Modulation of Macrophage Cell Death, Pyroptosis by Host Defense Peptide LL-37

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Pyroptosis is defined as a caspase-1 dependent cell death and mainly occurs in macrophages and dendritic cells, accompanied with the release of pro-inflammatory cytokines. Moreover, pyroptosis results in the cellular lysis and release of cytosolic contents, which induce the augmentation of inflammatory reactions. Of note, caspase-1 knockout (KO) causes a protective effect on the sepsis models and improves the survival of mice, in which the IL-1 β level was totally suppressed. Thus, caspase-1 activation and possibly pyroptosis play a major role in the mortality of sepsis. AMPs (antimicrobial peptides) are functioning in the first line of defense against invading pathogens. Cathelicidin family of AMPs are recognized in many mammalian species, and LL-37 is the only one human cathelicidin. Besides its broad spectrum of antimicrobial actions, LL-37 has a role in innate immune defense, such as regulation of inflammatory responses and wound healing. Interestingly, LL-37 displays the protective effect on the endotoxemia models. In this review, we introduce a newly identified inhibitory effect of LL-37 on macrophage pyroptosis *in vitro* and *in vivo*.

Key words: antimicrobial peptide, LL-37, pyroptosis, macrophage, sepsis

Introduction

Sepsis is a life-threatening organ dysfunction induced by an uncontrolled host response to infection¹⁾. In sepsis, over expression of inflammatory cytokines is a major mechanism for the multiple organ failure²⁾⁻⁴⁾. Recently, much attention has been focused on the mechanism for host cell death, which advances during sepsis and leads to the dysregulated inflammatory responses^{2) 5) 6)}.

Pyroptosis is a caspase-1 dependent cell death of macrophages and dendritic cells recognized in bacterial infection⁷⁾. During pyroptosis, the cells promptly produce and release extracellularly the proinflammatory cytokines (such as IL-1 β)^{7) 8)}. IL-1 β is a proinflammatory cytokine, which induces both local and systemic inflammatory/immune

responses⁹⁾, and acts cooperatively with other cytokines to cause tissue injury in sepsis¹⁰⁾. Pyroptosis is induced by the two distinct stimuli, microbial PAMPs (pathogen associated molecular patterns) (such as lipopolysaccharide/LPS) and endogenous DAMPs (damage associated molecular patterns) (such as ATP) (Figure-1)^{7) 8)}. TLRs (Toll like receptors) start a signaling that leads to cellular activation (including the upregulation of proinflammatory cytokines), in response to PAMPs⁸⁾. Furthermore, in responses to DAMPs, NLRs (Nod like receptors)/NLRPs (Nod like receptor proteins) are associated for the formation of inflammasome, where the active caspase-1 is converted from the procaspase-1⁸⁾. Lastly, IL-1 β is processed and released by the activated caspase-1, and cell death is induced^{8) 11)}. Of note, caspase-1

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Figure-1 The stimulation of PAMPs and DAMPs induces pyroptosis

Pyroptosis is induced by the two distinct stimuli, microbial PAMPs (e.g. LPS and lipoproteins) and endogenous DAMPs (e.g. ATP and uric acid). In response to PAMPs, TLRs trigger a signaling leading to cellular activation (such as the upregulation of proinflammatory cytokines). In contrast, in responses to DAMPs, NLRs are associated for the formation of inflammasome, where the active caspase-1 is converted for the procaspase-1. Finally, IL-1 β is processed, and cell death is induced by the activated caspase-1.

knockout (KO) causes a protective effect on several murine sepsis models and improves the survival of mice, where the IL-1 β level is completely depressed, and the organs (such as kidney and spleen) are protected^{(12)–(16)}. Thus, caspase-1 activation and possibly pyroptosis play a major role in the pathogenesis (organ failure) and mortality of sepsis^{(7) (17)}.

Antimicrobial peptides (AMPs), which are also called host defense peptides, function as the first line of defense against invading pathogens⁽¹⁸⁾. Cathelicidin family of AMPs are recognized in various mammalian species, and LL-37 is the only one human cathelicidin, which is cleaved from CAP18 (a cationic antimicrobial protein of 18-kDa) and primarily produced by neutrophils and epithelial cells⁽¹⁸⁾. Besides its broad spectrum of bactericidal activities, LL-37 exhibits a wide range of inflammatory/immune modulatory activities^{(18) (19)}. Of note, LL-37 suppresses TNF- α production by LPS-stimulated macrophages and improves the survival of LPS-induced endotoxin mice via the neutralization of LPS^{(20) (21)}. Thus, as a therapeutic strategy in sepsis, LL-37 is attracting researchers' attention. Interestingly, LL-37 reduces the LPS-induced apoptosis of endothelial cells by neutralizing the action of LPS and inhibits the spontaneous apoptosis of neutrophils via the purinergic receptor

X₇ (P2X₇) and formyl-peptide receptor-like 1 (FPRL1)^{(22) (23)}, suggesting that LL-37 is involved in the modulation of cell death. Based on these facts, we focus on the effect of LL-37 on a macrophage cell death, pyroptosis in sepsis and introduce our results in this review.

Inhibition of LPS/ATP-induced pyroptosis of macrophages by LL-37

LPS is a major constituent of the outer membrane of gram-negative bacteria and function as a potent inducer of inflammatory responses by macrophages. Thus, LPS is a key molecule in the pathogenesis of gram-negative sepsis^{(20) (24)}. LPS stimulates macrophages via the binding to the membrane receptors, CD14/TLR4⁽²⁵⁾. In addition, extracellular ATP can be released from the dead host cells⁽²⁶⁾. For example, the plasma ATP level reaches to 10 μ M in the CLP (cecal ligation and puncture) septic mice (2 μ M in the Sham mice)⁽²⁷⁾, and the peritoneal ATP level increases up to 1 μ M in the *E. coli*-induced septic mice (0.1 μ M in the Sham mice)⁽²⁸⁾. Then, ATP activates a purinergic nucleotide receptor P2X₇ to elicit the signal to induce the formation of inflammasome, where the active caspase-1 is converted from the

Figure-2 Effect of LPS and ATP on the pyroptosis of J774 cells

Macrophage-like cells (J774) were primed with 10 ng/ml LPS for 4 h, and then stimulated with 3 mM ATP for the indicated time intervals in the presence or absence of 20 μ M Ac-YVAD-CHO (a caspase-1 specific inhibitor). J774 cells were also incubated without LPS and ATP (Resting) or with LPS or ATP alone. Then, the supernatants were assayed for IL-1 β (A) and LDH (B). Data shows the mean $\pm$ SD. Values are compared between the absence and presence of Ac-YVAD-CHO. ** $p < 0.01$, *** $p < 0.001$. C. J774 cells were primed with 10 ng/ml LPS for 4 h and then stimulated with 3 mM ATP for 90 min. Then, the cells were stained with FAM-YVAD-fmk (a fluorescent labeled caspase-1 inhibitor for inflammasome staining, green) and Hoechst 33342 (for nuclear staining, blue). Arrowheads indicate the inflammasomes, which contained the activated caspase-1.

procaspase-1. Lastly, IL-1 β is processed and released by the activated caspase-1, and cell death (pyroptosis) is induced (Figure-1)²⁹⁾.

Thus, we use the LPS/ATP treatment as the typical inducers to assess the effect of LL-37 on macrophage pyroptosis *in vitro*. Mouse macrophage-like cells (J774) were primed with 10 ng/ml LPS, and then treated with ATP. LPS or ATP treatment alone did not essentially induce the production of IL-1 β and cell death (as assessed by LDH release); however, the treatment with both LPS and ATP induced the production of IL-1 β and cell death (Figure-2A and B). Of interest, Ac-YVAD-CHO (a caspase-1 specific inhibitor) suppressed both the LPS/ATP-induced release of IL-1 β and LDH, suggesting that the IL-1 β release

and cell death are caspase-1 dependent. In fact, we confirmed by immunohistochemical staining that LPS/ATP induces the activation of caspase-1 in inflammasome (Figure-2C). Moreover, the effect of LL-37 on the LPS/ATP-induced pyroptosis was investigated. Of importance, LL-37 substantially inhibited the LPS/ATP-induced IL-1 β production, cell death (LDH release)³⁰⁾. In addition, we confirmed by flow cytometry that LPS/ATP induces the caspase-1 activation, and LL-37 significantly suppresses the LPS/ATP-induced caspase-1 activation³⁰⁾.

To clarify the mechanism for the inhibitory action of LL-37, we assessed the action of LL-37 on the LPS binding to the cells. LL-37 potently inhibited the binding of LPS to its receptor CD14/TLR4 on

J774 cells³⁰⁾. Together these observations apparently indicate that LL-37 suppresses the LPS/ATP-induced pyroptosis via the inhibition of the LPS binding to its receptor (CD14/TLR4). Moreover, we confirmed that ATP stimulates the nucleotide receptor (P2X₇) to activate caspase-1 in J774 cells and the activation was suppressed by the P2X₇ antagonists (KN-62 and KN-93)³⁰⁾. Importantly, LL-37 substantially inhibited the ATP-induced caspase-1 activation³⁰⁾, indicating that LL-37 clearly inhibits the ATP-induced P2X₇ activation.

Together these observations indicate that the treatment with LPS/ATP induces the pyroptosis of macrophage-like cells, and that LL-37 suppresses the LPS/ATP-induced pyroptosis, which is associated with IL-1 β release, caspase-1 activation and cell death. In addition, LL-37 represses the LPS binding to the cells, and the ATP-induced P2X₇ activation, indicating that LL-37 inhibits the pyroptosis via both the neutralization of LPS action and the inhibition of the P2X₇ response to ATP (Figure-5, left panel).

Inhibition of the macrophage pyroptosis and improvement of the survival of septic mice by LL-37

Based on the above findings, we hypothesize that LL-37 may inhibit the pyroptosis of macrophages *in vivo*, thereby exhibiting the protective action on septic model. Thus, we intravenously administered LL-37 into a CLP septic model of mice, and evaluated the effect of LL-37 on pyroptosis by analyzing caspase-1 activation, cell death and IL-1 β release.

First, we evaluated the effect of LL-37 administration on the survival of CLP-operated mice. Interestingly, the administration of LL-37 (2 μ g/mouse) significantly improved the survival rate (Figure-3). Next, to evaluate the effect of LL-37 on pyroptosis of macrophages, peritoneal cells were harvested at 5 h after the CLP operation, and peritoneal macrophages were evaluated for pyroptosis by detecting caspase-1 activation and cell death (7AAD positive). As shown in Figure-4, caspase-1 activation was significantly higher in the CLP group than that in the Sham group. Similarly, pyroptosis (caspase-1-activated dead cells) was obviously induced in the CLP group. Of note, the

Figure-3 Effect of LL-37 on the survival of CLP septic mice

Mice were divided into the Sham ($\square$), CLP ($\bullet$) and LL-37 ($\circ$) groups. In the LL-37 group, mice were intravenously administered with 2 μ g per mouse LL-37, and the survival rates of the mice were monitored for 7 days. Survival data were analyzed using the Kaplan-Meier method and survival curves were compared using the log-rank test and Gehan-Breslow-Wilcoxon test in univariate analysis. * $p < 0.05$.

administration of LL-37 significantly suppressed the CLP-induced caspase-1 activation and pyroptosis of peritoneal macrophages (Figure-4B and C). Moreover, LL-37 modulated the levels of inflammatory cytokines (IL-1 β , IL-6 and TNF- α) both in the peritoneal fluids and sera and suppressed the activation of peritoneal macrophages (as evaluated by the increase in the intracellular levels of IL-1 β , IL-6 and TNF- α)³¹⁾. Furthermore, LL-37 reduced the bacterial burdens both in peritoneal fluids and blood samples³¹⁾. Thus, our present study suggests that LL-37 improves the survival of CLP septic mice by possibly suppressing the pyroptosis of macrophages, and inflammatory cytokine production by activated macrophages as well as bacterial growth (Figure-5, right panel).

Importantly, as reported above, the combination of LPS and ATP induced the pyroptosis of macrophages, which was inhibited by LL-37 via both the neutralization of LPS action and inhibition of the P2X₇ response to ATP. Since LPS and ATP are major inducer of caspase-1 activation and pyroptosis in the CLP septic mice in various septic models^{32) 33)}, we speculate that LL-37 suppresses the actions of LPS and ATP *in vivo* as well as *in vitro*, thereby inhibiting pyroptosis (caspase-1 activation) and increasing the survival of CLP septic mice.

Figure-4 Effect of LL-37 on the pyroptosis of peritoneal macrophages

Peritoneal cells were collected from mice of Sham, CLP and LL-37 groups at 5 h after the surgery. Thereafter, peritoneal cells were evaluated for pyroptosis by detecting caspase-1 activation (FLICA positive) and cell death (7AAD positive) of the peritoneal macrophages (F4/80 positive). In panel A, upper halves, right halves and upper-right quadrants show cell death, caspase-1 activation and pyroptosis (FLICA/7AAD-double positive), respectively, among peritoneal macrophages. Images are representative of 6-7 separate experiments. Panels B and C show the percentage of caspase-1 activation and pyroptosis, respectively. Data are the mean $\pm$ SEM, and values are compared between the CLP and LL-37 groups. * $p < 0.05$, ** $p < 0.01$.

Figure-5 Effect of LL-37 on pyroptosis of macrophages and sepsis

LPS and ATP treatment induces the pyroptosis of macrophages, and LL-37 inhibits the LPS/ATP-induced pyroptosis (associated with IL-1 β release, caspase-1 activation and cell death), by both the neutralization of LPS action and the inhibition of the P2X₇ response to ATP (left panel). The administration of LL-37 reduces the pyroptosis of macrophages, inflammatory cytokine production and bacterial growth, and improves the survival of CLP septic mice (right panel).

Conclusion

Pyroptosis is recently identified as a caspase-1 dependent cell death of macrophages and dendritic cells observed in bacterial infection⁷⁾. During pyroptosis, the cells promptly produce and release extracellularly IL-1 β , which acts cooperatively with other cytokines to trigger tissue injury in sepsis⁷⁾⁻¹⁰⁾. Of note, caspase-1 KO causes a protective effect on several murine sepsis models and improves the survival of mice, in which the IL-1 β level was totally reduced, and the organs (such as kidney and spleen) were protected¹²⁾⁻¹⁶⁾. Thus, caspase-1 activation and possibly pyroptosis play a major role in the pathogenesis (organ failure) and mortality of sepsis.

LL-37 is initially identified as an AMP¹⁸⁾, and participates in the innate immune system, with the abilities to protect the host from invasive microbial infections and neutralize gram-negative LPS, and now recognized as a multifunctional molecule linking the innate immune response to the adaptive immune system via the diverse immunomodulatory actions (such as induction of the cytokine and chemokine production, the immune cell chemotaxis, and the alteration of dendritic cell differentiation)¹⁸⁾⁻²¹⁾. In this review, we demonstrated an *in vitro* function of LL-37 to suppress the pyroptosis of macrophages via the neutralization of LPS action and the inhibition of P2X₇ response and an *in vivo* function of LL-37 to suppress the caspase-1 activation and pyroptosis of macrophages, using a polybacterial CLP sepsis model. As a therapeutic strategy, the blocking of LPS action (e.g. TLR4 KO) protects mice from systemic inflammation and tissue damage in endotoxemia³⁴⁾, and the blocking of ATP action (e. g. administration of a P2X₇ antagonist) improve the survival rate in *E. coli*-induced sepsis³⁵⁾. Thus, the present findings indicate that LL-37 can be a promising candidate for sepsis because it has potent biological functions, including the inhibition of pyroptosis, modulation of inflammatory cytokine production and antimicrobial activity (Figure-5).

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