the immunosuppressive action of T/H plasma. HMGB1 selectively binds to Ai-Peptide 3, supporting the possibility for direct interaction between HMGB1 and the third external loop of $gp91^{phox}$. *In vivo*, Ai-Peptides improved survival of mice subjected to lethal peritonitis. Taken together, plasma-dependent inhibition of neutrophil NOX2 appeared to be a suitable indicator of immunosuppression in patients with severe trauma. Given that $gp91^{phox}$ decoys protected the neutrophil respiratory burst, selected Ai-Peptides have therapeutic potential to reduce bacterial infections and end-organ injury associated with sepsis/trauma-induced immunosuppression.

1. Introduction

Trauma, blood loss, and severe infections are life-threatening conditions characterized by exaggerated immune responses and multi organ injury [1]. Limited therapeutic interventions are linked to high in-hospital mortality among critically ill patients [2,3]. This includes patients that initially survived trauma and sepsis, but died later during the immunosuppressive phase [4,5]. Immunosuppression is regarded as immune dysfunction frequently linked to dissemination of primary infections and increased risk of secondary, often nosocomial, infections [1]. Neutrophils are professional phagocytic cells that play a central role in innate immune responses to infection and tissue injury, as well as the wound healing/repair processes [6]. Importantly, neutrophil dysfunction is associated with immunosuppression [7–9]. The respiratory burst, a rapid production and release of reactive oxygen species (ROS), is a primary mechanism by which neutrophils utilize nicotinamide adenine dinucleotide phosphate oxidase (NADPH) oxidase (NOX) 2 to eradicate pathogenic bacteria [10]. NOX2 is a membrane-bound enzyme complex that consists of several subunits, including transmembrane $gp91^{phox}$ and $p22^{phox}$ (flavocytochrome b_{558}), cytosolic $p40^{phox}$, $p47^{phox}$, $p67^{phox}$ and regulatory Rac2 [11]. The respiratory burst is activated by bacterial peptide, formyl-Met-Leu-Phe, that stimulates rapid NOX2 complex assembly and production of superoxide ($O_2^{\bullet-}$) *via* NADPH-dependent oxygen reduction. NOX2 inactivation is highly disadvantageous for the host immune responses to infections, as seen in individuals with hereditary chronic granulomatous disease [12,

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13]. NOX2 deficiency also compromises the host's ability to kill bacteria in experimental models of polymicrobial intra-abdominal sepsis [14]. Interestingly, loss of NOX2 activity has also been implicated in increased severity of sterile inflammatory conditions, as evidenced in pre-clinical models of endotoxin-induced acute lung injury (ALI) [15,16]. Although NOX2 activity is frequently impaired in polymicrobial sepsis and among trauma survivors, the mechanism/s by which plasma mediators inhibit neutrophil NOX2 is not delineated.

Damage Associated Molecular Pattern (DAMP) proteins are host-derived mediators that affect immune responses to infection and trauma, but also contribute to autoimmune disorders and carcinogenesis [17]. HMGB1, one of the first discovered DAMPs, has been implicated in regulating innate and adaptive immunity and organ injury [18,19]. In sepsis, the extent of HMGB1 accumulation in plasma has been correlated with severity and mortality in critically ill patients [20]. We have previously reported that HMGB1 has an adverse impact on bacterial eradication and clearance of apoptotic cells, thus it may interfere with resolution of infections and organ injury [21,22]. While HMGB1 is a known mediator of lethality in patients with severe sepsis or trauma, less is known about its contribution to development of immunosuppression and susceptibility to secondary bacterial infections.

In this study, we tested if NOX2-decoy peptide strategy prevents inhibition of the neutrophil respiratory burst by plasma of trauma/hemorrhage patients, and if HMGB1 contributes to this neutrophil immunosuppressive phenotype. Besides mechanistic insights into the regulation of NOX2 activity by plasma of trauma patients, our study aims to test the therapeutic potential of Anti-Immunosuppression (Ai) Peptides, including improved survival of mice subjected to a lethal bacterial peritonitis.

2. Materials and methods

2.1. Human plasma

This study was approved by the UAB Institutional Review Board. Trauma patients were admitted to the UAB from December 2016 to December 2019. Total 68 plasma samples were collected from 31 patients (Supplementary Table 1) with severe trauma at the time of admission to hospital, and 3 or 7 days afterwords. Control plasmas were obtained from 12 healthy donors (HD). Patients included in the study were severely injured and met the local criteria for highest level trauma activation at our level I trauma center. Our site-specific criteria, reviewed by the American College of Surgeons, are based on systolic blood pressure of <90 mmHg, respiratory rate of <10 or >29/min, on the presence of advanced airway management (LMA, COMBITUBE, endotracheal tube), on a Glasgow Coma Score of <9 and mechanism of injury and on non-extremity gunshot wounds (neck, torso, abdomen, back or pelvic region). These criteria are used clinically to ensure the trauma team is present before these critically injured patients arrive at the emergency department. The research assistant was notified along with the trauma team. The goal was to rapidly treat these patients with severe hemorrhage who were nonmoribund, regardless of injury type.

Patients were excluded if they were <18 years old, consent was denied, not returned or not obtainable due to no family available for consenting. Also, if patients had a suspected inhalation injury or burns, a bleeding diathesis, were known to take anti-coagulant medications, had known liver disease, known pregnancy, were incarcerated, expected to expire within 1 h of admission or had a known do-not-resuscitate order prior to enrollment in the study. Of note, four patients with diabetes mellitus type 2 (T2DM) were also excluded because T2DM is associated with reduced (low) activation of NOX2. A blood sample (17.5 ml) was drawn after admission to the Emergency Department, but before fluid resuscitation and 3 and 7 days after admission to the hospital. Whole blood was collected *via* a central line in acid citrate dextrose (ACD) vacutainers. Plasma samples were separated and stored for up to 24 h at 4 °C. Next, plasma samples were frozen in liquid nitrogen, and kept at −80 °C for long-term storage.

2.2. Mice

All experiments were conducted in accordance with protocols approved by the UAB Institutional Animal Care and Use Committee. Male C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice 10–12 weeks of age were used for experiments. Mice were given food and water ad libitum and kept on a 12-h light-dark cycle. Mouse models for peritonitis, sepsis-induced immunosuppression, and bacterial killing *in vivo* are provided in the Supplementary Materials and Methods.

2.3. Gp91-decoy peptides

Ai-Peptides: PPKFFYTRKLL; VNARVNNSDPYSVALSELGDRQNESYLNFARKRIKNP; and RIVRGQTAESLAVHNITVCEQKISEWGKIKECPI, were designed to amino acid sequences corresponding to the three external transmembrane loops of $gp91^{phox}$, NOX2 subunit. In selected experiments N-terminal Biotin-tagged Ai-Peptides 1 and 2 were used. All peptides were synthetized and purified (HPLC, 95%) by Sigma-Aldrich (St. Louis, MO).

2.4. Neutrophil respiratory burst

The neutrophil respiratory burst was measured using cytochrome C reduction assay, as described previously [21]. Briefly, human neutrophils (blood neutrophils or HL-60 neutrophil cell line; 8×10^5) suspended in phenol free RPMI-1640 media (serum free; 200 μl) were incubated humidified incubator for 30 min, at 37 °C (5% CO_2). Next, HD or T/H plasma (500 μg/ml) was added to the neutrophil culture for an additional 30 min. Respiratory burst was measured after inclusion of Cytochrome C (160 μM) and PMA (16 μM). Cytochrome C reduction was recorded at Abs 550 nm for 10–15 min, using Evolution 201 UV–visible spectrophotometer (Thermo Fisher Scientific, Waltham, MA). To determine the effect of Ai-Peptides, plasma samples (500 μg/ml) were prepared in serum free media (RPMI-1640) and incubated with Ai-Peptides (0 or 2.5 μg/ml) for 30 min, followed by inclusion of neutrophils. The impact of HMGB1 (recombinant) on NOX2 activity was evaluated using mouse peritoneal (mature) isolated from 2 to 3 mice/per experiment. Peritoneal neutrophils (8×10^5 cells/sample) were incubated with HMGB1 (0 or 1 μg/ml) for 20 min at 37 °C, followed by stimulation with PMA (16 μM) and recording the NOX2 activity rates using cytochrome C reduction assay.

2.5. Neutrophil-dependent bacterial killing

Neutrophils (2.5×10^5) in serum free media (RPMI-1640) were pre-incubated with HD or T/H plasma (500 μg/ml) for 30 min at 37 °C (5% CO_2). Bacterial killing was initiated by inclusion of ampicillin-resistant *E. coli* (DH5α; ampicillin resistant; 5×10^5) with 2:1 bacteria/neutrophil ratio (500 μl total volume), and samples were incubated for an additional 90 min, similar to a previously described method [23]. Next, 20 μl of cell/bacterial suspension was incubated with 480 μl Triton X-100 (0.1%) for 10 min to lyse neutrophils. Serial dilutions in PBS were plated on Luria-Bertani agar with ampicillin and incubated overnight at 37 °C. Colony forming units (CFUs) were counted the following day. In selected experiments, T/H plasma (500 μg/ml) was pre-treated with Ai-Peptides (0 or 2.5 μg each/ml) for 30 min prior to inclusion of neutrophils.

2.6. Statistical analysis

Multigroup comparisons were performed using one-way ANOVA with Tukey's post hoc test. Values were normally distributed. For comparisons between two groups, statistical significance was

determined using the Student's *t*-test. Kaplan-Meier survival curve Log-rank (Mentel-Cox) test was used to determine mice survival. Analyses were performed with Microsoft Excel and Prism (GraphPad; version 8.4.2). A value of $P < 0.05$ was consider significant.

3. Results

3.1. Plasma-dependent inhibition of neutrophil-respiratory burst is associated with development of secondary infections among trauma survivors

Plasma samples were obtained from 31 T/H patients at the day of admission to hospital, and 3- or 7-days afterword. In total, 68 plasma samples were collected from trauma patients, and 12 healthy donors (HD). Patients were matched in regard to age between HD (35 ± 3.1 s.e.m.) *vs.* T/H (42 ± 7.9 s.e.m.) individuals. Gender distribution was 50% in HD, and 71% *vs.* 29% male/female in T/H group, due to a higher prevalence of injury among males (Supplementary Table 1). In the T/H group, correlation between injury severity score and secondary bacterial infections was significant, including pneumonia and intra-abdominal infections (34.1 ± 5.2 s.e.m.) *vs.* none (17.1 ± 2.07 s.e.m.), $^*P < 0.05$. (Student's *t*-test).

In the first set of experiments, we investigated if plasma of healthy donors and trauma/hemorrhage patients affect neutrophil respiratory burst, *ex vivo* (Fig. 1a). Respiratory burst was measured using a well-established, superoxide specific, cytochrome c reduction assay. Human neutrophils (differentiated HL-60) were incubated with plasma for 20 min followed by activation of NOX2 after stimulation with PMA. Cytochrome c reduction rates were recorded over indicated time (Fig. 1b). More than half of the plasma samples (51.4%) obtained from T/H patients effectively reduced the rate of NOX2 activity, as compared to HD groups (Fig. 1c). The inhibitory effects of T/H plasma was confirmed

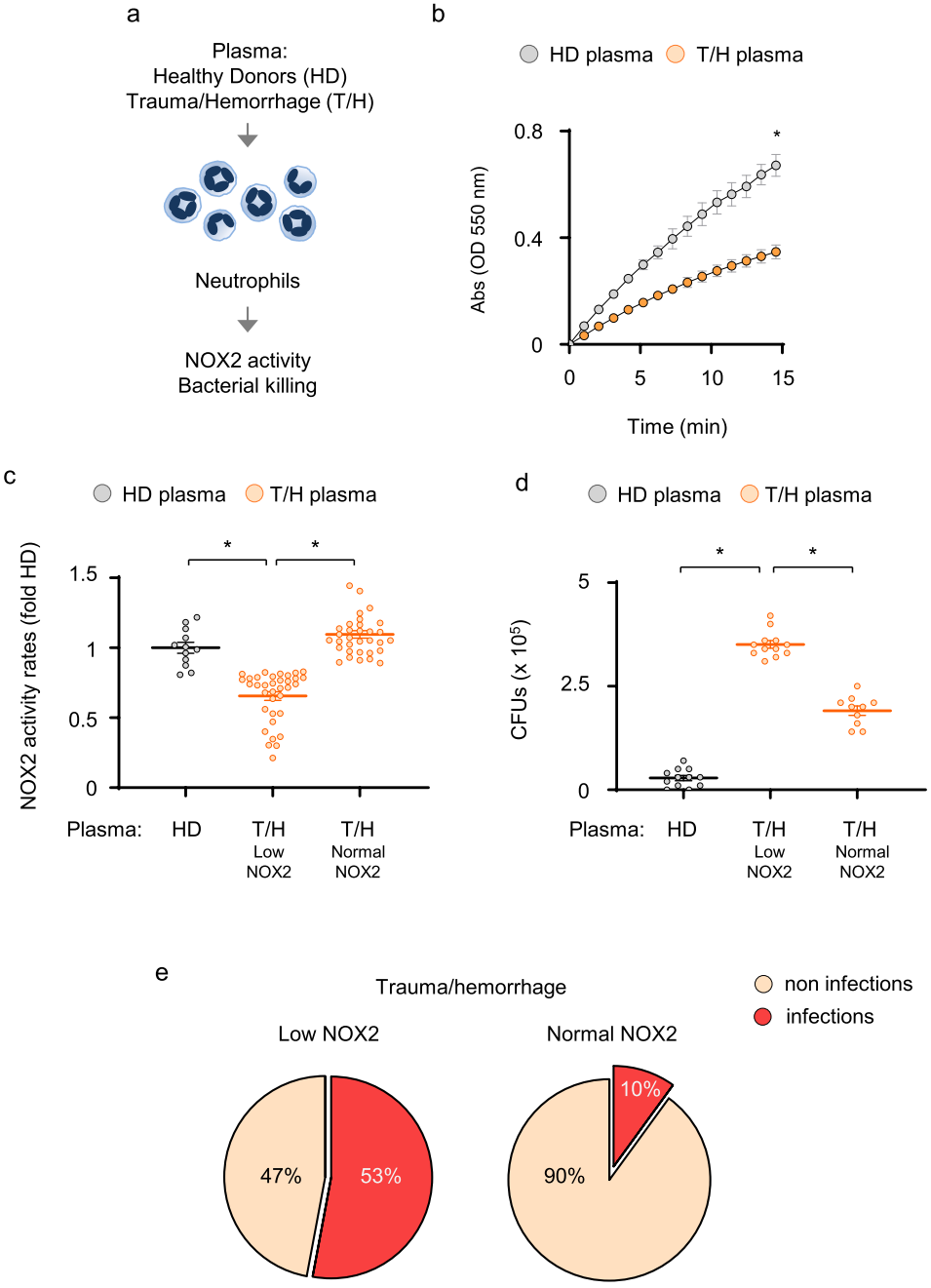

Fig. 1. Plasma-dependent inhibition of the neutrophil respiratory burst is associated with development of secondary infections among trauma survivors. (**a**) Outline of experiments for HD or T/H plasma, neutrophil NOX2 activity and bacterial killing. (**b**) Representative NOX2 activity rates in human neutrophils (HL-60) incubated with HD or T/H plasma. Data presented as means ± s.e.m, (Abs-absorbance), $n = 3$ technical repetitions using a selected plasma. $^*P < 0.05$ (Student's *t*-test). (**c**) A number HD and T/H plasma that elicited neutrophil NOX2 normal or low activity rates. Means ± s.e.m (fold HD), $n = 12$ HD plasma samples, $n = 68$ H/T group. $^*P < 0.05$ (ANOVA). (**d**) The effects of HD and T/H plasma on neutrophil-dependent *E. coli* killing, *ex vivo*. Data presented as neutrophil NOX2 activity rates (fold HD), in regards to T/H plasma that decreased rates (low NOX2), or had no effects (normal NOX2). CFU-colony forming units. Means ± s.e.m, $n = 12$ HD, $n = 12$ H/T-low NOX2, $n = 10$ H/T -normal NOX2 activation in response to T/H plasma. (**e**) Percentages of patients with bacterial infections, in regards to low versus normal neutrophil NOX2 activation by T/H plasma. Mean ± s.e.m., $n = 17$ low NOX2, $n = 10$ normal NOX2. $^*P < 0.05$ (ANOVA).

using normal blood PMNs from HD group (*Supplementary* Fig. S1a). While T/H plasma reduced the neutrophil respiratory burst, this effect is diminished after heat-inactivation of plasma for 10 min, at 95 °C (*Supplementary* Fig. S1b). Next, outcome of the T/H plasma inhibitory action was determined in regards to neutrophil-dependent bacterial killing. Consistent with the decrease levels in NOX2 activity, T/H plasma also diminished neutrophil capacity to kill *E.coli*, as evidenced by increased bacterial colony forming units (CFUs) on agar plates (Fig. 1d). These findings suggest that inhibitory impact of T/H plasma may be linked to development of bacterial infections in the T/H group. Additional analysis indicates that 53% of patients with inhibitory plasma developed bacterial infections, including pneumonia and UTI, as compared to 10% of trauma patients with modest or no effects of plasma (Fig. 1e). HD or T/H plasma did not alter neutrophil antioxidant responses, including NQO1 or GCLC levels and similar amounts of superoxide dismutase (SOD) were in HD and TH plasma (*Supplementary* Figs. S1c and d). These results suggest that specific mediators of immunosuppression in T/H plasma mediated decrease of NOX2 activity and bacterial infections among trauma survivors.

3.2. HMGB1 contributes to sepsis-induced immunosuppression

We next investigated if DAMPs, in particular HMGB1, affect NOX2 activity during sepsis-induced immunosuppression. Previous clinical studies have shown a prolong accumulation HMGB1 in circulation of sepsis survivors [24]. Mice with immunosuppression, *i.e.* seven days after CLP, had significant decrease of bacterial killing (*P. aeruginosa* strain K; PAK) in lungs (Fig. 2a), and immunosuppression was associated with a robust increase of HMGB1 levels in bronchoalveolar lavages (BALs), as compared to sham group (Fig. 2b, c). To test if HMGB1 affects bacterial killing, mice were subjected to intratracheal administration of recombinant HMGB1 (50 μg/mouse; i.t.) or vehicle (PBS), followed by exposure to lethal amounts of PAK (2×10^7/mouse, i.t.). Intratracheal injection of HMGB1 reduced the ability of host to kill PAK, as indicated by increased CFUs from lung homogenates (Fig. 2d). Because the PAK lethal dose, all mice dyed within 8 h after exposure to PAK, regardless of HMGB1 administration.

Additional, *ex vivo* results confirmed that, direct incubation of neutrophils with HMGB1 (disulfide) decreased the rate of NOX2 activity after stimulation with PMA (Fig. 2e). These results indicate that HMGB1 can decrease NOX2 activity and neutrophil antibacterial capacity during sepsis-induced immunosuppression.

3.3. Oxidized HMGB1 inhibits neutrophil respiratory burst

HMGB1 redox status (Fig. 3a) is known to affect its biological impact, including activation of chemotaxis by reduced form and cytokine production stimulated by disulfide HMGB1. HMGB1 preferentially forms a

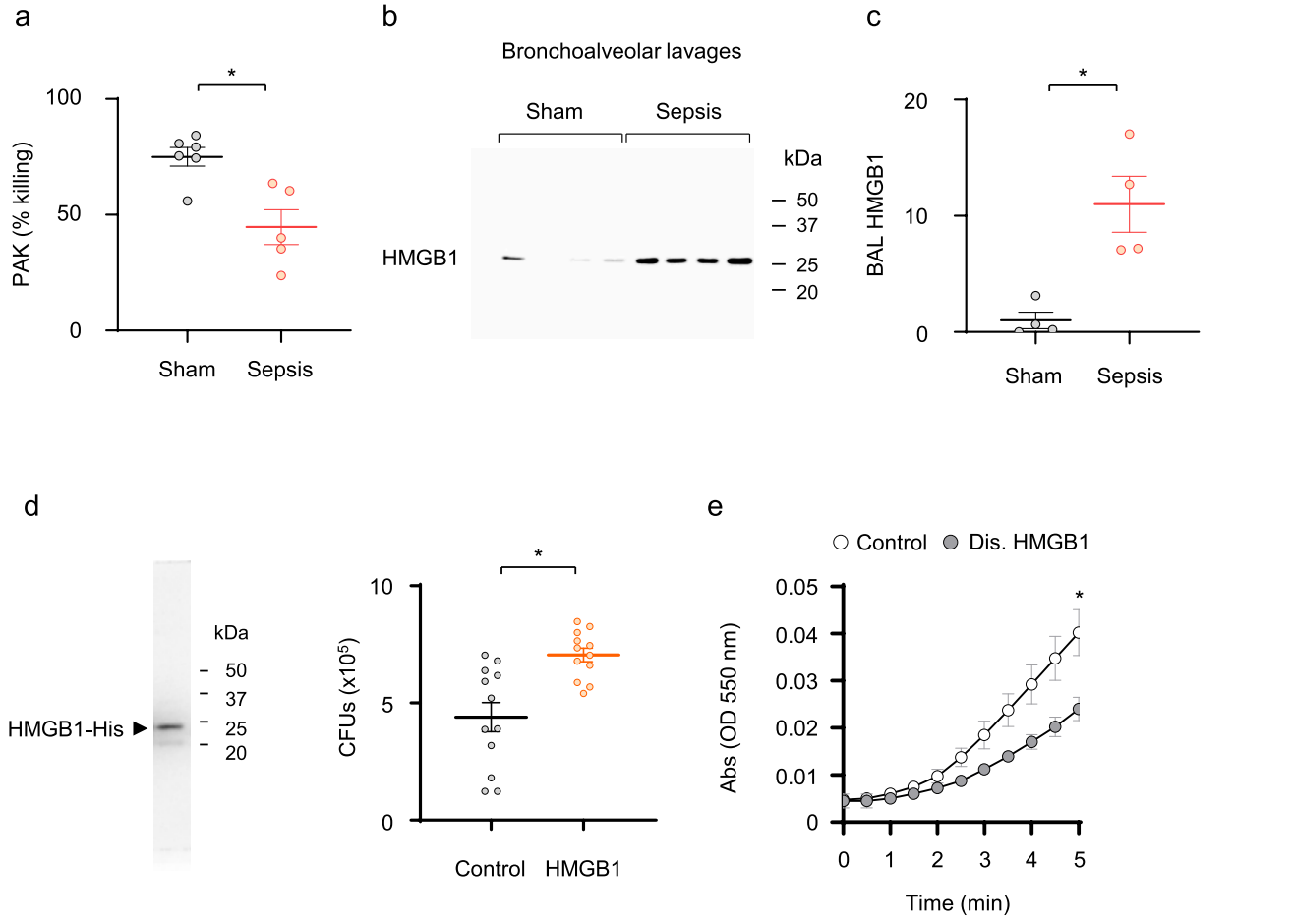

Fig. 2. HMGB1 contributes to sepsis-induced immunosuppression. (**a**) The extent of PAK killing in lungs of sham and mice subjected to CLP (sepsis). Data presented as mean ± s.e.m., $n = 5$–6. *$P < 0.05$ (ANOVA). (**b**,**c**) Western blot and optical bend densitometry of HMGB1 in BALs of indicated groups of mice. Mean ± s.e.m., $n = 4$. *$P < 0.05$ (ANOVA). (**d**) Representative western blot showing HMGB1 recombinant protein used for intratracheal installation (25 μg; i.t) in mice, followed by PAK-induced pneumonia. PAK (CFUs) in lung homogenates of control and HMGB1-treated mice are shown. Mean ± s.e.m., $n = 12$ mice per indicated groups. *$P < 0.05$ (Student's *t*-test). (**e**) Traces of NOX2 activity (PMA induced) in mouse peritoneal neutrophils that were incubated with or without recombinant Dis-HMGB1 (disulfide HMGB1; 1 μg/ml) for 20 min. Data presented as fold untreated, mean ± s.e.m., $n = 3$ technical repetitions per group. *$P < 0.05$ (Student's *t*-test).

disulfide bond between Cys_{23} and Cys_{45} within BOX A domain. Oxidative stress targets oxidation of cysteine thiol Cys_{106} in BOX B domain; this oxidation has been shown to diminished HMGB1 retention in the nucleus [27]. We tested if oxidized or reduced HMGB1 affects NOX2 activity. Oxidized HMGB1 (*s*-glutathionylated; GSS-HMGB1 adduct) and reduced HMGB1 (all reduced Cysteine thiols) were generated *ex vivo* (see *Materials and Methods*). GSS-HMGB1 adduct formation was confirmed using non-reduced/reduced PAGE and Western blot analysis (Fig. 3b). Reduced HMGB1 had no effects, as similar rate of NOX-dependent superoxide production was observed in control group.

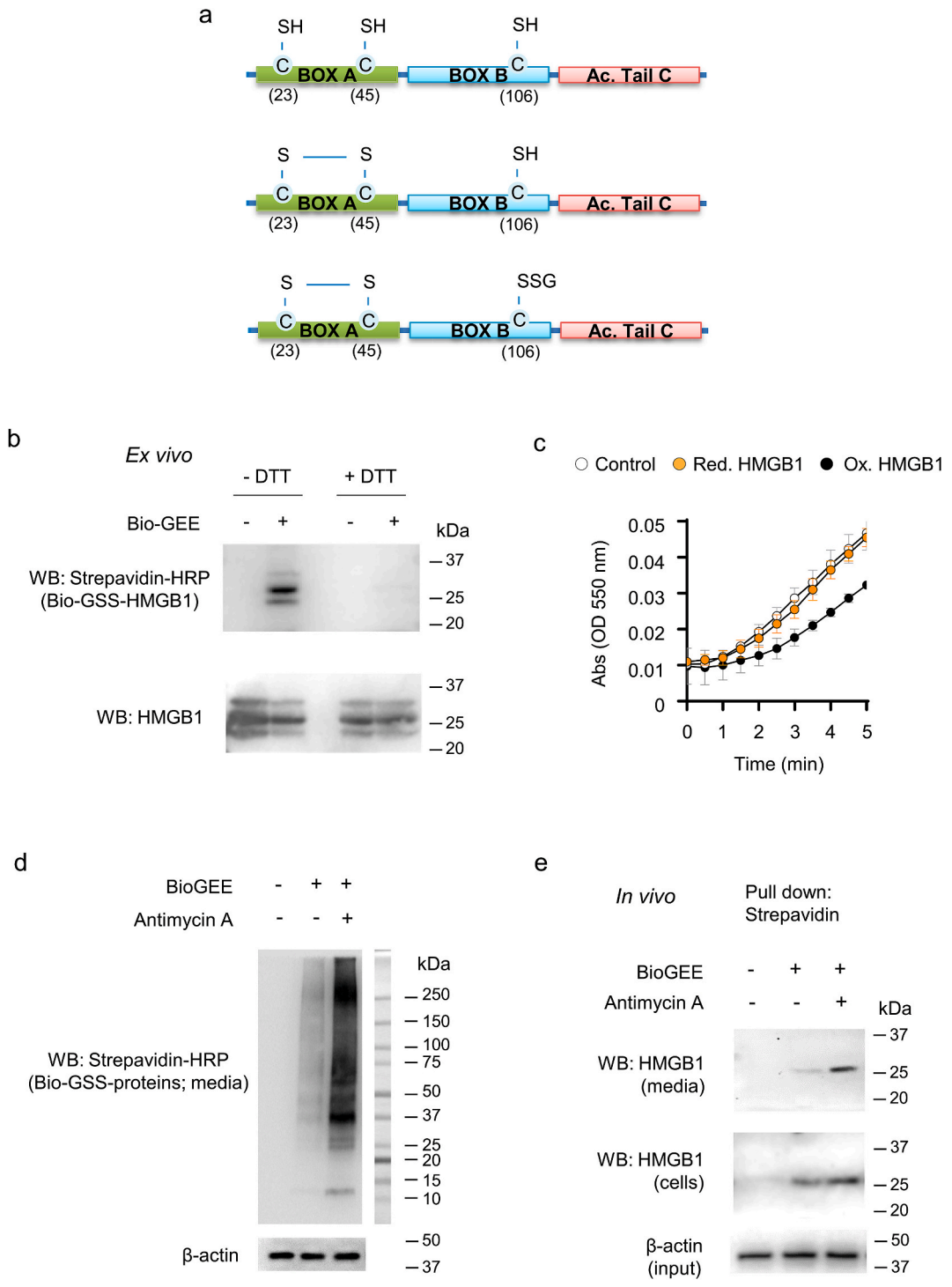

Fig. 3. Oxidized HMGB1 inhibits neutrophil respiratory burst. (**a**) HMGB1 domain organization and cysteine thiol oxidative modifications *via* *s*-glutathionylation. (**b**) Recombinant HMGB1 (disulfide) or Bio-GSS-HMGB1 (50 ng) was incubated with or without DTT followed by non-reducing SDS-PAGE. Representative western blots were developed using Streptavidin-HRP (upper) or anti-HMGB1 IgG (lower). (**c**) Traces of PMA-stimulated NOX2 activity in mouse peritoneal neutrophils that were treated with reduced or oxidized HMGB1 (0 or 1 μg/ml) for 20 min. Mean ± s.e.m., $n = 3$ technical repetitions per indicated groups. $^{*}P < 0.05$ (ANOVA). (**d**) The amounts of Bio-GSS-proteins in media from unaltered (control) and BioGEE-loaded mouse peritoneal macrophages that were treated with or without Antimycin A (5 μM) for 16 h. (**e**) Western blots showing Bio-GSS-HMGB1 adducts in media (upper) and cell lysates (middle) or β-actin (lower). Pull-down with Strepavidin-agarose was performed using cell lysates and media from macrophages that were treated as depicted in (d).

In turn, oxidized HMGB1 significantly diminished NOX2 activation (Fig. 3c). These findings indicate that besides disulfide, oxidized HMGB1 is implicated in development of immunosuppression, as a result of inhibition in the neutrophil respiratory burst. The biological relevance of HMGB1 oxidation (*s*-glutathionylation) can be associated with pro-oxidative conditions, including apoptosis that is linked to mitochondrial ROS production [25]. Indeed, exposure to Antimycin A, a pro-apoptotic compound that inhibits of mitochondrial electron transport chain complex III, robustly increased protein *s*-glutathionylation formation and release from macrophages (Fig. 3d), including the

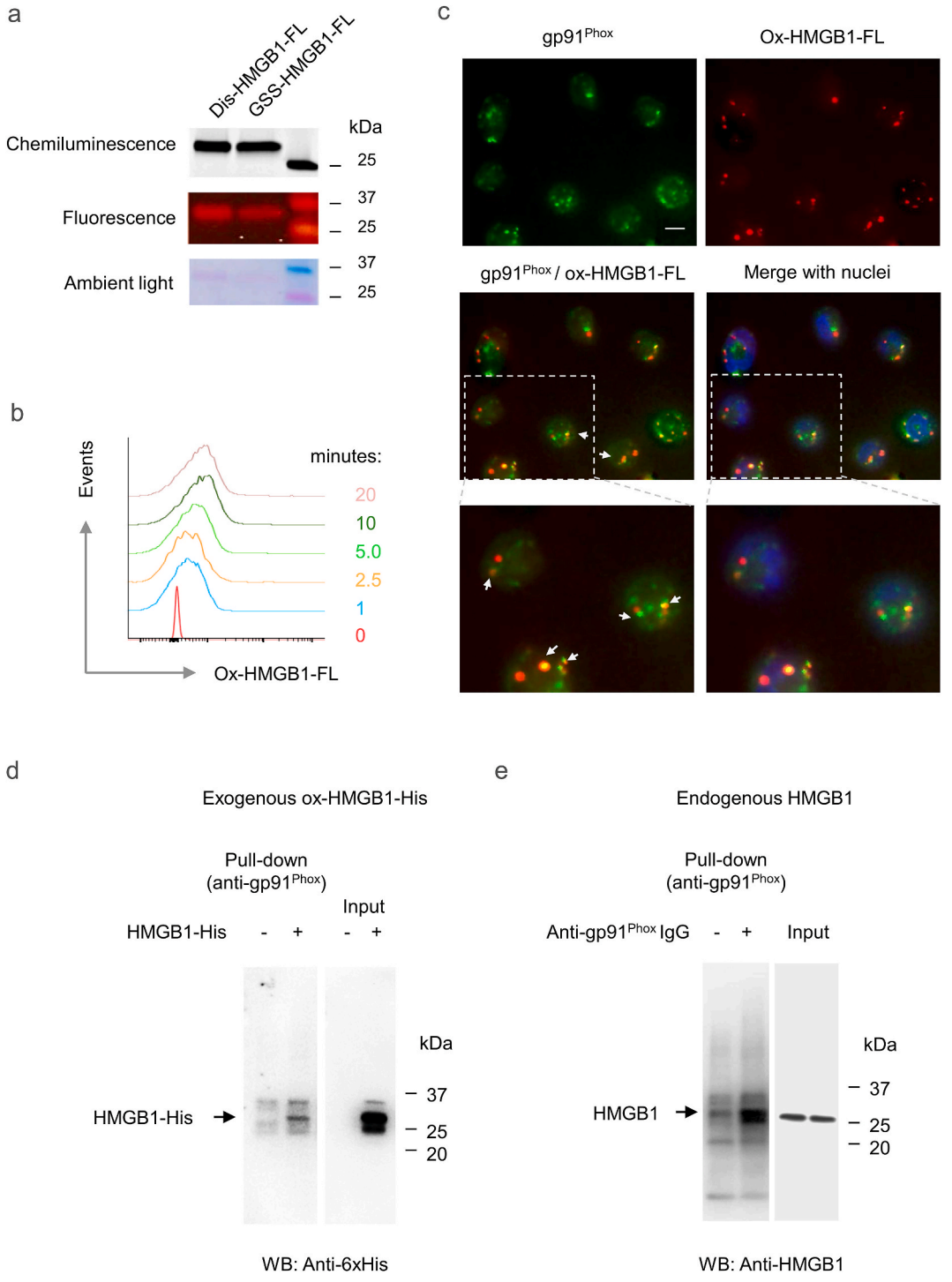

Fig. 4. HMGB1 binds to gp91^{phox} subunit of NOX2. (**a**) Western blot membrane shows fluorescence-labeled HMGB1 (HMGB1-FL) that was detected using chemiluminescence, fluorescence and ambient light imaging methods. (**b**) Flow cytometry analysis indicates time-dependent binding between oxidized HMGB1-FL and neutrophils. (**c**) Representative images show fluorescence patterns (live imaging) of oxidized HMGB1-FL and gp91^{phox} (anti-gp91^{phox} fluorescence IgG) in neutrophils. Selected regions indicated by dashed boxes are enlarged and display below. Ox-HMGB1-FL-red; gp91^{phox} IgG-green; nuclei-blue. Scale bar 20 μm. Arrows indicate co-localization (yellow fluorescence) between ox-HMGB1-FL and gp91^{phox}. (**d**) The amounts of HMGB1 recombinant after pull-down with anti-gp91^{phox} IgG (left membrane) and in cell lysates before immunoprecipitation (Input; right). Cell lysates were from neutrophils treated with oxidized 6His-tagged HMGB1 (0 or 1 μg/ml) for 30 min. (**e**) HMGB1 levels after pull-down with anti-gp91^{phox} IgG (left), and HMGB1 in cell lysates prior to immunoprecipitation (Input; right). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

GSS-HMGB1 (Fig. 3e). Furthermore, we confirmed HMGB1 nucleus-to-cytosol translocation and extracellular release, either from Antimycin A-treated macrophages or after Tunicamycin-mediated ER stress in alveolar epithelial cells (*Supplementary* Fig. S2). These results indicate that epithelial injury and macrophage apoptosis are associated with sepsis-induced lung injury, and that release of oxidized proteins, including HMGB1, can induce the neutrophil immunosuppression phenotype.

3.4. HMGB1 binds to gp91^{phox} subunit of NOX2

Given the ability of HMGB1 to decrease of neutrophil respiratory burst, we tested a possible interaction between GSS-HMGB1 and gp91^{phox}, a transmembrane subunit of NOX2. Fluorescent HMGB1 (GSS-HMGB1-FL) and florescent-conjugated anti-gp91^{phox} antibody were generated, as described in *Materials and Methods*. The GSS-HMGB1 adduct formation was confirmed using non-reducing PAGE and Western blot analysis (Fig. 4a). We established that oxidized HMGB1 is rapidly binding to the neutrophil surface membrane, as evidenced by flow cytometry analysis and time-dependent exposure of live neutrophils to HMGB1 (Fig. 4b). Image analysis indicates yellow fluorescence patterns that evidenced interaction between HMGB1 and gp91^{phox}, although HMGB1 can also bind gp91-independent sites (Fig. 4c). Pull-down experiments show more direct evidence that HMGB1 interacted with gp91^{phox} (Fig. 4d). Notably, endogenous HMGB1 in cell lysates can also bind to gp91^{phox} (Fig. 4e). Results obtained from co-immunoprecipitation experiment and imaging suggest that oxidized and disulfide HMGB1 inhibit NOX2 activity because of direct interaction with gp91^{phox}.

3.5. Gp91^{phox} decoy peptides disrupts inhibitory action of T/H plasma on neutrophil respiratory burst

Three external loops of gp91^{phox} were used to design NOX2 decoy Ai-Peptides (Fig. 5a and b). These peptides have relatively high amino acid homology between human and mice (*Supplementary* Fig. S3). Ai-Peptides were evaluated against T/H plasma inhibitory action on the neutrophil respiratory burst (Fig. 5c). We found that pre-incubation of T/H plasma with Ai-Peptide 1, 3 or simultaneous inclusion of Ai-Peptides 1–3, effectively preserved neutrophil respiratory burst, as compared to neutrophils that were treated with T/H plasma alone (Fig. 5d). In this setting, Peptide 2 had no effect. While use of peptide 1 or 3 alone were partially protected against T/H plasma, combined peptides showed a synergistic impact. The ability of Ai-Peptide 1 and 3 to prevent T/H plasma inhibitory action was consistent with improved bacterial killing by neutrophils *ex vivo*. Notably, while T/H plasma decreased neutrophil capacity to eradicate *E. coli*, this effect was diminished after incubation of plasma with Ai-Peptide 1, 3 or combined Ai-Peptides (Fig. 5e). Mechanistic studies revealed that HMGB1 is directly binding to Ai-Peptide 3, but not Ai-Peptide 1, as evidenced by ELISA assay that utilized biotinylated Ai-Peptides (Fig. 5f). This finding suggests that HMGB1 directly interacts with the third external loop of gp-91^{phox} subunit. These results indicate that NOX2 inhibition is partially dependent on HMGB1, as Ai-Peptide 1 or 3 alone provided incomplete protection against T/H plasma, and HMGB1 is exclusively binding to Ai-Peptide 3. This finding suggests that other molecules can be involved in inhibition of NOX2 by T/H plasma.

Finally, we tested if Ai-Peptides are able to improve mice viability in a murine model of intraperitoneal infections, *i.e. E. coli* -induced lethal peritonitis. Injection of *E. coli* (2×10^8/mouse; i.p.) resulted in rapid accumulation of HMGB1 in peritoneal cavity, and all mice died within 48 h (Fig. 5g and h). Importantly, delayed administration of Ai-Peptides, *i.e.* 2 h after *E. coli* injection, resulted in improved survival. Collectively, these findings suggest that Ai-Peptides have therapeutic potential to improve survival from infections, likely via disruption of the neutrophil immunosuppressive phenotype induced by HMGB1.

4. Discussion

This is the first study, to our knowledge, that demonstrates a benefit of gp-91^{phox} decoy peptide strategy to prevent development of neutrophil immunosuppression after severe sepsis and trauma. *First*, this possibility is supported by an effective implementation of Ai-Peptides to disrupt T/H plasma-inhibitory action on the neutrophil respiratory burst. *Second,* the plasma-induced neutrophil dysfunction is a clinically relevant issue, as this is associated with increased bacterial infections among trauma survivors. *Third*, we provide compelling evidence that Ai-Peptides reduce mortality in mice subjected to lethal intraperitoneal infections. *Fourth*, we identify that disulfide or oxidized HMGB1 are among relevant mediators of neutrophil immunosuppression. *Fifth*, mechanistic studies reveal that HMGB1-dependent inhibition of NOX2 is mediated by a direct interaction with gp-91^{phox}, likely *via* the third loop of gp-91^{phox} external domain.

Our results provide new mechanistic insights into HMGB1 and development of the neutrophil immunosuppressive phenotype. Previous studies have shown that release of oxidized HMGB1 has been linked to tissue injury and intrinsic apoptosis characterized by a robust production of ROS and RNS by mitochondria [26]. Notably, mitochondrial dysfunction in lungs of critically ill patients has been correlated with mortality rates [27,28]. Similarly, mitochondrial dysfunction immune cells has been associated with HMGB1 accumulation in BAL fluid of sepsis-immunosuppressed mice [29]. These studies suggest that HMGB1-mediated development of the neutrophil immunosuppressive phenotype could be indirectly trigger by mitochondrial dysfunction in trauma and sepsis. In this context, HMGB1 oxidation of Cys_{106} appeared to have a regulatory function, *e.g.* promoted HMGB1 nucleus-to-cytosol translocation and extracellular flux [30]. Unlike reduced and disulfide HMGB1, HMGB1 derived from apoptotic cells has been found in an oxidized state and with limited ability to activate immune responses [19,31]. Importantly, inflammatory conditions associated with sepsis have been shown to increase *s*-glutathionylation of HMGB1 [32]. Of note, while pro-oxidative environment in trauma is implicated in oxidation of HMGB1, plasma of T/H patients did not altered NRF2 signaling in neutrophils, *ex vivo*.

Previous studies, including our results, have indicated that extracellular HMGB1 interferes with bacterial killing and impairs neutralization of apoptotic cells, as well as clearance of cellular debris [21,22,33]. While our studies are focused on NOX2-dependent bacterial killing, NOX2 activation can also affect neutrophil pro-inflammatory responses and severity of organ injury, as observed in experimental models [15,16,34]. For example, mice deficient in p47^{phox} or gp91^{phox} subunit developed more severe response to endotoxin-mediated ALI [34]. A benefit of NOX2 activation is mediated by production of hydrogen peroxide (H_2O_2) that is utilized for bacterial eradication. However, H_2O_2 also appeared to have anti-inflammatory effects on neutrophils. This is related to the redox-dependent inhibition of the NF-κB signaling cascade in neutrophils [35–37]. Thus, preservation of NOX2 activity is not only crucial for eradication of pathogenic bacteria, but also to moderate neutrophil pro-inflammatory responses. Such observations indicate that Ai-Peptides affects NOX2-dependent bacterial killing, but also may prevent exaggerated neutrophil pro-inflammatory responses, a relevant issue associated with endotoxin-induced lung injury.

We demonstrated that gp91^{phox} decoy strategy prevents plasma-mediated decrease in the NOX2 activation. However, another strategy may utilize enzymatic capacity of serum glutathione peroxidase 3 (GPx-3), in particular to reverse HMGB1 glutathionylation. Notably, activation of GPx-3 could be relevant, as clinical studies have shown that GPx-3 bioactivity is diminished among patients with sepsis [38]. It is important to note that HMGB1 is not a sole mediator of immunosuppression. Other mechanisms of immunosuppression have been associated with inhibited responses to endotoxins and reduced phagocytosis [9,39]. Moreover, sepsis and trauma are known to have adverse impact on macrophages and T cells, including enhanced apoptosis, as well as

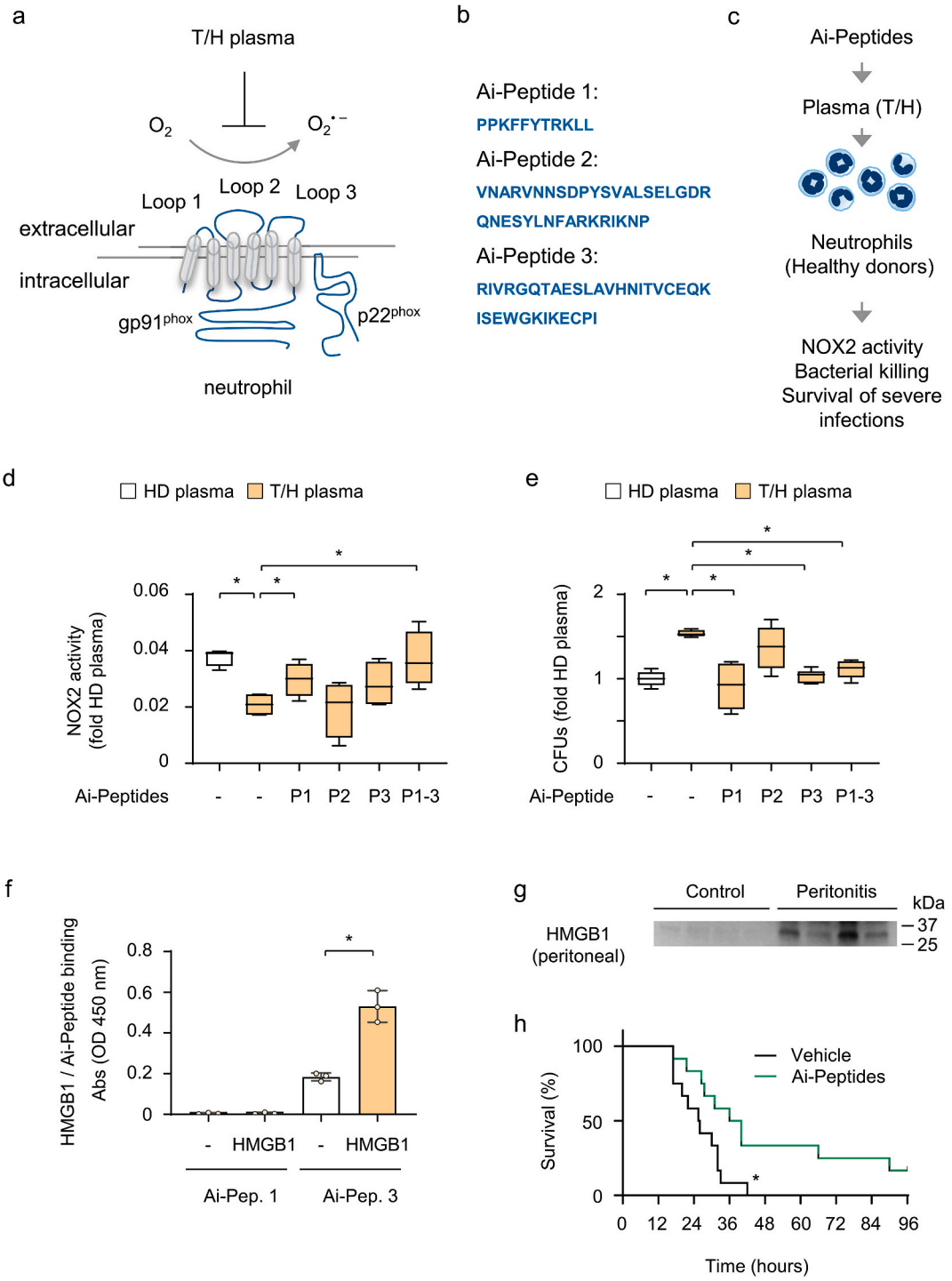

Fig. 5. Ai-Peptides prevent inhibition of neutrophil respiratory burst by T/H plasma. (**a**) Outline of T/H plasma-dependent inhibition of superoxide production by NOX2 in neutrophils. Gp91^{phox} transmembrane and p22^{phox} subunits are shown, with indication of gp91 external loops 1–3 that were used to design Ai-Peptides. (**b**) Ai-Peptides; amino acids sequence. (**c**) Outline to test the effects of Ai-peptides on T/H plasma-dependent inhibition of the neutrophil respiratory burst, bacterial killing and mice survival after lethal peritonitis. (**d**) The effects of Ai-Peptides on T/H plasma-mediated inhibition of neutrophil respiratory burst (NOX2 activity). T/H plasma was pre-incubated with Ai-Peptide 1, 2 or 3, (0 or 2.5 μg/plasma), or combined Ai-Peptides, for 30 min, followed by exposure of neutrophils to T/H plasma (with or without peptides) for additional 30 min. HD plasma (healthy donor, 500 μg/ml) was used as a control. NOX2 activity was determined using cytochrome c reduction assay. (**e**) Bacterial viability (CFUs; fold untreated HD plasma) after incubation of *E. coli* with neutrophils that were treated with T/H plasma and Ai-Peptides *in vitro*, as depicted in (d). (d,e) Data presented as mean ± s.e.m., $n = 5$ per each indicated group. *$P < 0.05$ (ANOVA). (**f**) HMGB1 (recombinant) and Ai-Peptide complex formation (ELISA). Mean ± s.e.m., $n = 3$. *$P < 0.05$ (Student's *t*-test). (**g**) HMGB1 levels in peritoneal lavages from control or mice subjected to *E. coli* (2×10^8; i.p.) -induced peritonitis for 4 h. Representative Western blot is shown, $n = 4$ lavages from indicated groups. (**h**) Improvement of survival in mice subjected to peritonitis and intraperitoneal injection of Ai-Peptides (combined Ai-Peptide 1, 2, and 3; 2.5 μg each/mouse; i.p.) or vehicle. Kaplan-Meier survival curve, $n = 12$ mice/groups. *$P < 0.05$.

accumulation of T-reg and myeloid-derived suppressor cells (MDSCs) [40,41]. Although HMGB1 promoted the neutrophil immunosuppressive phenotype, other mediators of immunosuppression are likely present in T/H plasma. This conclusion is supported by the ability of Ai-Peptide 1 to reduce the plasma inhibitory effect despite lack of its interaction with HMGB1.

NOX family enzymes are essential for host antimicrobial function, cellular redox signaling, gene expression, and recovery/wound healing [42–45]. However, oxidative stress can stimulate development of pathophysiological conditions, including cardiovascular complications and neurodegenerative disorders [46,47]. Harmful oxidants are involved, including hydroxyl radical and reactive nitrogen species that causes lipids, carbohydrates, protein oxidation (and aggregation), as well as DNA damage [48,49]. Although extracellular HMGB1 limits neutrophil respiratory burst and bacterial killing, it is possible that HMGB1 can also decrease neutrophil-driven oxidative stress and collateral damage. Despite the ability of free radicals to inflict passive damage, previous studies have shown that NOX2-derived ROS also diminished LPS-induced lung inflammation [34]. For example, heme oxygenase-1 has been shown to protect against NOX2-induced alcohol intoxication and burn injury [50]. Notably, selective NOXs, in particular NOX4-related oxidative stress is linked to in organ injury and fibrosis [51].

In summary, our study revealed a new mechanism by which HMGB1-dependent inhibition of NOX2 is linked to neutrophil immunosuppression. We suggest that $gp91^{phox}$ decoy peptides can be a therapeutic strategy to reduce neutrophil dysfunction and high risk of bacterial infections among trauma or sepsis survivors.

Author contributions

Concept and design: N.B.B. and J.W.Z. Experiments, data analysis and interpretation: M.H., E.J.B., N.B.B., A.S., J.W.Z. Drafting Figures and editing the manuscript: M.H, E.J.B., N.B.B., J.F.P., J.W.Z. Manuscript writing and revision: JWZ. We thank LaShun Horn, M.Sc. (Department of Anesthesiology, UAB) for coordination of blood/plasma sample collection.

Declaration of competing interest

Authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2020.101651.

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