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Antimicrobial Peptide LL-37 Ameliorates Murine Sepsis Through the Induction of Microvesicle (Ectosome) Release from Neutrophils

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Abstract

Neutrophils release microvesicles (ectosomes) upon stimulation. Interestingly, ectosome level is elevated in sepsis survivors. Previously, we revealed that LL-37, a human cathelicidin antimicrobial peptide, improves the survival of a murine cecal ligation and puncture (CLP) sepsis model. Thus, in this study, we elucidated the action of LL-37 on sepsis, by focusing on the effect of LL-37 on ectosome release in the CLP model. The results demonstrated that the ectosome level was elevated in CLP mice, and the level was further enhanced by the administration of LL-37, accompanied by reduced bacterial load. More importantly, ectosome-containing microvesicles isolated from LL-37-injected CLP mice contained a higher amount of antimicrobial proteins/peptides (such as lactoferrin and murine cathelicidin-related antimicrobial peptide), and exhibited higher antibacterial activity, compared with those from PBS-injected CLP mice, suggesting that LL-37 induces the release of ectosomes with antibacterial potential in vivo. In fact, LL-37 stimulated mouse bone marrow neutrophils to release ectosomes ex vivo, and the LL-37-induced ectosomes possessed antibacterial activity. Furthermore, the administration of LL-37-induced ectosomes reduced the bacterial load and improved the survival of CLP mice. These observations suggest that LL-37 induces the release of ectosome-containing antimicrobial microvesicles in CLP mice, thereby reducing the bacterial load and protecting the mice from lethal septic conditions.

Keywords

Ectosomes
Antimicrobial peptides
Neutrophils
Sepsis

1. Introduction

Sepsis is caused by an exaggerated biological response to infection and is associated with multiple organ failure and fatal shock [1]. Effective treatment for sepsis has not yet been established, and it is a serious disease with a high fatality rate of 25–50% even in developed countries [1]. During our search for an effective treatment for sepsis, we reported that human antimicrobial peptide LL-37 reduces symptoms and improves survival in a mouse model of sepsis caused by cecal ligation and puncture (CLP) [2,3].

On the other hand, when neutrophils are stimulated with formyl peptide fMLF, ectosomes, which are 0.1–1 μm in size, are released [4,5]. Ectosomes contain molecules derived from neutrophil granules (e.g. antimicrobial proteins) and molecules present on the plasma membrane of neutrophils (e.g. Ly6G, phosphatidylserine [PS]) [4,5]. Interestingly, the number of ectosomes in the blood is elevated in patients surviving sepsis [6,7], suggesting the role of ectosomes in sepsis pathogenesis. In the present study, we investigated the effect of LL-37 on a murine model of sepsis, focusing on the ectosome-releasing effect of LL-37 from neutrophils.

2. Decreased viable cell counts and increased ectosome levels in CLP mice treated with LL-37

CLP was performed on BALB/c mice (female, 7–8 weeks old), LL-37 (3 μg/mouse), or control PBS (phosphate-buffered saline) was injected into the tail vein [3]. Sham mice underwent only laparotomy without ligation and puncture. 14–18 h after CLP, physiological saline or HBSS (Hanks balanced salt solution, Ca^{2+} and Mg^{2+} free) was injected intraperitoneally and the peritoneal exudate was collected. Additionally, heparin blood samples were taken from the heart, and the number of viable bacteria in the peritoneal exudate and blood was examined by colony formation. As shown in **Figure 1**, the results showed that the number of viable bacteria in CLP mice treated with LL-37 was significantly lower than that in CLP mice treated with PBS. In contrast, no viable bacteria were detected in the abdominal exudates and blood of Sham mice (data not shown). To determine the number of ectosomes, the peritoneal fluid was centrifuged at 400 × g for 5 minutes, the supernatant was centrifuged at 10,000 × g for 10 minutes, and then at 100,000 × g for 1 hour. The precipitate (extracellular vesicle fraction) was then suspended in PBS. Blood was centrifuged at 5,000 × g for 20 minutes and plasma was prepared without platelets. Ectosomes in these fractions were fluorescently labeled with PE (phycoerythrin)-Ly6G and FITC-Annexin V (specifically bound to PS), and their numbers were measured in a flow cytometer. The results showed that the number of ectosomes in the abdominal cavity and plasma was significantly increased by CLP in both PBS- and LL-37-treated mice (**Figure 2**, PBS-treated Sham vs. PBS-treated CLP; LL-37-treated Sham vs. LL-37-treated CLP). Interestingly, the number of ectosomes in CLP-treated mice was

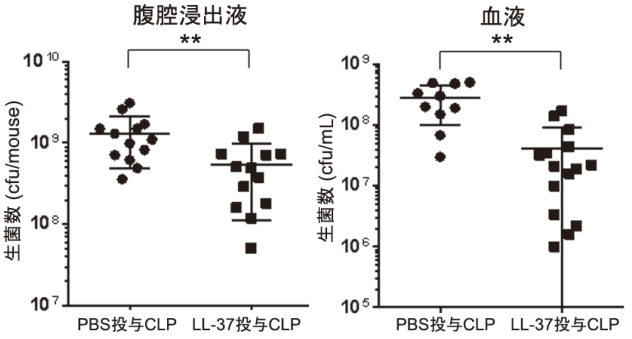

Figure 1. Effect of LL-37 administration on viable bacterial counts in CLP mice $^{**}P < 0.01$

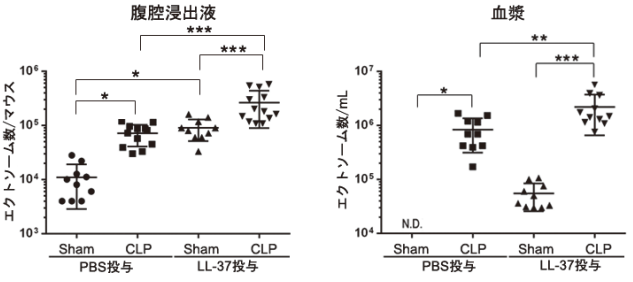

Figure 2. Effect of LL-37 treatment on ectosome counts in CLP mice $^{*}P < 0.05$, $^{**}P < 0.01$, $^{**}P < 0.001$, N. D.: not detected.

further increased by LL-37 administration (**Figure 2**, PBS-treated CLP vs. LL-37-treated CLP). In Sham mice, the number of ectosomes was also significantly increased by LL-37 treatment (**Figure 2**, PBS-treated Sham vs. LL-37-treated Sham).

3. Antimicrobial activity of extracellular vesicles (ectosomes) isolated from CLP mice treated with LL-37

As described above, an increase in the number of ectosomes and a decrease in the number of viable ectosomes were observed in LL-37-treated CLP mice compared to PBS-treated CLP mice, suggesting that LL-37 treatment releases ectosomes with antibacterial activity. Therefore, it was considered possible that LL-37 administration could release ectosomes with antimicrobial activity. Therefore, we incubated microvesicles (MVs) (1, 2.5, and 5 μg) prepared from the abdominal exudates of CLP mice treated with PBS or LL-37 with *E. coli* (5×10^3, log phase) isolated from the caecum of mice, and evaluated the antibacterial activity of these extracellular vesicles. The results showed that extracellular vesicles from PBS-treated CLP mice (PBS-CLP-MV) and extracellular vesicles from LL-37-treated CLP mice (LL-37-CLP-MV) both showed dose-dependent antimicrobial activity, and LL-37-CLP-MV exhibited significantly higher antibacterial activity than PBS-CLP-MV, as presented in **Figure 3A**. Furthermore, as ectosomes contain neutrophil-derived molecules [4,5], Western blotting of the antimicrobial proteins/peptides contained in the extracellular vesicles, shown in **Figure 3B**, revealed that LL-37-CLP-MV had significantly higher lactoferrin, myeloperoxidase, and cathelicidin-related antimicrobial peptide (CRAMP, mouse ortholog of LL-37-CLP-MV) compared to PBS-CLP-MV.

4. Ectosome release from neutrophils by LL-37

Having shown that LL-37 induces ectosome release in CLP mice, we next investigated whether LL-37 acts directly on neutrophils to release ectosomes. For this purpose, neutrophils (2×10^6) isolated from mouse bone marrow by density gradient centrifugation using Percoll [8]

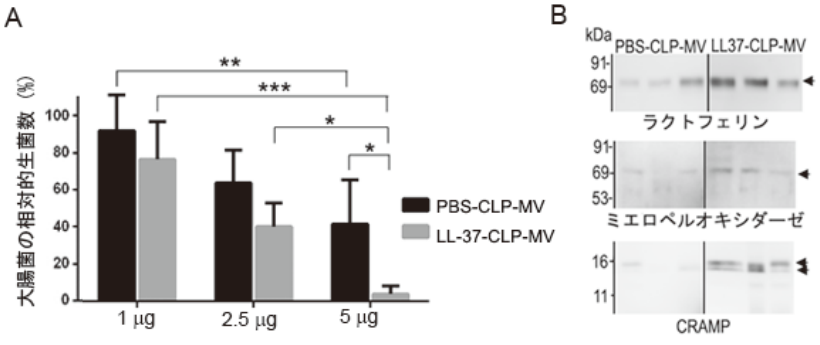

Figure 3. Antimicrobial activity (A) and antimicrobial proteins/peptides (B) of extracellular vesicle fractions prepared from CLP mice. (A) The number of viable *E. coli* cells, when incubated with PBS, is shown as 100, relative to the number of viable cells when incubated with extracellular vesicles. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. (B) Lactoferrin, myeloperoxidase, and CRAMP were detected by Western blotting.

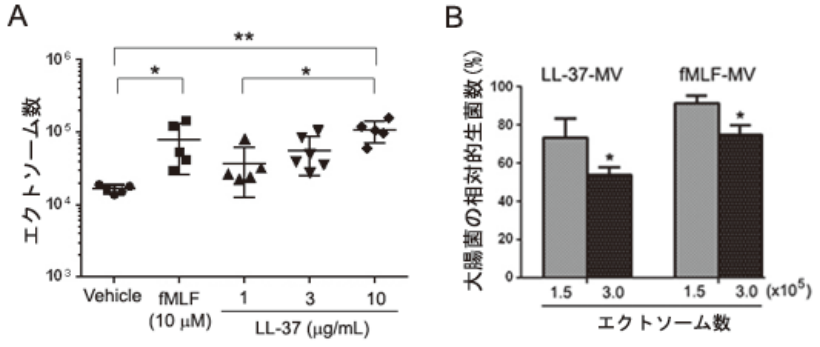

Figure 4. Ectosome release from neutrophils by LL-37 (A) and antibacterial activity of ectosome-containing extracellular vesicle fractions (B) *$P < 0.05$, **$P < 0.01$

were stimulated with fMLF or LL-37 in HBSS+ (Hanks balanced salt solution, containing Ca^{2+} and Mg^{2+}), and then incubated for 30 minutes at 37°C. The supernatant was then centrifuged at 500 × g for 5 minutes and at 3,000 × g for 5 minutes, and the number of ectosomes in the supernatant was measured by flow cytometry.

The results showed that fMLF stimulation induced ectosome release and that LL-37 also induced ectosome release in a concentration-dependent manner, as displayed in **Figure 4A**. Based on **Figure 4B**, when the extracellular vesicle fraction containing ectosomes was incubated with *E. coli*, the number of viable cells decreased in both extracellular vesicles released by fMLF (fMLF-MV) and by LL-37 (LL-37-MV), confirming that the extracellular vesicles containing ectosomes released from neutrophils have antibacterial activity.

5. Protective effect of extracellular vesicles (ectosomes) released from neutrophils stimulated with LL-37 on septic mice

Lastly, we assessed whether LL-37-MV has protective effects against sepsis. Two hours after CLP treatment of mice, LL-37-MV containing 3×10^5 ectosomes was administered intraperitoneally and the number of viable cells in the abdominal cavity and blood of mice was measured 24 hours later, and the survival of mice was monitored for 10 days. The results showed that the viable bacterial counts were significantly lower in CLP mice treated with LL-37-MV (**Figure 5A**) and the survival rate was significantly higher (**Figure 5B**) compared to CLP mice treated with PBS. In contrast, no viable bacteria were detected in the abdominal exudates or blood of Sham mice treated with PBS or LL-37-MV, and the survival rate was 100%, as shown in **Figure 5B**.

6. Conclusion

We have previously reported that the antimicrobial peptide LL-37 improves survival in CLP septicemic mice [2,3], and in the present study, we found that LL-37 reduced the number of viable bacteria in the abdominal cavity and blood of CLP mice and simultaneously increased the number of ectosomes. Furthermore, the extracellular vesicles isolated from LL-37-treated mice contained more neutrophil-derived antimicrobial active molecules (lactoferrin, myeloperoxidase, and CRAMP) and had higher antimicrobial activity than those from PBS-treated mice. Therefore, LL-37 may reduce the number of viable bacteria in the abdominal cavity and blood by increasing the number of ectosomes with antimicrobial activity in CLP mice, resulting in improved mouse survival. LL-37 was also found to act directly on neutrophils prepared from mouse bone marrow to induce ectosome release. Furthermore, ectosomes released from LL-37-stimulated neutrophils also had antimicrobial activity, and administration of these ectosomes to CLP mice reduced the number of viable bacteria in the abdominal cavity and blood and improved survival. These findings suggest that LL-37 acts protectively against sepsis by promoting the

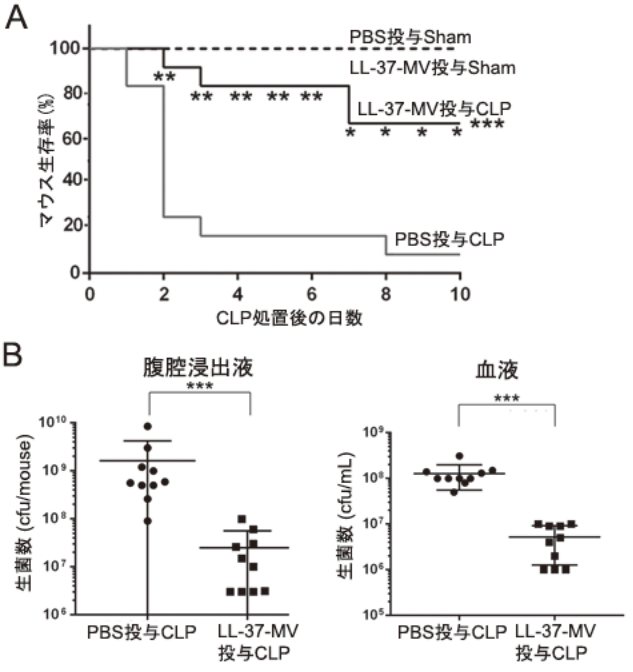

Figure 5. Protective effect of extracellular vesicles released from neutrophils stimulated with LL-37 against murine sepsis $*P < 0.05$, $**P < 0.01$, $***P < 0.001$

release of ectosomes with antimicrobial activity.

LL-37 has been shown to have multifaceted actions in sepsis. We have already shown that LL-37 acts on neutrophils to induce the release of neutrophil extracellular traps (NETs), which have antimicrobial activity [2], and on macrophages, which release inflammatory cytokines such as IL-1β to exacerbate inflammation [3]. Therefore, the search for substances like LL-37 that exert multifaceted effects in sepsis and the elucidation of their mechanisms of action will provide important information for the development of effective treatments for sepsis.

Disclosure statement

The authors declare no conflicts of interest.

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