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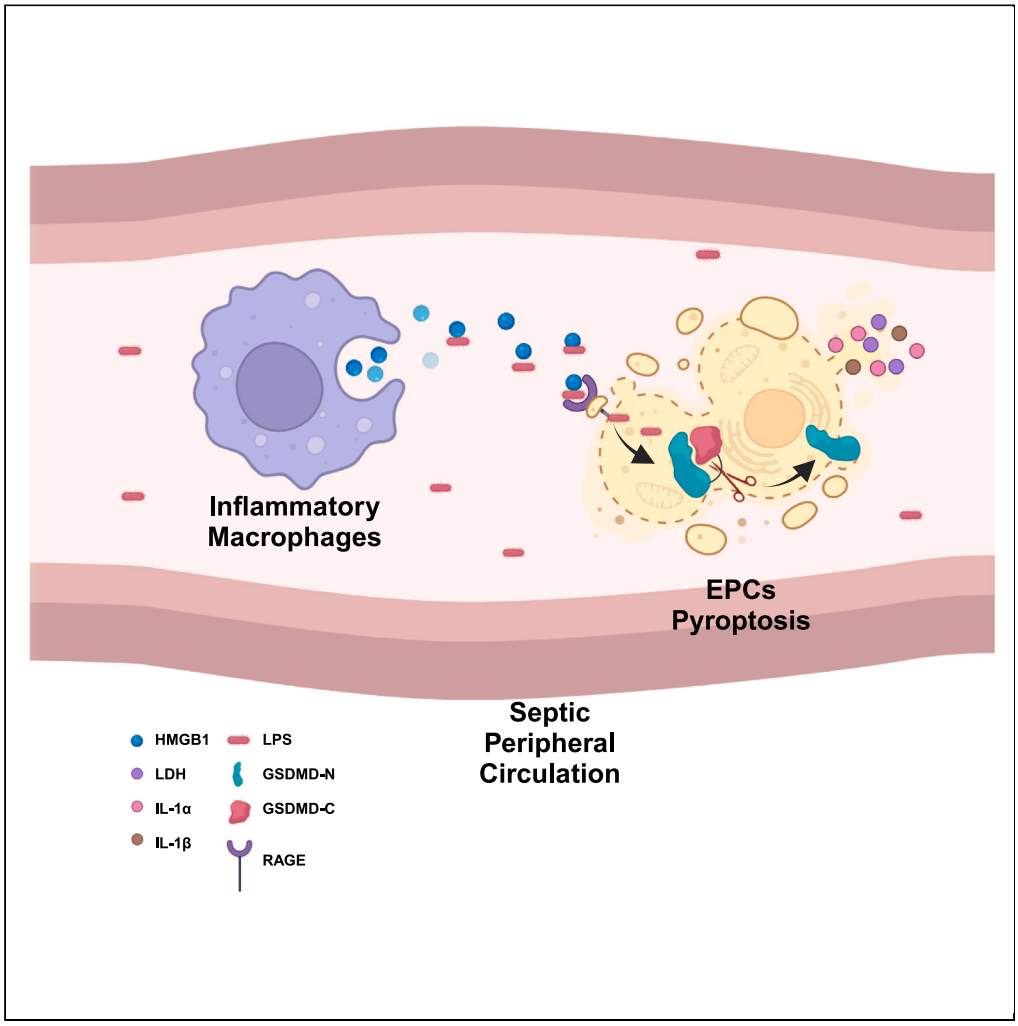

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Highlights

EPCs in peripheral blood of patients with sepsis undergo pyroptosis

Septic macrophages are associated with EPCs pyroptosis

EPCs in septic peripheral circulation undergo pyroptosis via the HMGB1/RAGE axis

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Macrophages-derived high-mobility group box-1 protein induces endothelial progenitor cells pyroptosis

Menghao Zeng,[1,2,4] Guibin Liang,[1,2,4] Fangfang Yuan,[1] Shanshan Yan,[1] Jie Liu,[1] and Zhihui He[1,2,3,5,*]

SUMMARY

Endothelial dysfunction is an important factor in the progress of sepsis. Endothelial progenitor cells (EPCs) are the precursor cells of endothelial cells and play a crucial role in the prognosis and treatment of sepsis. EPCs in the peripheral blood of patients with sepsis undergo pyroptosis, but the mechanism remains much of unknown. Serum high-mobility group box-1 (HMGB1) is significantly elevated in patients with sepsis, but whether it is related to EPCs pyroptosis is unknown. We used a cell model of sepsis *in vitro* to isolate EPCs for better observation. By detecting the pyroptosis-related indicators of EPCs and the level of release and acetylation of HMGB1 in inflammatory macrophages, it was found that HMGB1 released by inflammatory macrophages combined with receptor for advanced glycation end products (RAGE) is a key pathway to induce pyroptosis of EPCs.

INTRODUCTION

Sepsis is a life-threatening organ dysfunction caused by the host's dysregulated response to infection.[1] Endothelial dysfunction plays a major role in the pathogenesis of sepsis.[2] Endothelial progenitor cells (EPCs) are crucial as they are the precursors to vascular endothelial cells which line the inner surface of blood vessels.[3] During sepsis, EPCs are mobilized from bone marrow into peripheral circulation to repair the damaged endothelium.[4] Many studies have underscored the significance of EPCs in both the prognosis and treatment of sepsis.[2,5–7] Macrophages are pivotal immune cells which wield significant influence over the immune response during sepsis. They release abundant inflammatory mediators, including interleukin (IL)-6, tumor necrosis factor α, IL-1, high-mobility group box-1 (HMGB1).[8–10] However, the impact of macrophages on EPCs in sepsis remains rarely understood.

Programmed cell death, including apoptosis, necrosis, pyroptosis, necroptosis, and ferroptosis, participates in various pathophysiological processes of sepsis.[11–13] The research on pyroptosis in sepsis has attracted much attention in recent years. Pyroptosis, primarily mediated by the Gasdermins (GSDMs) family, is characterized by a dependence on inflammatory caspase accompanied by the release of numerous pro-inflammatory factors.[14,15] Currently, research on pyroptosis mainly focuses on immune cells, with less study on EPCs.

HMGB1 is a ubiquitous nuclear and cytosolic protein.[16] The extracellular HMGB1 is mainly released by monocytes and macrophages; it is considered as a signal of tissue injury[17] and even mediates lethality during endotoxemia or sepsis.[18] Receptor for advanced glycation end products (RAGE) is a signal transduction receptor. HMGB1 bound to RAGE could induce cell pyroptosis.[19,20] EPCs express high level of RAGE.[21] The issue of whether HMGB1 released from inflammatory macrophages could induce circulating EPCs pyroptosis during sepsis is still undisclosed.

Here, we analyzed blood samples from sepsis patients and incubated EPCs *in vitro* using supernatant from sepsis-associated inflammatory macrophages to investigate whether HMGB1 released from inflammatory macrophages could induce EPCs pyroptosis.

RESULTS

The number of EPCs with FAM-FLICA-activated caspase-1 and PI double-positive staining and the level of HMGB1 in serum were increased in sepsis patients

Activated caspase-1 plays an important role in mediating inflammation and pyroptosis.[22] Pyroptosis was characterized by the coexistence of active caspase-1 and propidium iodide (PI) positivity.[23] We found that the number of EPCs with FAM-FLICA-activated caspase-1 (green) and PI (red) double-positive staining from sepsis patients was significantly higher than that of healthy controls ($p < 0.01$) (Figures 1A and 1B). The level

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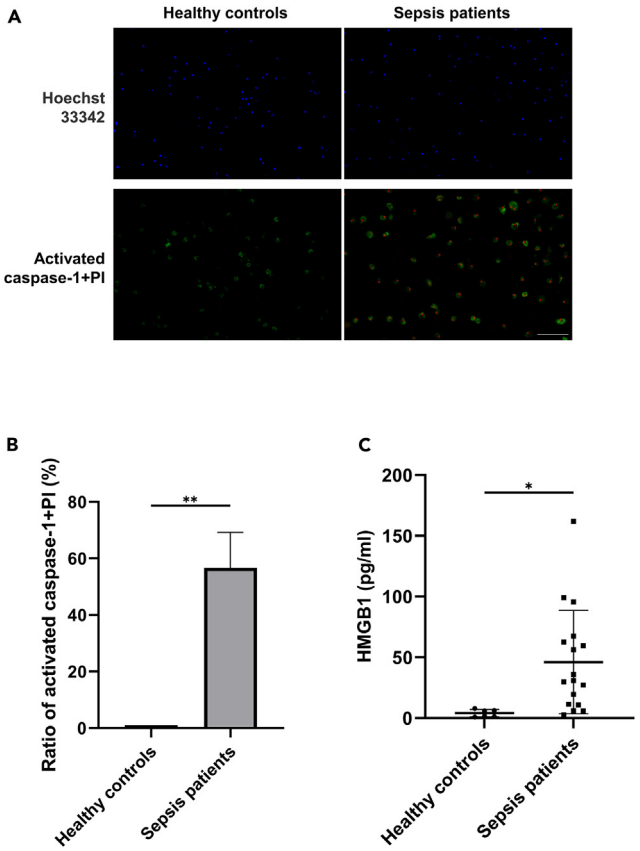

Figure 1. The number of EPCs with FAM-FLICA-activated caspase-1 and PI double-positive staining and the level of HMGB1 in serum were increased in sepsis patients

(A and B) EPCs with FAM-FLICA-activated caspase-1 and PI double-positive staining in sepsis patients and healthy controls. Scale bar: 100 μm.

(C) The level of HMGB1 in serum in sepsis patients and healthy controls. PI, propidium iodide; HMGB1, high-mobility group box-1. The data shown are representative of three independent experiments. Data are represented as mean ± SD. *p* values were determined using a two-tailed t test. **p* < 0.05, ***p* < 0.01.

of HMGB1 in serum in sepsis patients was also higher than that in healthy controls ($p < 0.05$) (Figure 1C). These results suggested that EPCs in the peripheral blood of sepsis patients undergo pyroptosis and the HMGB1 in serum may be involved in EPCs pyroptosis.

Septic mSN-induced EPCs pyroptosis required LPS

In order to investigate the effect of septic macrophages supernatant (mSN) on EPCs, EPCs were incubated with mSN alone or mSN plus 1 μg/mL Lipopolysaccharide (LPS) *in vitro* (Table 1). When EPCs were incubated with mSN alone, there was no morphological sign of cell death and no activated markers related to pyroptosis. When incubated with mSN plus 1 μg/mL LPS, EPCs showed cell membrane rupture, leakage of cellular contents, cellular swelling and deformation, and the appearance of necrotic vesicles in the group mSN-L_pN+1 μg/mL LPS (Figures 2A and 2D). At the same time, the uptake of PI (red) (Figure 2A) and FAM-FLICA-activated caspase-1 (green) (Figure 2B) and the formation of apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) -speck (red arrows) (Figure 2C) in EPCs were increased in the group mSN-L_pN+1 μg/mL LPS. Additionally, the cleavage of GasderminD (GSDMD) and IL-1β (Figures 2E, S2A, and S2C) in EPCs were observed in the group mSN-L_pN+1 μg/mL LPS, but not in other groups. The level of lactic dehydrogenase (LDH) ($p < 0.0001$) and IL-1β ($p < 0.0001$) in EPCs supernatant also increased in the group mSN-L_pN+1 μg/mL LPS (Figures 2F and 2G). There was no difference in the cleavage of GSDME in EPCs among these groups (Figures 2E and S2B). These results indicated that EPCs incubated with mSN-L_pN+1 μg/mL LPS suffered from pyroptosis by mediating the cleavage of GSDMD.

L_pN increased the release and acetylation level of HMGB1 in macrophages

The concentration level of HMGB1 in mSN and the protein expression level of HMGB1 in macrophages were detected by ELISA and western blot (WB), respectively. Results showed that both the concentration level of HMGB1 in mSN and the protein expression level of HMGB1 in macrophages in the group mSN-L_pN were significantly higher than those in other groups ($p < 0.0001$, $p < 0.01$) (Figures 3A–3C). The findings demonstrated that HMGB1 was released from macrophages into the extracellular space only in the case of L_pN intervention.

Table 1. The interventions for EPCs related to experimental model and study participant details

	1	2	3	4	5	6	7	8
mSN-Ctrl	-	-	+	-	-	+	-	-
mSN-L_p	-	-	-	+	-	-	+	-
mSN-L_pN	-	-	-	-	+	-	-	+
LPS (1 μg/mL)	-	+	-	-	-	+	+	+

mSN-Ctrl, macrophages supernatant-control group; mSN-L_p, macrophages supernatant-LPS priming group; mSN-L_pN, macrophages supernatant-LPS priming plus nigericin group.

Acetylation is the main form of HMGB1 exocytosis. The acetylation modification promotes HMGB1 transfer from nucleus to cytoplasm, facilitating its further release into the extracellular space.[24,25] Result of coimmunoprecipitation (coIP) showed that the acetylation level of HMGB1 in macrophages was up-regulated in group L_pN ($p < 0.001$) (Figures 3D and 3E). In addition, using an acetylase inhibitor to pretreat macrophages before L_pN intervention could reduce the acetylation level of HMGB1 in macrophages ($p < 0.01$) (Figures 3F and 3G).

EPCs pyroptosis relies on the release of HMGB1 from macrophages

In order to observe the impact of HMGB1 released from macrophages on EPCs pyroptosis, macrophages were treated with different concentrations (80, 160, 320 μg/mL) of glycyrrhizic acid (GA), direct inhibitor of HMGB1, for 2 h prior to L_pN stimulation. Then EPCs were incubated with GA-pretreated mSN-L_pN+1 μg/mL LPS as before. As expected, the cleavage of GSDMD in EPCs ($p < 0.05$) (Figures 4A and S3A) and the level of LDH ($p < 0.05$) (Figure 4B) in EPCs supernatant were decreased in a dose-dependent manner with the administration of GA. Similar results were observed when EPCs were incubated with siHMGB1-pretreated mSN-L_pN+1 μg/mL LPS ($p < 0.05$) (Figures 4C, 4D, and S3B–S3D).

Purified rHMGB1 (dithiothreitol [DTT]-reductive form) plus 1 μg/mL LPS also induced the cleavage of GSDMD in EPCs (Figures 4E and S3E) and the increased levels of LDH, IL-1β, and IL-1α in EPCs supernatant ($p < 0.0001$) (Figures 4F–4H).

HMGB1-RAGE axis induces EPCs pyroptosis

It is well known that RAGE is a high-affinity receptor for HMGB1.[26] HMGB1 internalization largely depends on RAGE.[27] The result of immunofluorescence showed that RAGE was highly expressed in EPCs (Figure 5A). Next, we pretreated EPCs with the RAGE inhibitor FPS-ZM1 (8, 16, 32 μM) and then incubated EPCs with mSN-L_pN+1 μg/mL LPS. The results displayed that the cleavage of GSDMD in EPCs (Figures 5B and S4) and the level of LDH (Figure 5C) in EPCs supernatant were down-regulated in a dose-dependent manner with the administration of FPS-ZM1 ($p < 0.05$). The result of coIP assay exhibited that in the group mSN-L_pN+1 μg/mL LPS, the binding of HMGB1 to RAGE was increased compared with the group mSN-L_pN ($p < 0.001$); this trend was consistent with what was seen in the cleavage of GSDMD in EPCs (Figures 5D and 5E).

DISCUSSION

The present study showed that compared with healthy controls, the number of circulating EPCs with FAM-FLICA-activated caspase-1 and PI double-positive staining and the level of HMGB1 in serum were increased in patients with sepsis. *In vitro*, EPCs were co-incubated with mSN alone or mSN plus 1 μg/mL LPS; only EPCs in the group mSN-L_pN+1 μg/mL LPS exhibited up-regulated uptake of PI and FAM-FLICA-activated caspase-1, as well as the cleavage of GSDMD and the formation of ASC-speck in EPCs. Furthermore, EPCs in the group mSN-L_pN+1 μg/mL LPS showed the morphological characteristics of pyroptosis and released amount of LDH and IL-1β. These results indicated that EPCs treated with mSN-L_pN+1 μg/mL LPS suffered from pyroptosis by mediating the cleavage of GSDMD. In the macrophage groups, compared with the group Ctrl, the acetylation level of HMGB1 and the release of HMGB1 from macrophages were up-regulated in the group L_pN. EPCs were incubated with mSN-L_pN+1 μg/mL LPS after pretreatment with GA or siHMGB1; the cleavage of GSDMD in EPCs and the level of LDH in EPCs supernatant were attenuated. Moreover, the release of LDH, IL-1α, and IL-1β from EPCs and the cleavage of GSDMD in EPCs intervened with purified rHMGB1 plus 1 μg/mL LPS were increased. Compared with the group mSN-L_pN, the combination of RAGE and HMGB1 on EPCs in the group mSN-L_pN+1 μg/mL LPS was significantly up-regulated. These results suggested that HMGB1 released by macrophages could induce EPCs pyroptosis by binding to RAGE.

More and more evidences revealed that pyroptosis plays a key regulatory role in sepsis.[28–30] The inflammasome triggers pyroptosis by activating the caspase family.[31] Activated caspases, such as activated caspase-1, caspase-3, and caspase-8, cleave its downstream GSDMs family, such as GSDMD and GSDME, resulting in the release of inflammatory factors and the activation of inflammasome.[32–34] Activated caspase-1 combined with dead cell dyes (such as PI) is one of the markers for pyroptosis.[35] During sepsis, HMGB1 is released in the late stage of inflammation and acts as an inflammatory mediator, mediating the tissue damage or cell death.[36] There are three main oxidation-reduction reaction (REDOX) forms of HMGB1, including mercaptan (reduced), disulfide (oxidized), and peroxide. Among them, oxidized HMGB1, usually oxidized by hydrogen peroxide, can induce the production of pro-inflammatory mediators in cells. Reduced HMGB1, usually reduced with DTT, acts as a chemokine and can bind to RAGE to induce cells pyroptosis.[19,37,38]

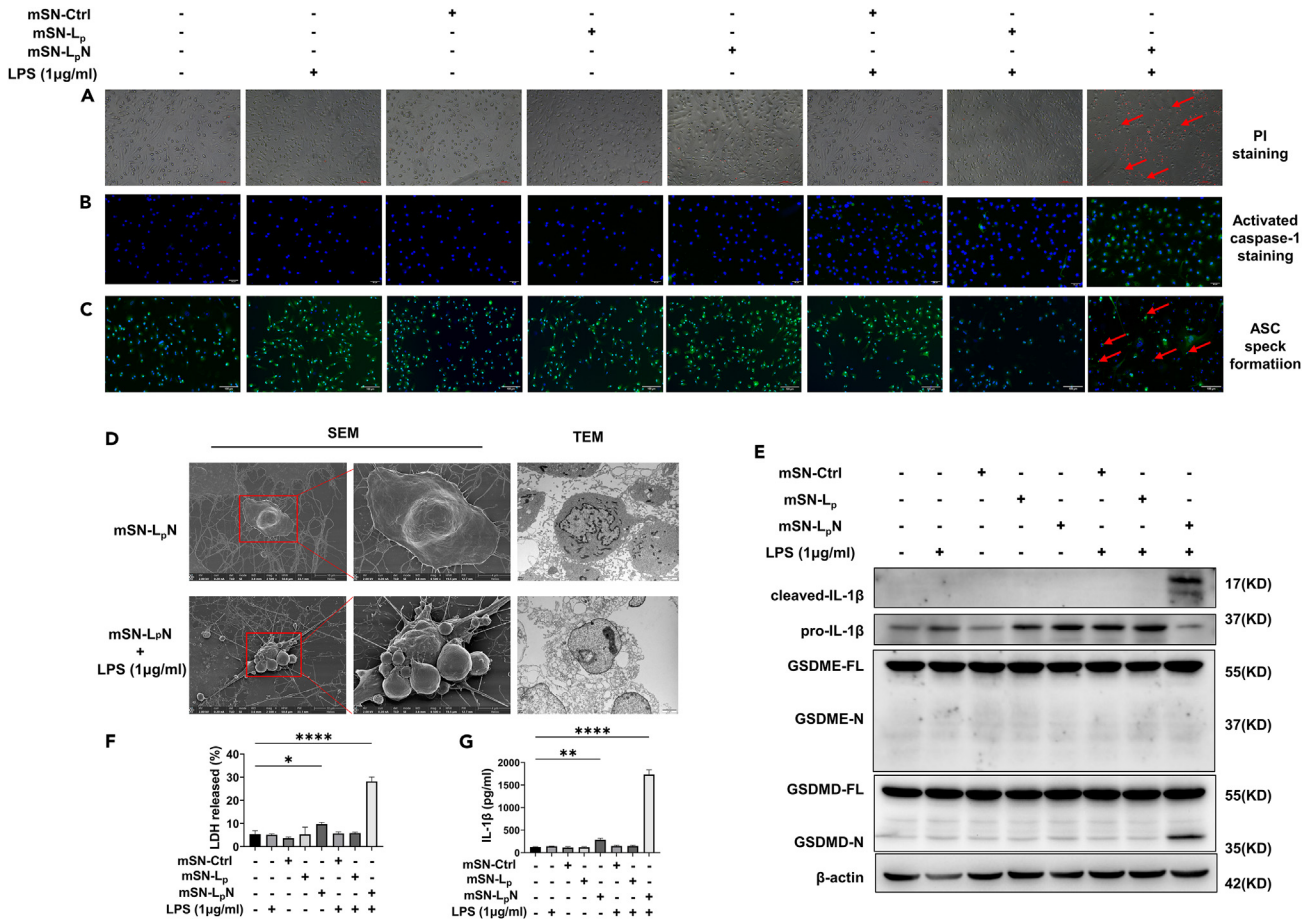

Figure 2. Septic mSN-induced EPCs pyroptosis required LPS

(A) EPCs morphological change and uptake of PI after incubated with mSN alone or mSN plus 1 μg/mL LPS. The group mSN-L_pN+1 μg/mL LPS was expanded and ruptured with prominent necrotic vesicles (red arrows), while other groups displayed no cell death characteristics. The uptake of PI (red) in the group mSN-L_pN+1 μg/mL LPS was much higher than that in other groups. Scale bar: 100 μm.

(B) Uptake of FAM-FLICA-activated caspase-1 (green) in EPCs after incubated with mSN alone or mSN plus 1 μg/mL LPS. Scale bar: 50 μm.

(C) The formation of ASC-speck (red arrows) in EPCs after incubated with mSN alone or mSN plus 1 μg/mL LPS. Scale bar: 100 μm.

(D) Electron microscopy images of EPCs in the group mSN-L_pN and mSN-L_pN+1 μg/mL LPS of EPCs. Scale bar: 10, 4, and 2 μm.

(E) The protein expression levels of GSDMD-FL, GSDMD-N, GSDME-FL, GSDME-N, pro-IL-1β, and cleaved-IL-1β in EPCs after incubated with mSN alone or mSN plus 1 μg/mL LPS.

(F) The level of LDH in EPCs supernatant.

(G) The level of IL-1β in EPCs supernatant. mSN, macrophages supernatant; Ctrl, control; L_p, LPS priming (100 ng/mL); N, nigericin (10 μM); GSDMD-FL, GasderminD-full length; GSDMD-N, GasderminD-N domain; GSDME-FL, GasderminE-full length; GSDME-N, GasderminE-N domain; SEM, scanning electron microscope; TEM, transmission electron microscope; PI, propidium iodide; IL-1β, interleukin-1β; LDH, lactic dehydrogenase. The data shown are representative of three independent experiments. Data are represented as mean ± SD. *p* values were determined using ANOVA analysis, $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$.

See also Figure S2.

Sepsis with systemic inflammation leads to extensive damage to the vascular endothelium.[39] EPCs are heterogeneous cell population which can specifically differentiate into endothelial cells.[4] During sepsis, the considerable damage to endothelial cells prompts the mobilization of EPCs from bone marrow into peripheral blood, where they contribute to vascular endothelium repair.[40] The increased number and functional integrity of EPCs in peripheral blood are important for the prognosis of sepsis.[5,41] However, in sepsis, elevated levels of inflammatory mediators, chemokines, reactive oxygen species, and other factors can impair EPCs function including mobilization, migration, and signaling. These detrimental effects could lead to EPCs pool depletion or EPCs insufficiency or both.[42,43] The uncoordinated development between the quantity of EPCs and the ability to regenerate and clone formation may be one of the reasons for the difficulty in recovering from endothelial dysfunction in sepsis. Our results showed that circulating EPCs underwent pyroptosis, which may be one of the reasons for EPCs impairment in sepsis.

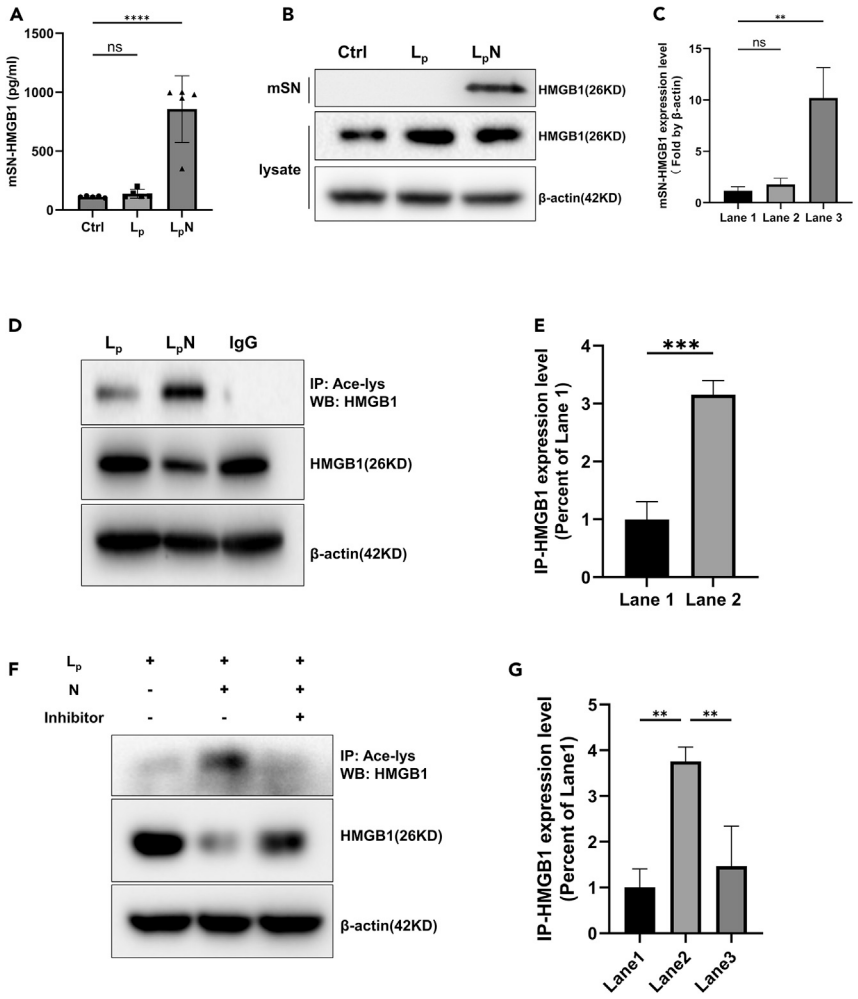

Figure 3. L_pN increased the release and acetylation modification of HMGB1 in macrophages

(A) The concentration level of HMGB1 in mSN detected by ELISA.

(B and C) The protein expression level and statistical analysis of HMGB1 in macrophages detected by WB.

(D and E) The acetylation level and statistical analysis of HMGB1 in macrophages.

(F and G) The acetylation level and statistical analysis of HMGB1 in macrophages after using acetylase inhibitor to pretreat macrophages before L_pN intervention. mSN, macrophages supernatant; HMGB1, high-mobility group box-1; Ctrl, control; L_p, LPS (100 ng/mL); N, nigericin; IP, immunoprecipitation. WB was performed using ImageJ software for gray value analysis and β-actin as the standard. The data shown are representative of three independent experiments. Data are represented as mean ± SD. *p* values were determined using ANOVA analysis, ns means no significant, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

In sepsis, macrophages release varies of inflammatory cytokines including HMGB1.[10] Acetylation is a common protein modification, and post-translational acetylation of lysine residues in HMGB1 is a major mechanism for HMGB1 release from activated macrophages during inflammation.[44,45] Researches showed that HMGB1 was released into the circulation by necrotic cells and binds to LPS, inducing lysosomal lysis and ultimately pyroptosis.[46,47] However, the activation conditions for the release of HMGB1 by macrophages *in vitro* are still controversial. Bonaldi et al. believed that LPS intervention alone could cause macrophages to release a large amount of HMGB1.[48] However, Volchuk et al. proposed that the release of HMGB1 requires LPS plus the second signal, like nigericin.[8] Results of the present study showed that macrophages release HMGB1 into the supernatant when intervened with L_pN, while L_p alone could not induce the HMGB1 release. This result is inconsistent with the finding of Bonaldi et al.[48] but consistent with Volchuk et al.[8] The reason for the different voices may be related to the purity of LPS used by researchers. In the present study, ultrapure LPS was utilized; none of any contaminated bacterial proteins, lipids, or nucleic acids could induce cell activation, so macrophages need to be stimulated by a second signal to release HMGB1.

The inflammasome activates caspase-1, in most cases, requiring ASC to catalyze proteolytic cleavage of GSDMs and drive pyroptosis.[49] The inflammasome induces the assembly of ASC in the cytoplasm and promotes ASC-speck released from pyroptotic cells and remains stable for several days, which is considered to be a hallmark of inflammasome assembly.[49–51] GSDMD is a member of the GSDMs family, and it is also

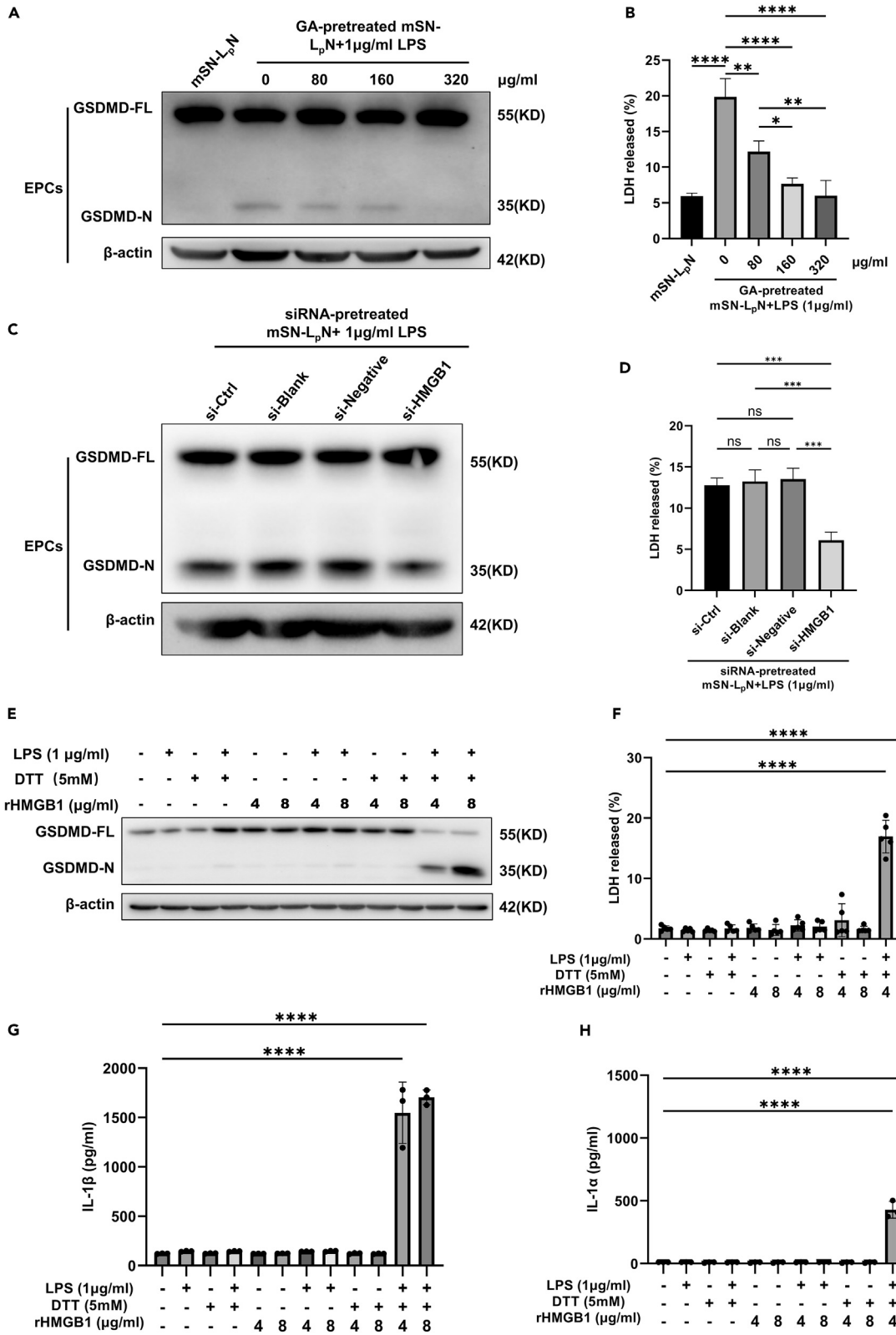
A
GA-pretreated mSN-LpN+1μg/ml LPS
mSN-LpN
0
80
160
320
μg/ml
GSDMD-FL
EPCs
GSDMD-N
β-actin
55(KD)
35(KD)
42(KD)
B
LDH released (%)
mSN-LpN
GA-pretreated mSN-LpN+LPS (1μg/ml)
C
siRNA-pretreated mSN-LpN+ 1μg/ml LPS
si-Ctrl
si-Blank
si-Negative
si-HMGB1
D
ns
siRNA-pretreated mSN-LpN+LPS (1μg/ml)
E
LPS (1 μg/ml)
DTT (5mM)
rHMGB1 (μg/ml)
F
LPS (1μg/ml)
DTT (5mM)
rHMGB1 (μg/ml)
G
IL-1β (pg/ml)
H
IL-1α (pg/ml)

Figure 4. EPCs pyroptosis relies on the release of HMGB1 from macrophages

(A) The protein expression level of GSDMD-N in EPCs when incubated with GA-pretreated mSN-L_pN+1 μg/mL LPS.

(B) The level of LDH in EPCs supernatant when incubated with GA-pretreated mSN-L_pN+1 μg/mL LPS.

(C) The protein expression level of GSDMD-N in EPCs when incubated with siHMGB1-pretreated mSN-L_pN+1 μg/mL LPS.

(D) The level of LDH in EPCs supernatant when incubated with siHMGB1-pretreated mSN-L_pN+1 μg/mL LPS.

(E–H) The level of GSDMD-N in EPCs and the level of LDH, IL-1β, and IL-1α in EPCs supernatant when EPCs were treated with purified rHMGB1 plus 1 μg/mL LPS. mSN, macrophages supernatant; Ctrl, control; L_p, LPS priming (100 ng/mL); N, nigericin (10 μM); GSDMD-FL, GasderminD-full length; GSDMD-N, GasderminD-N domain; GA, glycyrrhizic acid; DTT, dithiothreitol; rHMGB1, recombinant high-mobility group box-1; IL-1β, interleukin-1β; IL-1α, interleukin-1α; LDH, lactic dehydrogenase. WB was performed using ImageJ software for gray value analysis and β-actin as the standard. The data shown are representative of three independent experiments. Data are represented as mean ± SD. p values were determined using ANOVA analysis, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

See also Figure S3.

the earliest identified executive protein of pyroptosis.[31,52] Full-length GSDMD cleaved into GSDMD-C and GSDMD-N domain by activated caspase.[53,54] The GSDMD-N domain exhibits a specific affinity for liposomes, allowing it to bind to the lipid cell membrane and form cell pores, a process known as cell perforation. This mechanism is a crucial step in the onset of pyroptosis.[14,55] Accordingly, the cleavage of GSDMD is considered to be an important indicator of cell pyroptosis.[14,56] In the present study, we utilized activated caspase-1 and PI double staining, the formation of ASC, and the cleavage of GSDMD instead of GSDME combined with morphological changes and inflammatory cytokine release levels in cells to detect septic EPCs pyroptosis.

Our earlier study has displayed that EPCs in peripheral blood of patients with sepsis had pyroptosis.[57] In the present study, we also found that the number of circulating EPCs with FAM-FLICA-activated caspase-1 and PI double staining in sepsis patients was much higher than that in healthy controls. This result proved again that circulating EPCs in sepsis patients underwent pyroptosis. Besides, the level of HMGB1 in serum in sepsis patients was significantly higher than that in healthy controls. Therefore, we want to investigate whether HMGB1 is associated with EPCs pyroptosis in sepsis. In order to re-create the inflammatory environment surrounding EPCs in sepsis, EPCs were incubated with mSN alone or mSN+1 μg/mL LPS. The results revealed that mSN alone failed to induce EPC pyroptosis unless a higher concentration of 1 μg/mL LPS was added. This may be due to the fact that the medium containing L_pN had been discarded and replaced with endothelial cell growth medium-2 (EGM-2) medium before the collection of mSN. The collected supernatant at this time contained only the secreta from macrophages. mSN- L_pN added with additional high-dose 1 μg/mL LPS can match up with the inflammatory environment in which circulating EPCs are exposed.

HMGB1 is composed of the A box, B box, and C tail domains. Among them, the B box plays the role of pro-inflammatory activity of HMGB1.[58,59] GA, a specific inhibitor of HMGB1, can directly bind to B box to inhibit the bioactivity and mitotic activity of HMGB1.[60,61] RAGE has been implicated in the onset and progression of pathologies associated with aging, cellular stress, and inflammation.[62] HMGB1 is known to be able to signal through RAGE.[63,64] It has been shown that EPCs expressed more RAGE in sepsis patients compared to the control groups and the level of RAGE was associated with patients survival.[65] Our results also showed that RAGE was highly expressed in EPCs, and the binding of HMGB1 to RAGE was significantly up-regulated in the conditions of EPCs pyroptosis. Pretreating macrophages with a HMGB1 inhibitor or pretreating EPCs with a RAGE inhibitor before EPCs incubated by mSN-L_pN+1 μg/mL LPS could reduce the level of pyroptosis-related proteins in EPCs and alleviate EPCs pyroptosis. Together, these results suggested that EPCs pyroptosis in sepsis is mediated by HMGB1/RAGE signaling.

Summarily, the present study demonstrated that macrophages treated by L_pN released a large amount of HMGB1 which induced EPCs pyroptosis by binding to the RAGE receptor on EPCs. Previous studies on pyroptosis of sepsis mainly focused on immune cells. The present study aimed to investigate the biological characteristics of EPCs during sepsis, which may be one of the reason for the decline of vascular endothelial repair function in sepsis. The results of the present study may provide a direction for the treatment of severe infections such as sepsis. On the other hand, the study explored the impact of macrophages on EPCs in sepsis, which may be helpful for the in-depth study of "crosstalk" between immune cells and non-immune cells.

Limitations of the study

There are some limitations in the present study. Firstly, the composition of mSN interfered with L_pN is very complicated, and only the HMGB1/RAGE pathway has been studied. The issue of whether there are other factors or pyroptosis signals involved in EPCs pyroptosis is uncertain and requires further investigation. Secondly, other types of programmed cell death that play an important role in the pathogenesis of sepsis, including apoptosis and necrotic apoptosis, were not covered in this study. All of these need in-depth investigation in the future.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Zhihui He, E-mail: hzh703@csu.edu.cn.

Materials availability

This study did not generate new unique reagents.

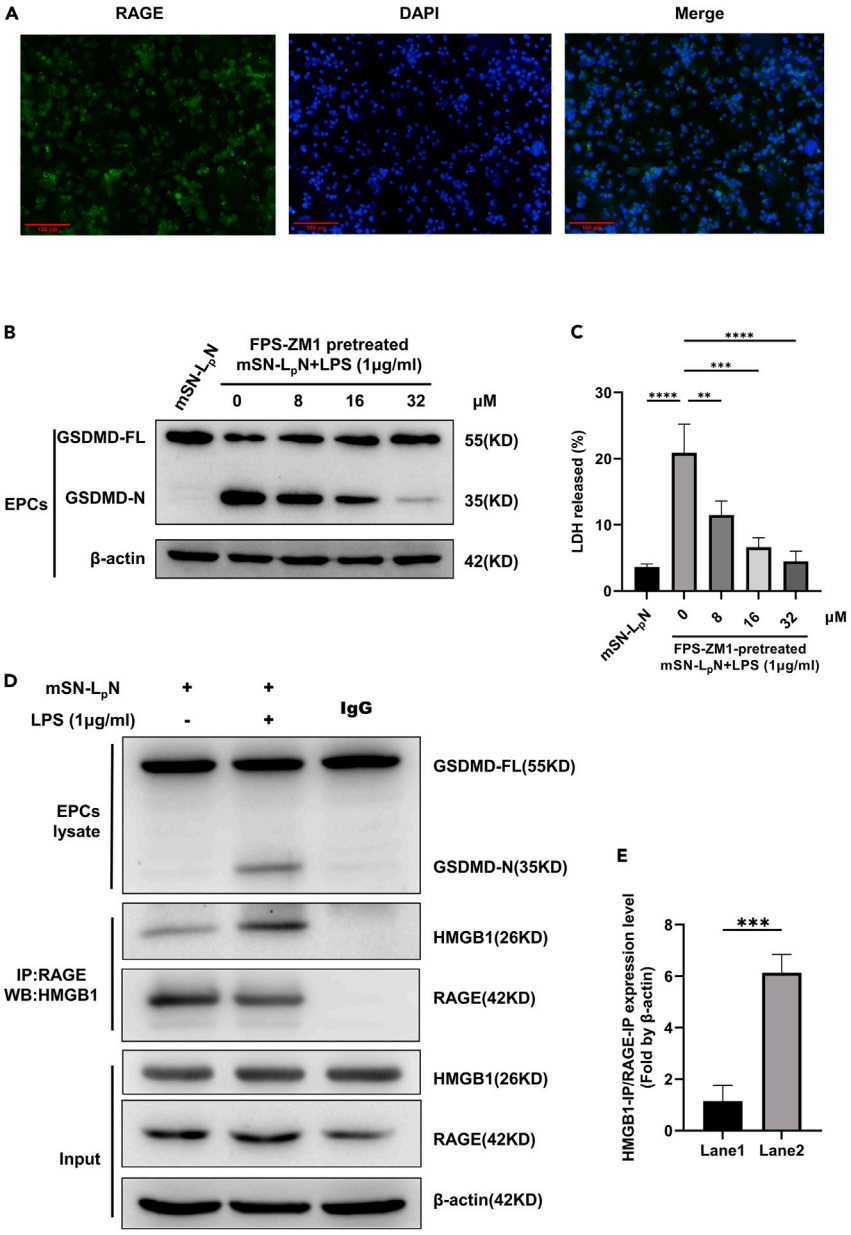

Figure 5. HMGB1-RAGE axis induces EPCs pyroptosis

(A) The expression level of RAGE in EPCs. Scale bar: 100 μm.

(B) The protein expression level of GSDMD-N in EPCs when pretreated with FPS-ZM1 before incubated with mSN-L_pN+1 μg/mL LPS.

(C) The level of LDH in EPCs supernatant when pretreated with FPS-ZM1 before incubated with mSN-L_pN+1 μg/mL LPS.

(D and E) Representative images and statistical analysis of coIP result. RAGE, receptor for advanced glycation end products; HMGB1, high-mobility group box-1; DAPI, 4′,6-diamidino-2-phenylindole; mSN, macrophages supernatant; Ctrl, control; L_p, LPS priming (100 ng/mL); N, nigericin (10 μM); GSDMD-FL, GasderminD-full length; GSDMD-N, GasderminD-N domain; IP, immunoprecipitation; LDH, lactic dehydrogenase. WB was performed using ImageJ software for gray value analysis and β-actin as the standard. The data shown are representative of three independent experiments. Data are represented as mean ± SD. *p* values were determined using a two-tailed t test and ANOVA analysis, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

See also Figure S4.

Data and code availability

- All data generated or analyzed during this study are included in this published article and supplemental information.
- No new code was generated in this study.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

M.Z. and G.L. are co-first authors. They contributed equally to this work, conceived the study, performed the experiment and data analysis, and drafted the manuscript. F.Y., S.Y., and J.L. carried out the data collection and analysis. Z.H. conceived the study and its design and critically revised the manuscript. All authors read and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-RAGE	Santa cruz	Cat# sc-365154; RRID:AB_10707685
Mouse monoclonal anti- Ac-lysine	Santa cruz	Cat# sc-32268; RRID:AB_627898
Goat polyclonal anti-IL-1β	R&D Systems	Cat# AF-401-NA; RRID:AB_416684
Rabbit monoclonal anti-ASC	Cell SignalingTechnology	Cat# 67824; RRID:AB_2799736
Mouse monoclonal anti-β-actin	Cell SignalingTechnology	Cat# 3700; RRID:AB_2242334
Rabbit monoclonal anti- GSDMD	Abcam	Cat# ab209845; RRID:AB_2783550
Rabbit monoclonal anti-HMGB1	Abcam	Cat# ab79823; RRID:AB_1603373
Rabbit monoclonal anti-GSDME	Abcam	Cat# ab215191; RRID:AB_2737000
Biological samples		
Peripheral blood of sepsis patients and healthy controls	Department of Critical Care Medicine, Xiangya Third Hospital	N/A
Chemicals, peptides, and recombinant proteins		
Recombinant Human HMGB1 protein, CF	R&D Systems	Cat# 1690-HMB-050
Critical commercial assays		
LDH Cytotoxicity Assay Kit	Beyotime	Cat# C0017
IL-1 alpha Mouse Uncoated ELISA Kit	eBioscience	Cat# 88-5019-22
IL-1 beta Mouse Uncoated ELISA Kit	eBioscience	Cat# 88-7013A-86
Mouse HMGB-1(Highmobility group protein B1) ELISA Kit	elabscience	Cat# E-EL-M0676
BCA kit	Thermo Scientific	Cat# 23227
FAM-FLICA-activated caspase-1 detection kit	Amyjet Scientific	Cat# CT-97
Experimental models: Cell lines		
Immortalized bone marrow-derived macrophages (iBMDM)	Lab stock	N/A
Oligonucleotides		
siRNA sequences for mouse HMGB1 (sense5′-3′: CAAGGCUCGUUAUGAAAGATT, antisense5′-3′ UCUUUCAUAACGAGCCUUGTT)	This paper	N/A
Software and algorithms		
Graphpad Prism 9.5	GraphPad Software	https://www.graphpad.com/
ImageJ	N/A	https://imagej.net/

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Clinical samples

Seventeen patients were recruited from the Third Xiangya Hospital of Central South University and met the diagnostic criteria for sepsis.[66] Six healthy subjects were recruited as the control. Written informed consent of each subject has been obtained for this study (NO. Q24809). All included patients were between 18 and 80 years of age, and they had not received other surgical interventions prior to enrollment. The genders of the patients were comparable. All patients were Asians.

Animals

8-weeks old male C57BL/6 mice were purchased from Hunan SJA Laboratory Animal Co. ltd (Changsha, China). All animals were feeded in specific pathogen free conditions under constant temperature and humidity control in the Department of Experimental Animals, Central South University. All animal experiments are conducted in accordance with the Institutional Animal Care and Use Committee of Central South University (CSU-2022-0047).

Serum preparation

Fasting peripheral blood samples were taken and centrifuged within 1 hour (h) at 700 ×g for 10 minutes (min). The supernatant was collected and stored at −80°C.

Isolation, culture, and identification of EPCs

EPCs were derived from mouse bone marrow or human peripheral blood as previously described.[67,68] In brief, mononuclear cells were isolated from the bone marrow of C57BL/6 mice aged 8 weeks or human peripheral blood and seeded in culture plates incubated with EGM-2 complete medium (CC-3162, LONZA, Switzerland). Every 3–4 days, we replaced the old medium with fresh medium and removed the cells those unattached to the wall. Cells were harvested on the 7-10th day of culture.

EPCs were identified by double staining with 1,1 ′-dioctadecyl-3, 3, 3′, 3- tetramethyl indocarbocyanine perchlorate-labeled acetylated low-density lipoprotein (DiI-Ac-LDL, L3483, Thermo, USA) and fluorescein isothiocyanate-labeled ulex europaeus agglutinin-1 (FITC-UEA-1, L9006, Sigma, USA), and cells with double-positive staining was defined as EPCs (Figure S1).

Macrophages preparation and intervention

Healthy male C57BL/6 mice with 8 weeks old were injected with 3mL sterile 3% thioglycollate broth intraperitoneally to elicit peritoneal macrophages. 72 h later, cells were collected by lavage of the peritoneal cavity with 15mL of RPMI medium 1640 (GIBCO, USA). After being washed, cells were resuspended in RPMI medium 1640 supplemented with 10% fetal bovine serum and antibiotics (GIBCO, USA).

Peritoneal macrophages (2 ×10^6 cells per well) plated in 6-well plates were intervened with ultrapure LPS priming (L_p, 100 ng/mL) for 4 h alone or L_p for 3 h and then plus nigericin (Nig (N), 10 μM) for 1h (L_pN). As thus, macrophages were divided into group control (Ctrl), group L_p and group L_pN. Macrophages supernatant (mSN) in each group was collected and centrifuged at 400×g for 5 min and prepared for the subsequent EPCs incubation.

EPCs intervention and groups

EPCs were cultured with each group of mSN or mSN plus 1 μg/mL of ultrapure LPS. Therefore, EPCs were divided into eight groups: Blank, 1 μg/mL LPS, mSN-Ctrl, mSN-L_p, mSN-L_pN, mSN-Ctrl+1 μg/mL LPS, mSN-L_p+1 μg/mL LPS and mSN-L_pN+1 μg/mL LPS (Table 1). After being cultured for 2 h, EPCs were lysed to obtain total protein which was quantified and subsequently used for western blot analysis.

METHOD DETAILS

PI staining

Each group of EPCs were washed with PBS for 3 times, PI dye with a v/v ratio of 1:4000 was added, and the cells were incubated at 37°C for 20 min and observed under fluorescence microscope (Nikon Ti2-U, Japan).

LDH release assay

Level of LDH in cell culture supernatant was determined using LDH Cytotoxicity Assay Kit (C0017, Beyotime) according to the manufacturer's instructions. Briefly, EPCs supernatant was collected and centrifuged at 400×g for 5 min. Then, 120 μL supernatant was mixed with 60 μL LDH working reagent and incubated in dark at room temperature for 30 min. The absorbance was measured at 490 nm.

ELISA assay

Levels of IL-1β, IL-1α and HMGB1 in cell culture supernatant and HMGB1 in serum were determined using quantitative ELISA kits according to the manufacturer's instructions. In short, EPCs supernatant or serum in sepsis patients were collected and added to the pre-coated well plates. The well plates were incubated at room temperature for 2 h and cleaned with washing solution for 3 times. Then the detection antibody and HRP-antibody were added to the well plates successively and the well plates were incubated for 1 h and 30 min respectively. After being washed, the luminescent solution and termination solution were added to the well plates. The absorbance was measured at 450 nm.

WB

Samples were separated by 10–15% SDS-PAGE and transferred onto PVDF membranes (Millipore). Antibodies to mouse RAGE (sc-365154, Santa cruz, USA), Ac-lysine (sc-32268, Santa cruz, USA), GSDME (ab215191, Abcam, UK), IL-1β (AF-401-NA, R&D Systems, USA), GSDMD (ab209845, Abcam, UK) were used at 1:1000 dilution; anti-mouse HMGB1(ab79823, Abcam, UK) was used at 1:5000 dilution.

ASC-speck detection

EPCs in each group were fixed in 4% paraformaldehyde for 15 min followed by permeabilization with 0.1% Triton X-100 for 10 min. The slices were blocked with PBS containing 3% bovine serum albumin (BSA) for 1 h, followed by anti-ASC antibody (67824, CST, USA) and DAPI (C0065, Solarbio, China) staining. Cells were visualized by fluorescence microscope.

coIP

After being cultured, EPCs was lysed in IP buffer (#9803, CST, USA) containing protease inhibitor cocktail for 30 min to obtain total proteins. Proteins were then incubated with anti-RAGE or anti-Ac-lysine antibody overnight at 4°C with rotation. The next day, we added 20 μL pre-washed protein A/G agarose beads (sc-2003, Santa cruz, USA) into the samples. 3 h later, the samples were washed five times with IP buffer. After being extensively washed with a diluted lysis buffer, the lysates was used for WB analysis.

RNA interference assay

Immortalized bone marrow-derived macrophages (iBMDMs) were seeded in 6-well plates (1×10^6 cells per well), then transfected with siRNA using Lipofectamine RNAiMAX (13778075, Thermo, USA) according to the manufacturer's instructions. The siRNA sequences for mouse HMGB1 (sense5′-3′: CAAGGCUCGUUAUGAAAGATT, antisense5′-3′ UCUUUCAUAACGAGCCUUGTT) and the negative control (sense5′-3′: UUCUCCGAACGUGUCACGUTT, antisense5′-3′: ACGUGACACGUU. CGGAGAATT) were chemically synthesized by SuZhou GenePharme Co., Ldt., China.

Immunofluorescence

The expression of RAGE on EPCs was detected by immunofluorescence. The slices were washed with PBS and fixed with 4% paraformaldehyde for 30 min. Then the slices were sealed by 3% BSA for 1 h, followed by anti-RAGE and DAPI staining. Cells were visualized by fluorescence microscope.

FAM-FLICA-activated caspase-1 and PI double staining

Mononuclear cells were isolated from the peripheral blood or mice bone marrow and cultured with growth stimulating and differentiation factors. After culture on days 7–10, the cells reached 80% fusion and were harvested for subsequent treatment.

EPCs were seeded in 48-well (2.5×10^6 cells per well) culture plates, and then cells were tested for intracellular FAM-FLICA-activated caspase-1 and PI staining according to the manufacturer's instructions. In short, 1× FLICA buffer was added to the medium and incubated at 37°C. 1 h later, cells were washed with washing buffer 3 times and then stained with Hoechst 33342 for 5 min. Next, cells were stained with PI for 5 min and then fixed for 30 min and visualized by fluorescence microscope.

QUANTIFICATION AND STATISTICAL ANALYSIS

Each experiment was repeated at least three times. Data were analyzed using GraphPad Prism 9.5 (GraphPad Software, USA), and all data were expressed as mean ± standard deviation (SD). WB was performed using ImageJ software for gray value analysis. ANOVA analysis was used to compare the mean of the normal distribution data among groups, and two-tailed Student's t test was used to compare the difference of the mean between the two groups. Differences were considered significant when *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.