

HMGB1-induced NLRP3 Inflammasome Participating in Platelet Activation and Thrombocytopenia in Heatstroke

Huimei Yin

Guangzhou Military Command General Hospital: People's Liberation Army General Hospital of Southern Theatre Command <https://orcid.org/0000-0002-7721-6382>

Ming Wu

Shenzhen Second People's Hospital

Yong Lu

Chenzhou No 1 People's Hospital

Xinghui Wu

Guangzhou Military Command General Hospital: People's Liberation Army General Hospital of Southern Theatre Command

Baojun Yu

Shenzhen Bao An Peoples Hospital

Ronglin Chen

Longgang Central Hospital of Shenzhen

Jiefu Lu

First People's Hospital of Foshan

Huasheng Tong (✉ fimmuths@163.com)

Department of Intensive Care Unit, General Hospital of Southern Theatre Command, Guangzhou 510010, China <https://orcid.org/0000-0001-6916-2487>

Research

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Abstract

Background: Previous studies have suggested that NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome plays an important role in heat stroke (HS). As a common complication in HS, thrombocytopenia has been widely considered as a good predictor of HS-related mortality. However, little is known about the relationship between inflammasome and thrombocytopenia as well as platelet activation in HS.

Methods: We established a rat HS model to investigate the roles of NLRP3 inflammasome in both platelet activation and thrombocytopenia, platelet activation was reflected with Flow cytometry while thrombocytopenia was measured by platelet count. The colocalization of NLRP3 inflammasome was detected by confocal fluorescence microscopy. Mitochondrial-derived reactive oxygen species (ROS) were detected using the molecular probes. Plasma HMGB1 levels were measured by ELISA.

Results: Activation of the inflammasome was detected in platelet of rats in HS. Elevated ROS activated NLRP3 inflammasome in HS group could significantly induce platelet activation and thrombocytopenia. The upregulated P-selectin (CD62P) and decreased platelet count triggered by NLRP3 inflammasome were attributed to the high mobility group box protein 1 (HMGB1) in plasma. Moreover, inhibition of HMGB1, caspase-1, NLRP3, or ROS in rats with HS suppressed platelet activation and the decline of platelet count. Similar results were obtained when the receptor toll-like receptor 4 (TLR4) / advanced glycation end product (RAGE) was blocked.

Conclusions: This study indicated that platelets were activated by NLRP3 inflammasome through TLR4/RAGE/HMGB1 signaling pathway. The NLRP3 inflammasome might be the potential target for HS treatment.

Background

Heat stroke (HS), a life-threatening disease with the core body temperature rising above 40.5°C, often develops multiple organ dysfunction syndrome, which is dominated by central nervous system dysfunction and causes high rates of mortality and disability [1, 2]. Thrombocytopenia, a common phenomenon that occurs within 24 h after HS onset, could prognose the higher mortality rates [3]. However, little is known about the mechanism of thrombocytopenia in HS.

Platelets, mainly involved in hemostasis and thrombosis, also play important roles as inflammatory cells in innate and adaptive immune responses [4, 5]. The NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome is involved both in the initiation of many inflammatory responses and thrombosis under many insults [6, 7]. A recent in vitro study showed that the NLRP3 inflammasome contributed to platelet activation, aggregation, and consequently thrombus formation [7]. Hottz et al. found that activated platelets, with increased expression of P-selectin (CD62P), could activate NLRP3 inflammasome and release increased interleukin-1 β (IL-1 β), which is associated with thrombocytopenia in patients with dengue fever [7]. IL-1 β , an important inflammatory factor mediated by NLRP3-dependent

caspase-1 activation, has been linked to increased endothelial permeability [8,9], thrombosis, and dysregulated hemostasis in dengue fever [10]. Significantly increased IL-1 β levels had been found in HS [11]. However, whether IL-1 β induced by NLRP3 inflammasome is involved in platelet activation and thrombocytopenia in HS is not yet clear.

High mobility group box protein 1 (HMGB1), a typical damage-related molecular pattern molecule, is often transferred from the nucleus to the cytoplasm and can be extracellularly released under many stimuli [12]. We previously found that elevated plasma HMGB1 levels in early HS patients prognosed the severity and mortality of disease [13]. More importantly, Gen et al. [11] found that HS induced liver injury via HMGB1-induced NLRP3 inflammasome as well as the release of IL-1 β . It is well known that HMGB1 plays a role in regulating platelet activation, microparticle secretion, adhesion, and thrombus formation [14,15]. Mice lacking HMGB1 in platelets exhibited increasing bleeding time and reduced formation of thrombus, platelet aggregation, inflammation, and organ damage during experimental trauma/hemorrhagic shock [14]. Unfortunately, whether HMGB1-induced NLRP3 inflammasome participates in platelet activation and thrombocytopenia in HS is still unknown.

In this study, using a rat HS model, we demonstrated that HMGB1-induced NLRP3 inflammasome was activated via toll-like receptor 4 (TLR4) and the receptor for advanced glycation end product (RAGE) signaling pathway, and contributed to platelet activation and thrombocytopenia. The findings of this study suggest that targeting the inflammasome may represent a novel therapeutic strategy to recover from a depletion of platelets in HS.

Materials And Methods

Experimental animals

Because estrogen has certain effects on HS pathogenesis and organ injury [16], only adult male, pathogen-free Sprague-Dawley (SD) rats (Experimental Animal Center of the General Hospital of Southern Theatre Command, license number for animal experimentation: SCXK, GuangDong 2016-0041), weighing 220 to 250 g, were used in this study. The rats received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication 86-23, 1985 revision). We adhered to the guidelines for animal care of the General Hospital of Southern Theatre Command. All procedures were approved by the Institutional Animal Care and Use Committee before the experiments were conducted.

Rat model of HS

Rats were housed for 6 h at ambient temperature ($25^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$) with a humidity of $35\% \pm 5\%$ and were randomly divided into 21 groups, with 6 rats per group. To induce HS, rats were placed in a prewarmed incubator maintained at $39.5^{\circ}\text{C} \pm 0.2^{\circ}\text{C}$ with a relative humidity of $60.0\% \pm 5.0\%$. The rectal temperature (Tr) was monitored at 10-min intervals using a thermocouple (BW-TH1101; Biowill, Shanghai, China), which was inserted 6.5 cm into the rectum of each rat. The time point at which the Tr reached

43°C was considered a reference point of HS onset [11]. The rats were removed from the incubator after HS onset, transferred to room temperature (22.0°C ± 0.5°C) with ice-cold saline (0.09%, 20ml) and were fed with adequate food and water. The rats in control group were sham-heated at a temperature of 25°C ± 0.5°C and a humidity level of 35% ± 5%. Ethyl pyruvate (EP, 50 mg/kg body weight, MCE, USA) was injected intraperitoneally (i.p.) to inhibit HMGB1 release, or phosphate buffered solution (PBS) were injected as a negative control before rats subjected to heat stress. A neutralizing antibody against HMGB1 (3 mg/kg body weight, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or nonimmune control IgG were immediately injected i.p. into the rats before heat stress. In some rats, TLR4 neutralizing antibody (8 µg/kg body weight, Abcam, Cambridge, MA, USA), RAGE neutralizing antibody (80 µg/kg body weight, R&D, USA), or both were injected i.p. before rats subjected to heat stress. Other rats were injected i.p. with the caspase-1 inhibitor Ac-Tyr-Val-Ala-Asp-chloromethylketone (ac-YVAD-cmk, 0.3 mg/kg body weight, Enzo Biochem Inc., New York, USA) or PBS containing 2.8% dimethyl sulfoxide (DMSO) as a control before rats subjected to heat stress. Some rats were given MCC950 (20 mg/kg body weight, Selleckchem, Houston, TX, USA), a specific blocker of NLRP3, 1 h before heat stress. Some rats were injected i.p. with the antioxidant N-acetylcysteine (NAC, 300 mg/kg body weight, MCE) immediately before heat stress.

Blood collection and platelet count

Rats were anesthetized by inhalation of isoflurane. Blood was collected from the abdominal aorta, and 50 µL of whole blood was collected into a sodium citrate anticoagulation tube to measure the platelet count using a blood cell analyzer (BC-3000, Mairui, Shenzhen, China).

Platelet isolation

Platelets were isolated as described previously [17]. Briefly, 3 mL of anticoagulant blood samples were centrifuged at 150 g for 15 min to obtain platelet-rich plasma (PRP), and about 3/4 of the PRP was carefully pipetted into 2 volumes of modified Tyrode buffer (12 mM NaHCO₃, 138 mM NaCl, 5.5 mM glucose, 2.9 mM KCl, 2 mM MgCl₂, 0.42 mM NaH₂PO₄, 10 mM HEPES, pH 7.4). The mixture was centrifuged at 37°C at 500 g for 8 min in the presence of PGE1 (0.1 µg/mL, Cayman Chemical) and apyrase (1 U/mL, Sigma-Aldrich), washed twice with CGS buffer (120 mM sodium chloride, 12.9 mM trisodium citrate, 30 mM D-glucose, pH 6.5), and resuspended in modified Tyrode buffer.

Flow cytometric analyses

Freshly isolated platelets (10⁶ to 10⁷) were resuspended in 1 mL of modified Tyrode buffer, and treated with 0.2% triton before to measure intracellular cytokines. A minimum of 10,000 events per gate was acquired using a flow cytometer (BD LSR Fortessa). P-selectin (CD62P) was determined using the anti-CD62P monoclonal antibody (1:1000, Biolegend, USA), and TLR2 (toll-like receptor 2), TLR4, and RAGE were assessed with antibodies that target TLR2 (1:500, Abcam), TLR4 (1:1000, Abcam), and RAGE (1:1000, Abcam). NLRP3 expression was evaluated by an NLRP3 antibody (1:1000, Abcam), and activation of caspase-1 was assessed by the fluorescent probe green fluorescent-labeled inhibitor of

caspase-1 (FLICA, 1:100, Immunochemistry Technologies, Bloomington, MN), which irreversibly binds to activated caspase-1. IL-1 β was evaluated by the anti-IL-1 β antibody (1:1000, Biorbyt). Mitochondrial-derived reactive oxygen species (ROS) were detected using the molecular probes Mito-SOX (for mitochondria O₂^{•-}, 1:1000, Invitrogen), DHE (for cytoplasmic O₂^{•-}, 1:1000, Invitrogen), and DCF-DA (for H₂O₂, 1:1000, Invitrogen). All indices were doubly labeled with a phycoerythrin- or fluorescein isothiocyanate-conjugated CD61 monoclonal antibody (1:1000, Biolegend) to label platelets. The results were analyzed using FlowJo software 7.6.

Confocal fluorescence microscopy

Immunofluorescence for NLRP3 and apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC) was performed according to previous research [18]. Platelets attached on a poly-L-lysine-coated coverslip were fixed with pre-cooled methanol for 20 min, washed twice with PBS (135 mM NaCl, 4.7 mM KCl, 10 mM Na₂HPO₄, 2 mM NaH₂PO₄, pH 7.4), and blocked with 10% fetal calf serum (FCS) in PBS. Then, platelets were incubated with goat anti-rat NLRP3 antibody (1:1000, Abcam) or rabbit anti-rat ASC antibody (1:500, Santa Cruz) for 30 min, washed twice with PBS, and labeled with corresponding Dlight 488 and Dlight 594 conjugated secondary antibodies (1:1000, Invitrogen) for 30 min respectively. Preparations were analyzed on a laser scanning confocal microscope (Fluo View FV1000, Olympus), and FV1000 operations software was used for recording.

Plasma HMGB1 level measurement

Plasma HMGB1 levels were measured by a commercially available HMGB1 enzyme-linked immunosorbent assay kit according to the manufacturer's instructions (Shino-Test Corporation, Sagamihara, Japan).

Statistical analysis

All data were presented as the mean \pm SD unless stated otherwise. Statistical significance was determined with the least significant difference t test or one-way analysis of variance (ANOVA) using GraphPad Prism 5.0 software (GraphPad, San Diego CA, USA). $P < 0.05$ was considered statistically significant.

Results

HS-induced platelet activation and thrombocytopenia

The platelet count began to decline at 3 h after HS onset (Fig. 1A), whereas the expressions of IL-1 β (Fig. 1D) and CD62P (Fig. 1E) in platelets increased progressively during the same period after HS onset. All changes reached peak at 9 h after HS onset. The platelet parameters such as mean platelet volume (MPV) and platelet distribution width (PDW) were assessed. MPV (Fig. 1B) and PDW (Fig. 1C) at 3 h and 6 h after HS were within normal ranges, with no statistical differences comparing to the sham group.

However, both values increased significantly compared to those in the control group after 9 h of HS ($P < 0.01$). Therefore, for the remaining experiments, we observed heat-stressed animals only at the time point of 9 h after HS onset.

Activation of NLRP3 inflammasome in platelets from rats with HS

The association of NLRP3 and ASC as well as caspase-1 represents activation of the inflammasome. The colocalization of NLRP3 (green) and ASC (red) in platelets was observed with a confocal microscopy (Fig. 2C). In addition, flow cytometric analyses showed that NLRP3 (Fig. 2A) and cleaved caspase-1 (Fig. 2B) increased at 3 h after HS onset and reached peak levels at 9 h after HS onset. All results indicate the HS-inducing NLRP3 inflammasome activation in platelets.

NLRP3 inflammasome mediates platelet activation and thrombocytopenia in HS

To investigate whether HS-activated NLRP3 inflammasome mediates platelet activation and thrombocytopenia, we injected rats i.p. with MCC950, a selective inhibitor of the NLRP3 inflammasome, before the rats being subjected to heat stress. The results showed that inhibited NLRP3 inflammasome significantly down-regulated the levels of cleaved caspase-1 (Fig. 3A), IL-1 β (Fig. 3B) and CD62P (Fig. 3C) and reduced the drop in platelet count (Fig. 3D) at 9 h after HS onset. Moreover, pharmacological inhibition of caspase-1 activity with ac-YVAD-cmk also markedly down-regulated the expression of IL-1 β (Fig. 3E), CD62P (Fig. 3F) and the drop in platelet count (Fig. 3G) 9 h after HS onset.

HMGB1 is involved in platelet NLRP3 inflammasome activation in HS rats

Previous studies found that increased extracellular HMGB1 levels early in HS were negatively correlated with its prognosis [11,19]. HMGB1 activates the NLRP3 inflammasome in liver cells, which in turn promotes hepatocyte pyroptosis [11]. Consistent with previous studies (13), we found that the concentration of HMGB1 in plasma of HS rats gradually increased (Fig. 4A). We also unraveled the role of HMGB1 in inducing NLRP3 inflammasome activation, platelet activation, and thrombocytopenia in HS. We injected rats i.p. with EP to inhibit HMGB1 release and used anti-HMGB1 neutralizing antibody to block the effect of HMGB1 before submitting rats to HS.

EP inhibiting or neutralizing antibody against HMGB1 significantly reduced the expression of NLRP3 (Fig. 4B) and cleaved caspase-1 (Fig. 4C) at 9 h after HS onset. Notably, levels of IL-1 β (Fig. 4D), expression of CD62P (Fig. 4E), and thrombocytopenia (Fig. 4F) at 9 h after HS onset were significantly alleviated after pretreatment with EP and anti-HMGB1 antibody. These results indicate that HMGB1 plays an important role in the activation of the NLRP3 inflammasome in platelets and participate in platelet activation and thrombocytopenia in HS.

In the early stages of sterile inflammation, HMGB1 activates the NLRP3 inflammasome through TLR4 or RAGE receptors [20]. As shown in Figures 4G-I, expressions of TLR2, TLR4, and RAGE in platelets were gradually increased after HS onset. To define the receptors that mediate HMGB1-induced NLRP3

inflammasome activation, we applied anti-TLR4 and anti-RAGE neutralizing antibodies before heat exposure. Either anti-TLR4 or anti-RAGE neutralizing antibody significantly inhibited HS-induced NLRP3 (Fig. 4J) and cleaved caspase-1 (Fig. 4K) expression in platelets at 9 h after HS onset. In addition, the combination of anti-TLR4 and anti-RAGE neutralizing antibody induced greater inhibition of NLRP3 and cleavage of caspase-1 expression. Synchronous changes were also observed in HMGB1-induced upregulated expression of IL-1 β (Fig. 4L), CD62P (Fig. 4M), and thrombocytopenia (Fig. 4N) at 9 h after HS.

These data indicate that both TLR4 and RAGE receptors are involved in activation of NLRP3 inflammasome in platelet and consequently are associated with platelet activation and thrombocytopenia in HS.

HMGB1 activates platelet NLRP3 inflammasome by upregulating ROS in HS rats

ROS mainly include the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical [$HO\cdot$; 21]. Many studies confirmed that ROS can be regulated by HMGB1 [22,23], and is the key factor affecting the activation of NLRP3 inflammasome [24-26]. However, the effect of HS-induced ROS on NLRP3 inflammasome is not yet clearly understood yet.

To study the changes of ROS in platelets and the effect of ROS on the HMGB1-induced NLRP3 inflammasome in HS, we used a fluorescence probe to detect the expression of mitochondrial $O_2^{\bullet-}$, cytoplasmic $O_2^{\bullet-}$, and H_2O_2 in platelets of rats with HS. We found that mitochondrial $O_2^{\bullet-}$ (Fig. 5A), cytoplasmic $O_2^{\bullet-}$ (Fig. 5B), and H_2O_2 (Fig. 5C) increased at 3 h after HS onset. Inhibiting HMGB1 significantly reduced the level of ROS, which indicates that HMGB1 is involved in high-level of ROS in HS (Fig. 5D-F).

To explore the relationship between ROS and the NLRP3 inflammasome activation, we pretreated rats with the antioxidant NAC. In platelets, NAC significantly inhibited the expression of NLRP3 (Fig. 5G) and cleaved caspase-1 (Fig. 5H) at 9 h after HS onset. Similar results were observed for IL-1 β (Fig. 5I), CD62P (Fig. 5J), and thrombocytopenia (Fig. 5K). These results indicate that ROS is responsible for platelet NLRP3 inflammasome-induced activation and thrombocytopenia in HS.

Discussion

Thrombocytopenia is commonly observed and associated with poor clinical outcomes in HS (3). The mechanism by which HS induces thrombocytopenia and platelet activation remains unclear. Here, we uncovered an important role of the NLRP3 inflammasome in mediating platelet activation and thrombocytopenia in a rat HS model. We demonstrated that high levels of extracellular HMGB1 induced by HS activate the NLRP3 inflammasome through TLR4 and RAGE pathway. This activation in turn promotes platelet activation and thrombocytopenia (Fig. 6).

Gader previously demonstrated that thrombocytopenia appeared in patients with mild HS and that it was more pronounced when body temperatures increased above 41°C. Furthermore, when the temperature increased within a certain range, the aggregation rate of platelets were also increased [27]. Platelet aggregation plays a key role in hemostasis and thrombosis, and it may be involved in HS-induced thrombocytopenia as well. On the basis of our results, we speculate that HS-induced thrombocytopenia is possibly caused by platelet activation and depletion.

MPV and PDW are indices of platelet size in circulation, MPV measures the average platelet volume, while PDW describes the variation in platelet size [28], both of which are markers of platelet activation and thrombocytopenia [29,30]. In this experiment, we found that MPV and PDW in rats with HS did not change at 3 h but were increased significantly at 9 h after HS onset. Previous study showed that thrombocytopenia in patients with HS without damage to megakaryocytes in bone marrow [27]. Combined with the changes in platelet counts and platelet activation, we speculate that thrombocytopenia in rats with HS may not be related to impaired platelet production in bone marrow but is mainly caused by peripheral platelet consumption.

Zhuang et al. found that heat stress facilitated the procession of Px-caspase-1 (a caspase gene) expression, an important effector molecule of the NLRP3 inflammasome [31]. One team in our institute previously found that HMGB1-induced NLRP3 inflammasome activation in liver cells during HS was associated with liver damage [11]. Hottz et al. [7] also reported NLRP3 inflammasome activation in platelets of patients with dengue fever. Pranav et al. [32] found that NLRP3 inflammasome affects platelet activation, aggregation, and thrombosis. Therefore, it is speculated that the HMGB1-induced NLRP3 inflammasome could mediate platelet activation and thrombocytopenia in HS. We observed that the expressions of NLRP3, ASC, and cleaved caspase-1 in platelets in rats with HS were increased early during HS onset and remained increased thereafter (Fig. 2A, B, C). By laser confocal microscopy, we confirmed that the NLRP3 inflammasome assembly presented at 9 h after HS onset (Fig. 2A). All these results reflect the assembly and activation of NLRP3 inflammasome in HS. Consistent with the results of a previous study [32], we found that inhibition of NLRP3 and cleavage of caspase-1 alleviated platelet activation and thrombocytopenia (Fig. 3), indicating that the NLRP3 inflammasome possibly plays an important role in platelet activation and thrombocytopenia.

HMGB1, a class of non-histone chromosomal binding protein present in eukaryotic cells, has been extensively studied as a transcription factor and growth-promoting factor [12] and an important molecule that can activate NLRP3 inflammasome [33]. Our previous studies showed that plasma HMGB1 was significantly elevated at an early stage in HS, and was associated with more critical disease [13]. Inhibiting HMGB1 release or blocking HMGB1 can alleviate liver sinusoidal endothelial damage and reduce thrombosis in HS [19,34]. In this experiment, we also found that inhibiting HMGB1 can inhibit the activation of the NLRP3 inflammasome, platelet activation, and thrombocytopenia in HS. Previous studies have showed that HMGB1 promotes inflammation via the main receptors, such as TLR2 and TLR4 [35,36]. Because there was no anti-TLR2 neutralizing antibody for rats, we only tested the effects of TLR4 and RAGE receptors. It has been reported that RAGE receptors are involved in mediating biological

functions outside the cell and the binding of the receptors to their ligands cannot independently conduct intracellular signaling pathways but requires interactions with other receptors [37,38]. Different to the results of a prior study that visfatin-induced disruption on junction proteins of mouse vascular endothelial cells was conducted through the HMGB1-RAGE, rather than the HMGB1-TLR4 pathway [39], our results indicate that both TLR4 and RAGE receptor-specific antibodies can inhibit activation of the NLRP3 inflammasome, platelet activation and thrombocytopenia. Also, anti-TLR4 antibody pretreatment significantly shortened the heat exposure and time to HS onset in rats. These results suggest that HMGB1 activates the NLRP3 inflammasome via TLR4 and RAGE receptors and participates in platelet activation and thrombocytopenia.

ROS have an important role in the homeostasis of aging and death of platelets in internal and external environments [23], which are involved in platelet activation, adhesion, and aggregation and cause platelet apoptosis [21]. Studies have confirmed that HS can lead to an explosive increase in intracellular ROS [40]. In our experiments, we found that 3 kinds of ROS in the platelets of rats with HS increased progressively with time (Fig. 5A, B, C). HMGB1 can upregulate intracellular levels of ROS in many diseases including HS [41]. We found that inhibition or blockade of HMGB1 could partially inhibit the production all of 3 kinds of ROS (Fig. 5D,E), suggesting a mechanism of HMGB1-induced ROS in the rat model of HS. ROS induced by HMGB1 is known to be a key mechanism for triggering NLRP3 inflammasome formation and activation in response to many exogenous stimuli [25]. Many researchers in recent years have shown that elevated intracellular ROS promote the activation of the NLRP3 inflammasome [42,43]. We found that NAC pretreatment could inhibit the expression of platelet NLRP3 and cleaved caspase-1 in rats with HS, indicating that ROS can upregulate the activation of the NLRP3 inflammasome in platelets.

Consistent with previous studies, we found that NAC could inhibit platelet activation and thrombocytopenia in rats with HS. Mitochondria is the main source of ROS, and a large amount of ROS can damage mitochondria, causing changes in mitochondrial membrane permeability that in turn triggers autophagy, apoptosis, and necrosis [44,45]. Here, we observed the ultrastructural changes of platelets in HS by transmission electron microscopy and found mitochondria swelling, cristae vague, autophagy (supplementary Fig. A), and platelet necrosis (supplementary Fig. B). However, whether the thrombocytopenia in rats with HS is related to platelet death caused by ROS and which type of death is dominant needs further study.

Pyroptosis, a caspase-1 dependent programmed cell death [11], possibly occurs in platelets because of caspase-1 activation and thrombocytopenia in rats with HS. During the time course of the study, thrombocytopenia and caspase-1 activation showed opposite trends. Typically, both activated caspase-1 and PI positive are both used to define pyroptosis in eukaryotic cells; however, because platelets have no nuclei, we cannot use this definition to describe pyroptosis of platelets. However, the relationship between cleaved caspase-1 and thrombocytopenia implies that a link may exist between caspase-1 and platelet death. It has been reported that HS can cause hepatocyte pyroptosis [11]. In this study, by transmission electron microscopy, we found a catheter expansion, a vacuole as one of the characteristics of

pyroptosis, in the platelets of rats at 9 h after HS onset (supplementary Fig. C). However, whether HS can cause platelet pyroptosis is still unknown and needs further research.

Conclusions

The elevated HMGB1 in HS via both of TLR4 and RAGE receptors induced high level of ROS, which activates NLRP3 inflammasome, ultimately aggravating inflammatory response, platelet activation and thrombocytopenia (**Figure 6**).

Abbreviations

NLRP3: NOD-like receptor family pyrin domain containing 3; HS: Heat stroke;

ROS: Reactive oxygen species; CD62P: P-selectin; HMGB1: High mobility group box protein 1; TLR4: Toll-like receptor 4; RAGE: Advanced glycation end product; IL-1 β : Interleukin-1 β ; SD: Sprague-Dawley; Tr: Rectal temperature; EP: Ethyl pyruvate; PBS: Phosphate buffered solution; DMSO: Dimethyl sulfoxide; ac-YVAD-cmk: Ac-Tyr-Val-Ala-Asp-chloromethylketone; NAC: N-acetylcysteine; PRP: Platelet-rich plasma; ROS: Reactive oxygen species; ASC: Apoptosis-associated speck-like protein containing a caspase-recruitment domain; FCS: Fetal calf serum; MPV: Mean platelet volume; PDW: Platelet distribution width

Declarations

Ethics approval and consent to participate

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the General Hospital of Southern Theatre Command of PLA.

Consent for publication

Not applicable.

Availability of data and materials

All materials are commercially available, and the datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

YHM conducted the experiments. YHM, LY, LJF and WXH collected and analyzed the data. WM, CRL, YBJ and THS gave technical or material support. YHM and THS designed this research, review and edited the manuscript.

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References

1. Kosgallana AD, Mallik S, Patel V, Beran RG, et al. Heat stroke induced cerebellar dysfunction : A "forgotten syndrome". *World J Clin Cases*. 2013; 1(8):260–261.
2. Epstein Y, Yanovich R. Heatstroke. *N Engl J Med*. 2019; 2019: 2449-2459.
3. Pan ZG, Shao Y, Liu YN, Gu ZT, Zhang XQ, Xu YQ, Su L, et al. Relationship between early coagulability parameters at admission and outcome in patients with severe heatstroke. *Chinese Critical Care Medicine*. 2013; 25: 725-728.
4. Vieira-de-Abreu A, Campbell RA, Weyrich AS, Zimmerman GA. Platelets: versatile effector cells in hemostasis, inflammation, and the immune continuum. *Semin Immunopathol*. 2012; 34(1):5-30.
5. Semple JW, Italiano JE Jr, Freedman J, et al. Platelets and the immune continuum. *Nat Rev Immunol*. 2011; 4: 264-274.
6. Martinon F. Signaling by ROS drives inflammasome activation. *Eur J Immunol*. 2010; 40: 616-619 .
7. Hottz ED, Lopes JF, Freitas C, Valls-de-Souza R, Oliveira MF, Bozza MT, et al. Platelets mediate increased endothelium permeability in dengue through NLRP3-inflammasome activation. *Blood*. 2013; 20: 3405-3414.
8. Bozza FA, Cruz OG, Zagne SM, Azeredo EL, Nogueira RM, Assis EF, et al. Multiplex cytokine profile from dengue patients: MIP-1beta and IFN-gamma as predictive factors for severity. *BMC Infect Dis*. 2008;8:86.
9. Houghton-Triviño N, Martín K, Giaya K, Rodríguez JA, Bosch I, Castellanos JE. Comparison of the transcriptional profiles of patients with dengue fever and dengue hemorrhagic fever reveals differences in the immune response and clues in immunopathogenesis. *Biomedica*. 2010; 30(4):587–597.

10. Suharti C, van Gorp EC, Setiati TE, Dolmans WM, Djokomoeljanto RJ, Hack CE, et al. The role of cytokines in activation of coagulation and fibrinolysis in dengue shock syndrome. *Thromb Haemost.* 2002; 1: 42-46.
11. Geng Y, Ma Q, Liu YN, Peng N, Yuan FF, Li XG, et al. Heatstroke induces liver injury via IL-1 β and HMGB1-induced pyroptosis. *J Hepato.* 2015; (3): 622-633.
12. Kang R, Chen R, Zhang Q, Hou W, Wu S, Cao L, et al. HMGB1 in health and disease. *Mol Aspects Med.* 2014;40: 1-116.
13. Tong HS, Tang YQ, Chen Y, Qiu JM, Wen Q, Su L. Early Elevated HMGB1 Level Predicting the Outcome in Exertional Heatstroke. *The Journal of Trauma: Injury, Infection, and Critical Care.* 2011; 71: 808-814.
14. Vogel S, Bodenstein R, Chen Q, Feil S, Feil R, Rheinlaender J, et al. Platelet-derived HMGB1 is a critical mediator of thrombosis. *J Clin Invest.* 2015; 125: 4638-4654.
15. Vallance TM, Zeuner MT, Williams HF, Widera D, Vaiyapuri S. Toll-Like Receptor 4 Signalling and Its Impact on Platelet Function, Thrombosis, and Haemostasis. *Mediators Inflamm.* 2017; 2017: 1-13.
16. Chen SH, Chang FM, Niu KC, Lin MY, Lin MT. Resuscitation from experimental heat stroke by estrogen therapy. *Crit Care Med.* 2006; 34(4):1113-8.
17. Qiao J, Wu X, Luo Q, Wei G, Xu M, Wu Y, et al. NLRP3 regulates platelet integrin α IIb β 3 outside-in signaling, hemostasis and arterial thrombosis. *Haematologica.* 2018; 103(9):1568-1576.
18. Feng W, Chang C, Luo D, Su H, Yu S, Hua W, et al. Dissection of autophagy in human platelets. *Autophagy.* 2014; 10(4):642-651.
19. Tong H, Tang Y, Chen Y, Yuan F, Liu Z, Peng N, et al. HMGB1 activity inhibition alleviating liver injury in heatstroke. *J Trauma Acute Care Surg.* 2013; 74(3):801-807.
20. Xiang M, Shi X, Li Y, Xu J, Yin L, Xiao G, et al. Hemorrhagic shock activation of NLRP3 inflammasome in lung endothelial cells. *J Immunol.* 2011; 187(9):4809-4817.
21. Leytin V. Apoptosis in the anucleate platelet. *Blood Rev.* 2012; 26(2):51-63.
22. Tsung A, Klune JR, Zhang X, Jeyabalan G, Cao Z, Peng X, et al. HMGB1 release induced by liver ischemia involves Toll like receptor 4 dependent reactive oxygen species production and calcium-mediated signaling. *J Exp Med.* 2007; 204(12):2913-2923.
23. Pietraforte D, Vona R, Marchesi A, de Jacobis IT, Villani A, Del Principe D, et al. Redox Control of Platelet Functions in Physiology and Pathophysiology. *antioxid Redox Signal.* 2014; 21(1):177-193.

24. Dostert C, Pétrilli V, Van Bruggen R, Steele C, Mossman BT, Tschopp J. Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. *Science*. 2008; 320(5876):674-677.
25. Bae JY, Park HH. Crystal structure of NALP3 protein pyrin domain (PYD) and its implications in inflammasome assembly. *J Biol Chem*. 2011; 286(45):39528-36.
26. Zhou R, Yazdi AS, Menu P, Tschopp J. A role for mitochondria in NLRP3 inflammasome activation. *Nature*. 2011; 469(7329):221-225.
27. Gader A. Heat Stroke and Platelets. *Platelets*. 1992; 3(3):125-128.
28. Jackson SR, Carter JM. Platelet volume: Laboratory measurement and clinical application. *Blood*. 1993; 104-113.
29. Bessman JD, Gilmer PR, Gardner FH. Use of mean platelet volume improves detection of platelet disorders. *Blood Cells*. 1985; 127-135.
30. Park Y, Schoene N, Harris W. Mean platelet volume as an indicator of platelet activation: methodological issues. *Platelets*. 2002; Aug-Sep;13(5-6):301-6.
31. Zhuang HM, Wang KF, Miyata T, Wu ZJ, Wu G, Xie LH. Identification and expression of caspase-1 gene under heat stress in insecticide-susceptible and -resistant *Plutella xylostella* (Lepidoptera: Plutellidae). *Mol Biol Rep*. 2011; 38(4):2529-2539.
32. Murthy P, Durco F, Miller-Ocuin JL, Takedai T, Shankar S, Liang X, et al. The NLRP3 inflammasome and bruton's tyrosine kinase in platelets co-regulate platelet activation, aggregation, and in vitro thrombus formation. *Biochem Biophys Res Commun*. 2017; 483(1):230-236.
33. Park JS, Gamboni-Robertson F, He Q, Svetkauskaite D, Kim JY, Strassheim D, et al. High mobility group box 1 protein interacts with multiple Toll-like receptors. *Am J Physiol Cell Physiol*. 2006; 290(3):C917-924.
34. Hagiwara S, Iwasaka H, Goto K, Ochi Y, Mizunaga S, Saikawa T, et al. Recombinant thrombomodulin prevents heatstroke by inhibition of High-mobility group box 1 protein in sers of rats. *Shock*. 2010; 34(4):402-6.
35. Park JS, Svetkauskaite D, He Q, Kim JY, Strassheim D, Ishizaka A, Abraham E. Involvement of Toll-like Receptors 2 and 4 in Cellular Activation by High Mobility Group Box 1Protein.*J Biol Chem*. 2004; 279: 7370-7.
36. Park JS, Gamboni-Robertson F, He Q, Svetkauskaite D, Kim JY, Strassheim D, et al. High mobility group box I protein interacts with multiple Toll-like receptors. *Am Physiol Cell Physiol*. 2006; 3: C917-24.

37. Degryse B, Bonaldi T, Scaffidi P, Müller S, Resnati M, Sanvito F, et al. The high mobility group(HMG)boxes of the nuclear protein HMGL induce chemotaxis and cytoskeleton reorganization in rat smooth muscle cells. *J Cell Biol.* 2001; 6: 1197-206.
38. Dumitriu IE, Bianchi ME, Bacci M, Manfredi AA, Rovere-Querini P. The secretion of HMGB I is required for the migration of maturing dendritic cells. *Leukoc Biol.* 2007; 1: 84-91.
39. Chen Y, Pitzer AL, Li X, Li PL, Wang L, Zhang Y. Instigation of endothelial Nlrp3 inflammasome by adipokine visfatin promotes inter-endothelial junction disruption: role of HMGB1. *J Cell Mol Med.* 2015; 19(12):2715-2727.
40. McAnulty SR, McAnulty L, Pascoe DD, Gropper SS, Keith RE, Morrow JD, et al. Hyperthermia increases exercise-induced oxidative stress. *Int J Sports Med.* 2005; 3: 188-192.
41. Tang D, Kang R, Zeh HJ 3rd, Lotze MT. High-Mobility Group Box 1, Oxidative Stress, and Disease. *Antioxid Redox Signal.* 2011; 7: 1315-1335.
42. Abais JM, Xia M, Zhang Y, Boini KM, Li PL. Redox Regulation of NLRP3 Inflammasomes: ROS as Trigger or Effector? *Antioxid Redox Signal.* 2011; 14(7):1315-1335.
43. Han S, Cai W, Yang X, Jia Y, Zheng Z, Wang H, et al. ROS-Mediated NLRP3 Inflammasome Activity Is Essential for Burn-Induced Acute Lung Injury. *Mediators Inflamm.* 2015; 2015:720457.
44. Andrassy M, Volz HC, Igwe JC, Funke B, Eichberger SN, Kaya Z, et al. High-Mobility Group Box-1 in Ischemia-Reperfusion Injury of the Heart. *Circulation.* 2008; 117(25):3216-3226.
45. Tang D, Shi Y, Kang R, Li T, Xiao W, Wang H, et al. Hydrogen peroxide stimulates macrophages and monocytes to actively release HMGB1. *J Leukoc Biol.* 2007; 741-747.

Figures

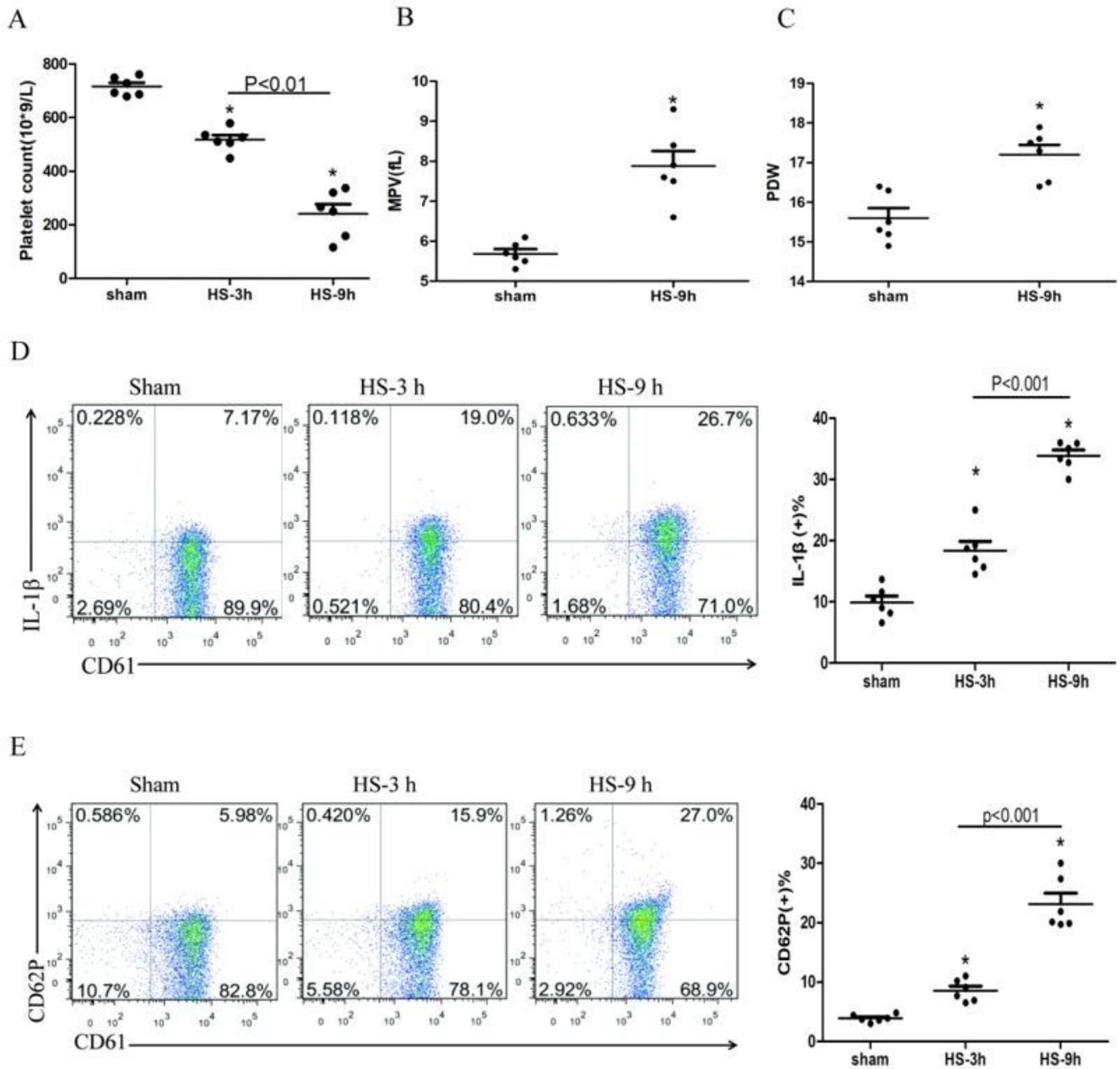


Figure 1

HS-induced platelets activation and thrombocytopenia. (A) Thrombocytopenia in HS rats. * $p < 0.01$ vs sham. (B) Changes of MPV in HS rats. * $p < 0.01$ vs sham. (C) Changes of PDW in HS rats. * $p < 0.01$ vs sham. (D) Significant increases of IL-1 β in HS rats. * $p < 0.01$ vs sham. (E) Significant increases of CD62P in HS rats. * $p < 0.01$ vs sham.

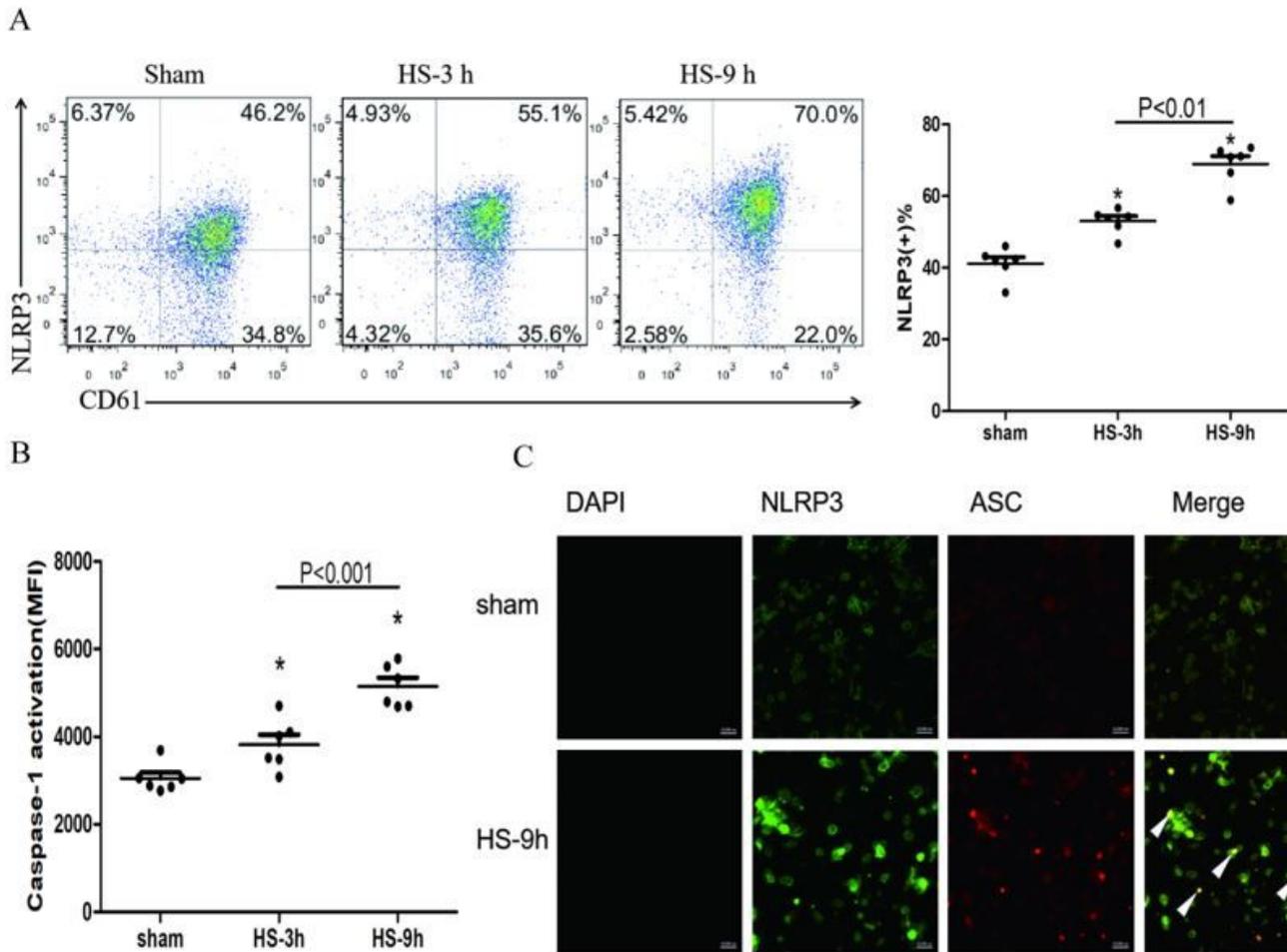


Figure 2

NLRP3 inflammasome activation in platelets in HS rats. (A) NLRP3 expression in HS rats. * $p < 0.01$ vs sham. (B) caspase-1 activation in HS rats. * $p < 0.01$ vs sham. (C) NLRP3 Inflammasome assembly in platelets of rat at 9 h after HS onset. NLRP3 (green) and ASC (red) and Merge (yellow) were observed by a laser confocal microscope. The white triangular arrows point to the NLRP3 inflammasome, suggesting the presence of NLRP3 and ASC in platelets from rats with HS. Bars represent 10,000 nm.

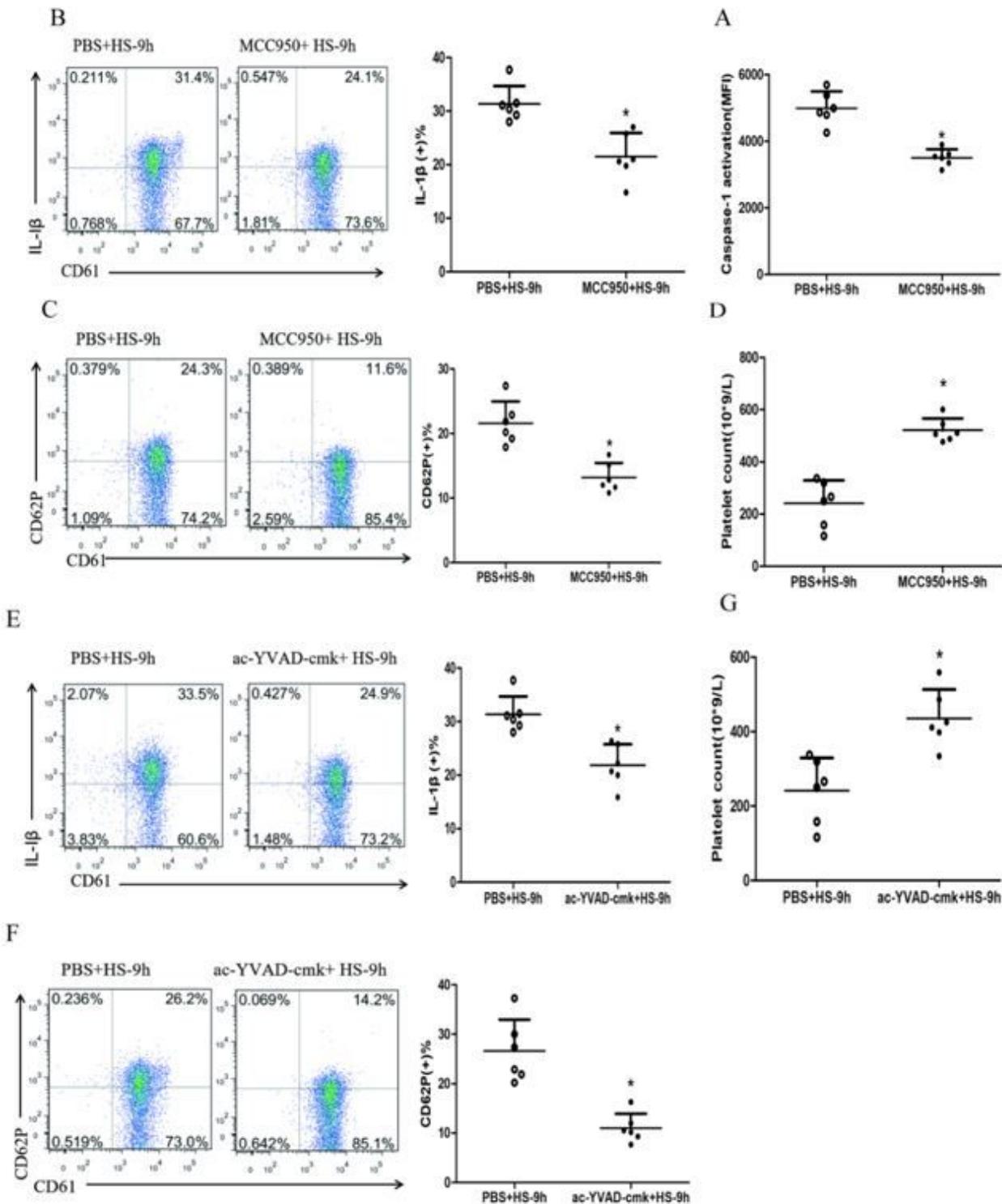


Figure 3

NLRP3 inflammasome mediates platelet activation and thrombocytopenia in HS rats. (A) Effect of MCC950 on caspase-1 activation in platelet of HS-9 h rats. * $P < 0.01$ vs PBS+HS-9 h. (B) Effect of MCC950 on IL-1 β in platelet of HS-9 h rats. * $P < 0.01$ vs PBS+HS-9 h. (C) Effect of MCC950 on CD62P in platelet of HS-9 h rats. * $P < 0.01$ vs PBS+HS-9 h. (D) Effect of MCC950 on platelet count of HS-9 h rats. * $P < 0.01$ vs PBS+HS-9 h. (E) Effect of ac-YVAD-cmk on IL-1 β in platelet of HS-9 h rats. * $P < 0.01$ vs PBS+HS-9 h. (F)

Effect of ac-YVAD-cmk on CD62P in platelet of HS-9 h rats. *P<0.01 vs PBS+HS-9 h. (G) Effect of ac-YVAD-cmk on platelet count of HS-9 h rats. *P<0.01 vs PBS+HS-9 h.

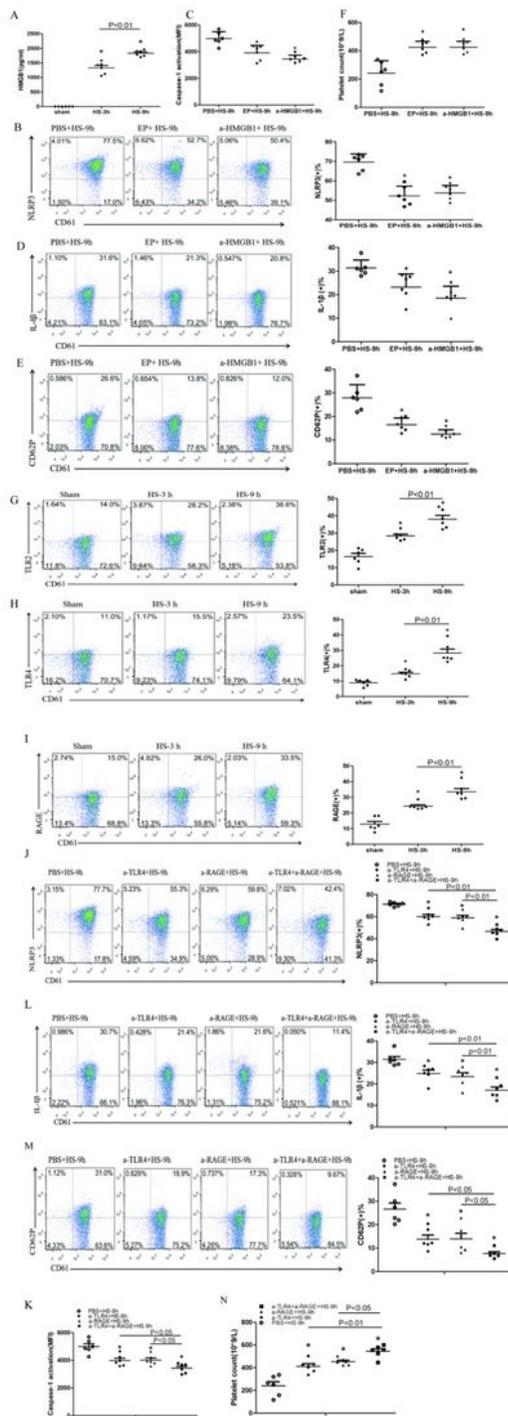


Figure 4

HMGB1 participates in platelets NLRP3 inflammasome activation in HS rats. (A) HMGB1 levels in plasma of HS rats. *P<0.01 vs sham. (B) Effects of EP and a-HMGB1 on NLRP3 expression in platelets of HS-9 h rats. *P<0.01 vs PBS+HS-9 h. (C) Effects of EP and a-HMGB1 on caspase-1 activation in platelets of HS-9

h rats. *P<0.01 vs PBS+HS-9 h. (D) Effects of EP and a-HMGB1 on IL-1 β in platelets of HS-9 h rats. *P<0.01 vs PBS+HS-9 h. (E) Effects of EP and a-HMGB1 on CD62P expression in platelets of HS-9 h rats. *P<0.01 vs PBS+HS-9 h. (F) Effects of EP and a-HMGB1 on platelet counts in HS-9 h rats. *P<0.01 vs PBS+HS-9 h. (G) TLR2 expression in platelets of HS rats. *P<0.01 vs sham. (H) TLR4 expression in platelets of HS rats. *P<0.01 vs sham. (I) RAGE expression in platelets of HS rats. *P<0.01 vs sham. (J) Effects of a-TLR4 and a-RAGE on NLRP3 expression in platelets of HS-9 h rats. *P<0.01 vs PBS+HS-9 h. (K) Effects of a-TLR4, a-RAGE on caspase-1 activation in platelets of HS-9 h rats. *P<0.01 vs PBS+HS-9 h. (L) Effects of a-TLR4, a-RAGE on IL-1 β in platelets of HS-9 h rats. *P<0.01 vs PBS+HS-9 h. (M) Effects of a-TLR4, a-RAGE on CD62P in platelets of HS-9 h rats. *P<0.01 vs PBS+HS-9 h. (N) Effects of a-TLR4, a-RAGE on platelet count in HS-9 h rats. *P<0.01 vs PBS+HS-9 h.

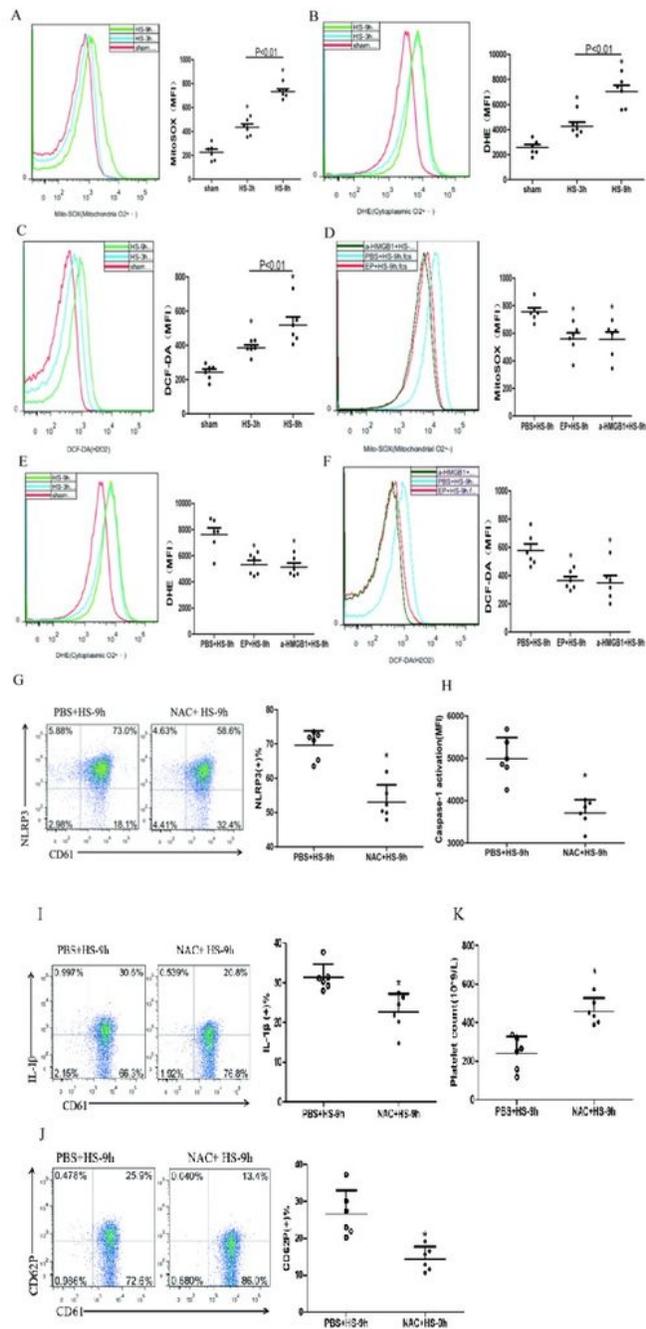


Figure 5

HMGB1 activating platelets NLRP3 inflammasome by upregulating ROS in HS rats. (A) Mito-SOX levels in HS rats. *p < 0.01 vs sham. (B) DHE levels in HS rats. *p < 0.01 vs sham. (C) DCF-DA levels in HS rats. *p < 0.01 vs sham. (D) Effects of EP and a-HMGB1 on Mito-SOX in platelets of HS-9 h rats. *P < 0.01 vs PBS+HS-9 h. (E) Effects of EP and a-HMGB1 on DHE in platelets of HS-9 h rats. *P < 0.01 vs PBS+HS-9 h. (F) Effects of EP and a-HMGB1 on DCF-DA in platelets of HS-9 h rats. *P < 0.01 vs PBS+HS-9 h. (G) Effects

of NAC on NLRP3 in platelets of HS-9 h rats. *P<0.01 vs PBS+HS-9 h. (H) Effects of NAC on caspase-1 activation in platelets of HS-9 h rats. *P<0.01 vs PBS+HS-9 h. (I) Effects of NAC on IL-1 β in platelets of HS-9 h rats. *P<0.01 vs PBS+HS-9 h. (J) Effects of NAC on CD62P in platelets of HS-9 h rats. *P<0.01 vs PBS+HS-9 h. (K) Effects of NAC on platelet count of HS-9 h rats. *P<0.01 vs PBS+HS-9 h.

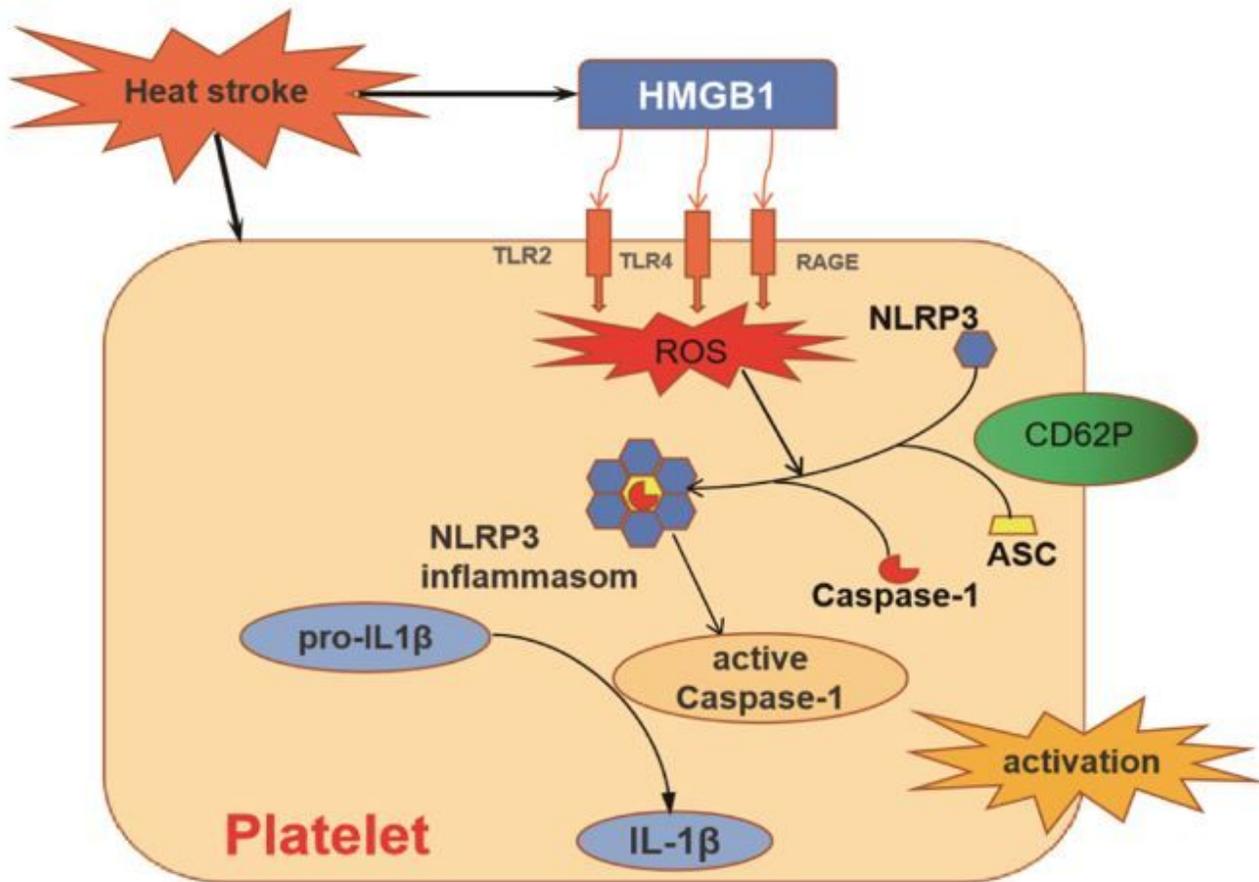


Figure 6

In the model, HS-induced HMGB1 in plasma acting through TLR4 and RAGE signaling induces high level of ROS, which activates NLRP3 inflammasome, which in turn promotes IL-1 β release, platelet activation and thrombocytopenia.

Supplementary Files

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