Original Paper

Antimicrobial Peptide LL-37 and IDR-1 Ameliorate MRSA Pneumonia *in Vivo*

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Key Words

MRSA • LL-37 • IDR-1 • JNK • Akt • Inflammation

Abstract

Background: The only human cathelicidin, LL-37, and the innate defense regulator peptide IDR-1, which have been proven to have antimicrobial activity, represent essential elements of immunity. Our previous study showed that the peptide LL-37 was protective *in vitro* to attenuate LTA-induced inflammatory effects. Methicillin-resistant staphylococcus aureus (MRSA) causes a multitude of serious and sometimes life-threatening diseases around the globe. However, the effect of LL-37 and IDR-1 in MRSA-induced pneumonia is unknown. In the present study, we explored the potential of LL-37 and IDR-1 in ameliorating MRSA-induced pneumonia *in vivo*. **Methods:** C57BL/6 mice were randomly divided into four groups and perfused intratracheally with PBS, peptide, MRSA and MRSA plus peptide, respectively. Pulmonary tissue pathology, ELISA and quantitative RT-PCR were employed. The relative signal pathways were further explored by western blot analysis. **Results:** Pathological analysis of the lung tissue sections demonstrated that, when compared with the MRSA-treated group, both the LL-37 and IDR-1 could ameliorate the MRSA-induced pneumonia. The phosphorylation of JNK and Akt were markedly decreased in the peptide plus MRSA-treated group compared with the MRSA-treated group. Furthermore, both of them also reduced TNF- α and IL-6 production in the bronchoalveolar lavage fluid (BALF) and serum *in vivo*. **Conclusion:** We report the first evidence of peptides inhibiting inflammation, decreasing the release of inflammatory cytokines and restoring pulmonary function *in vivo*. The antimicrobial peptide LL-37 and IDR-1 could ameliorate MRSA-induced pneumonia by exerting an anti-inflammatory property and attenuating pro-inflammatory cytokine release, thus providing support for the hypothesis that both innate and synthetic peptides could protect against MRSA *in vivo*.

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Introduction

The discovery of antibiotics is one of the greatest successes in modern medicine, but the excessive use of such drugs has already caused selection for resistant microorganisms. Methicillin-resistant staphylococcus aureus (MRSA) causes a multitude of serious and sometimes life-threatening diseases around the globe [1]. Surprisingly, the estimated lethality rate due to MRSA infections exceeds that of the rate due to HIV in the United States [2]. MRSA infections have become one of the most frequent causes of hospital- and community-associated infections. The combination of their resistance to methicillin and other β -lactam antibiotics makes MRSA infections difficult to treat and results in the high infiltration of MRSA in hospital and community settings [3]. Therefore, the increasing resistance to conventional antibiotics and the challenges posed by the “superbugs” have driven the need for novel antimicrobial drugs [4].

Antibacterial peptides are an integral part of the host defense that provides immediate protection against bacterial invasion. The only peptide of the cathelicidin family that is found in the human body is LL-37, which has been shown to have broad antibacterial effects against both Gram-positive and Gram-negative bacteria [5]. Our previous study showed that the peptide LL-37 can attenuate LTA-induced inflammatory effects by inhibiting the activation of MAPK and the Akt signal pathways while decreasing the pro-inflammatory cytokine levels *in vitro* [6]. LL-37 also has the ability to neutralize lipopolysaccharides (LPS) *in vitro* and *in vivo* [7], inhibit and destroy bacterial biofilms [8], and kill fungi [9]. It works as an antiviral agent [10]. IDR-1, one type of synthetic innate defense regulator, has its protective activity against bacterial infections mediated entirely through its effects on the host immunity [11]. Ultimately, both of them can act as chemokines and modulate and/or stimulate immune cells to the site of infection [10, 11].

Protein kinase B, also known as Akt, is involved in different cell responses such as cell survival, cell proliferation and gene expression [12-14]. Akt is phosphorylated during activation, the phosphorylation at Ser-473 depends on PI3K activity [15]. Akt could be directly activated by LPS [16], yet it can regulate LPS-activated signal pathways [17]. For example, Akt could increase the expression of the activator protein-1 (AP-1) -dependent transcription induced by LPS [17]. Both of the Akt and MAPK signal pathways play an important role in inflammation [6]. Mammals express multiple MAPK signal pathways to make coordinated and integrated responses to diverse stimuli [18], the majority of them are recruited by stress and inflammatory stimuli rather than by mitogens [19]. The c-Jun NH₂-Terminal Kinases (JNKs) that belong to the MAPK family are activated by growth factors, environmental stresses and pro-inflammatory stimuli [20]. The JNKs are the major Ser/Thr kinases responsible for the recruitment of the heterodimeric transcription factor AP-1 [21, 22]. AP-1 is also a pivotal transactivator of a number of genes recruited by inflammation and stress [23].

Materials and Methods

Media and reagents

LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES-COOH) and IDR-1 (KSRIVPAIPVSL) were synthesized using F-moc chemistry at SaiBaisheng biotechnology (Beijing China). The purity of them was 97.14% and 96.77% (HPLC), respectively. Akt^{Ser473} antibodies and p-Akt^{Ser473} were purchased from Cell Signaling Technology (USA), β -actin, JNK and p-JNK were purchased from Santa Cruz biotechnology (USA), respectively. Tumor necrosis factor alpha (TNF- α) and interleukin (IL)-6 ELISA kit were purchased from R&D Systems (Germany).

Mice

6-8 weeks old C57BL/6 mice weighting 25-33g were obtained from Vital River company (Beijing China) and maintained in the conditions as previously described [24]. All the experiments were approved

by the Beijing Hospital, Ministry of Health Animal Use and Care Committee and to the Guide for Care and Use of Laboratory Animals (NIH Publication #85-23, revised 1996).

Bacterial strains and lung infection model

Bacterial strains: American Type Culture Collection (ATCC, Manassas, VA) strain (29213) of methicillin-resistant staphylococcus aureus were investigated in this study. The sample was plated on 5% sheep blood Columbia agar plates overnight and then two colonies of MRSA were inoculated into 2 ml broth culture and hatched overnight at 37°C. The next day, 100 µL of overnight MRSA culture (stationary phase) was inoculated into 5ml of fresh broth culture and then subjected to shaking (170rpm) at 37°C for another 2.5 hours to acquire Log-phase (exponential bacterial growth) MRSA using a constant temperature oscillator. The log-phase MRSA suspension was centrifuged at 1000×g for 10 minutes, and then the supernatant was discarded. Bacteria were resuspended and diluted into sterile phosphate-buffered saline (PBS, PH7.4) to achieve a concentration of approximately 6.0×10^8 colony-forming units/ml.

Study protocols: (i) lung infection model Mice were intratracheally inoculated with 30 µl of the above described MRSA for lung infection model. (ii) peptide treatment The mice were randomized to receive intratracheal LL-37 or IDR-1 with immediate bacterial challenge. Then they were returned to cages and euthanized after 24hours [25]. Blood, BALF, lung tissues were sampled for further analysis. Each left lung was fixed in formalin and embedded in paraffin following standard procedures [26].

ELISA

TNF-α and IL-6 levels in the serum and BALF of the C57BL/6 mice transtracheal perfused with PBS, LL-37, IDR-1, MRSA, MRSA plus LL-37 or MRSA plus IDR-1 and untreated mice were quantified using a commercially available ELISA kit (R&D Systems) according to manufacturer's instructions.

RNA isolation and quantitative real-time PCR analysis

RNA was extracted from lung tissue using the Trizol (Invitrogen) as described previously [27] and suspended in RNase-free water. The concentration of total RNA was obtained using spectrophotometer (Eppendorf) and diluted to 100 ng/ul. 2 µl RNA was used according to the manufacturer's instructions (one step SYBR PrimeScript RT-PCR Kit II, China) by quantitative real-time PCR Detection System (BIO-RAD, USA). The samples were evaluated in triplicates and normalized by quantification of β-actin. The amount of SYBR Green was measured at the end of each cycle.

Primer sequences: β-actin: 5'-GGA AGG GCA CCA GGA GT-3' (forward) and 5'-TGC AGC CCC GGA CAT CTA AG-3' (reverse); IL-6: 5'-TGT GCA ATG GCA ATT CTG AT-3' (forward) and 5'-CAG AGG AAA TTT TCA ATA GGC-3' (reverse); TNF-α: 5'-TCT TCT CAT TCC TGC TTG TGG-3' (forward) and 5'-GAG GCC ATT TGG GAA CTT CT-3' (reverse).

Western blot

To determine the protein content in the lungs tissue, cytoplasmic proteins were prepared as previously described [28]. Protein bands were incubated overnight with p-JNK and p-Akt^{Ser473} antibodies and then measured with anti-phospho-JNK and anti-phospho-Akt^{Ser473} antibodies at a dilution of 1:1000 on a rotating platform at 4°C. Equal loading of samples was confirmed by immunoblotting of β-actin. The membrane of each step was rinsed in TBST (pH7.6). Proteins were detected using the appropriate HRP-conjugated secondary antibodies. Image J software (National Institutes of Health, NIH) was used to perform densitometric analysis.

Statistical analysis

Data were expressed as means ± SEM. Statistical comparisons used one-way ANOVA followed by the Bonferroni procedure for multiple-group comparisons, P-value<0.05 was considered statistically significant.

Results

MRSA could induce pneumonia in C57BL/6 mice

To determine the appropriate concentration and dosage of MRSA that would cause the inflammatory effect *in vivo*, we used different concentrations and dosages of MRSA to

Fig. 1. We used untreated (A), 30 μ l of sterile PBS (B), 30 μ l of MRSA (C) treated C57BL/6 mice as control, and (D) is 0.6 mg/kg IDR-1 plus 30 μ l of MRSA. The animals were euthanatized after 24 hours. The perfusion of all of the reagents was performed within 15 seconds followed by 5 minutes of vertical processing for each mouse. HE staining, original magnification, $\times 20$.

Fig. 2. We used different dosages of LL-37 (A) and IDR-1 (B) plus 30 μ l of MRSA to treat C57BL/6 mice intratracheally. The animals were euthanatized after 24 hours. According to the histopathology grading, we chose 0.8 mg/kg of LL-37 and 0.6 mg/kg of IDR-1 for further study, respectively. The perfusion of all of the reagents was performed within 15 seconds followed by 5 minutes of vertical processing for each mouse. HE staining, original magnification, $\times 20$.

treat the C57BL/6 mice. High concentrations or dosages could cause acute deaths or poor outcomes. At the concentration and dosage of MRSA of 6.0×10^8 CFU/ml and 30 μ l (Fig. 1C), we noted significant inflammation with infiltration of lymphocytes and neutrophils, the disappearance of normal alveolar structures, obvious incrustation of the alveolar walls and many alveolar cavities filled with effusion. The perfusion of MRSA was performed within 15 seconds, followed by 5-minute vertical processing for each mouse. The animals were euthanized after 24 hours.

LL-37 and IDR-1 could effectively ameliorate the MRSA-induced pneumonia

To investigate the effect of peptides in the MRSA-induced pneumonia model, we treated animals with different doses of peptides. We used 0–2 mg/kg of LL-37 (Fig. 2A) and IDR-1 (Fig. 2B) plus 30 μ l of MRSA to perfuse the C57BL/6 mice intratracheally. A single intratracheal dosage of 0.8 mg/kg LL-37 and 0.6 mg/kg IDR-1 (Fig. 1D) could effectively ameliorate the MRSA-induced pneumonia. Nevertheless, with a dosage of more than 2 mg/kg, both LL-

Fig. 3. Inflammatory effects of C57BL/6 mice intratracheally perfused with 30 μ l MRSA (ATCC 29213) of 6.0×10^8 CFU/ml, MRSA plus peptide LL-37 or MRSA plus IDR-1 treated mice were euthanatized after 24 hours, while chemically synthesized LL-37 and IDR-1 treated C57BL/6 mice were as control. Perfusion of all reagents were performed within 15 seconds following by vertical processing of 5 minutes of each mouse. The effects of LL-37 and IDR-1 on parenchyma (A) and airway (B) inflammation are illustrated. Original magnification, $\times 20$.

Fig. 4. LL-37 attenuates IL-6 and TNF- α expression after MRSA treatment. RT-PCR of (A) IL-6 and (B) TNF- α mRNA in control (control), chemically synthesized LL-37 treated (LL-37), 30 μ l MRSA treated (MRSA), and MRSA plus LL-37 double treated (MRSA+LL-37) C57BL/6 mice (** $p < 0.01$ vs. control, ## $p < 0.01$ compared with the MRSA+LL-37 group). ELISA of IL-6 (C) (E) and TNF- α (D) (F) expression in control (control), chemically synthesized LL-37 treated (LL-37), 30 μ l MRSA treated (MRSA), and MRSA and LL-37 double treated (MRSA+LL-37) C57BL/6 mice in BALF and serum ($n = 3$, ** $p < 0.01$ vs. control, ## $p < 0.01$ compared with the MRSA+LL-37 group).

37 and IDR-1 will not show any protective effects *in vivo*. Accordingly, the C57BL/6 mice were intratracheally given the indicated dosage of the peptides and were then immediately challenged with 30 μ l of MRSA, while untreated (Fig. 1A), only PBS (Fig. 1B) or MRSA (Fig.

Fig. 5. IDR-1 attenuates IL-6 and TNF- α expression after MRSA treatment. RT-PCR of (A) IL-6 and (B) TNF- α mRNA in control (con), chemically synthesized IDR-1 treated (IDR-1), 30 μ l MRSA treated (MRSA), and MRSA plus IDR-1 double treated (MRSA+IDR-1) C57 mice (n=6, **p<0.01 compared with the control group; ##p<0.01 compared with the MRSA+IDR-1 group). ELISA of (C) (E) IL-6 and (D) (F) TNF- α expression in control (con), chemically synthesized IDR-1 treated (IDR-1), 30 μ l MRSA treated (MRSA), and MRSA and IDR-1 double treated (MRSA+IDR-1) C57 mice in BALF and serum (n=3, **p<0.01 compared with the control group; ##p<0.01 compared with the MRSA+LL-37 group).

1C) treated group were used as a control. The perfusion of all of the reagents was performed within 15 seconds followed by 5-minute vertical processing for each mouse.

LL-37 and IDR-1 could attenuate MRSA-induced pneumonia by decreasing IL-6 and TNF- α release in vivo

Notably, both IL-6 and TNF- α are important inflammatory cytokines during inflammation. We measured their mRNA expression by quantitative PCR in lung tissue and their level in serum and BALF by ELISA, respectively. In the MRSA-treated group, the inflammatory cytokine IL-6 mRNA was 2.5 ± 0.5 folds (Fig. 4A), while the TNF- α mRNA was 2.5 ± 0.4 folds (Fig. 4B) when compared with the control group. In the MRSA-treated group, the IL-6 and TNF- α expression increased to 426.9 ± 20.2 ng/L by approximately 6.6 folds (Fig. 4C) and 75.0 ± 6.7 ng/L by approximately 2.2 folds (Fig. 4D) when compared with the basal level of 65.1 ± 10.7 ng/L and 33.9 ± 1.8 ng/L in the BALF, 151.0 ± 15.1 ng/L by approximately 8.0 folds (Fig. 4E) and 75.6 ± 10.6 ng/L by approximately 3.8 folds (Fig. 4F) when compared with the control level of 18.9 ± 7.8 ng/L and 19.9 ± 0.9 ng/L in the serum, respectively. In the BALF and serum groups, the IL-6 and TNF- α levels in the MRSA plus LL-37 (Fig. 4) or IDR-1 (Fig. 5) double treated group decreased significantly compared with

Fig. 6. LL-37 inhibits MRSA induced inflammatory reactions by regulating the phosphorylation of Akt. LL-37 attenuates the phosphorylation of Akt (A) and JNK (B) *in vivo* assayed by Western blot (** $p < 0.01$ vs. control, ## $p < 0.01$ compared with MRSA+LL-37 group, $n = 5$).

Fig. 7. IDR-1 inhibits MRSA induced inflammatory reactions by regulating the phosphorylation of Akt and JNK. IDR-1 attenuates the phosphorylation of Akt (A) and JNK (B) *in vivo* assayed by Western blot (* $p < 0.05$ vs. control, ** $p < 0.01$ vs. control, ## $p < 0.01$ compared with MRSA+IDR-1 group, $n = 5$).

that of the MRSA-treated group. These results confirmed that both LL-37 and IDR-1 could attenuate MRSA-induced inflammatory reactions through the downregulation of IL-6 and TNF- α expression *in vivo*.

LL-37 and IDR-1 inhibit the phosphorylation of JNK and Akt

To investigate the probable signal transduction pathways of LL-37 (Fig. 6 A and B) and IDR-1 (Fig. 7 A and B), we tested the activation of JNK and Akt in the lung tissue. In the MRSA-treated group, the phosphorylation of JNK and Akt increased sharply when compared with that of the control, while peptides treatment could obviously inhibit the phosphorylation of JNK and Akt. LL-37 and IDR-1 may quickly recruit the immune cells to eliminate the invading pathogens, protecting the individual from sustained and potentially excessive inflammatory responses. Thus, we conclude that both LL-37 and IDR-1 could modulate immune response and ameliorate inflammatory diseases (such as MRSA-induced pneumonia) by inhibiting JNK and Akt signal transduction pathways *in vivo*.

Discussion

The primary aim of this study is to establish the experimental mice models of staphylococcal pneumonia to investigate the potential therapeutic role of peptides LL-37 and IDR-1. Many industrialized countries have confirmed that methicillin-resistant strains account for approximately 25-50% of infectious *S. aureus* isolates in the hospital. Hospital-associated MRSA strains are often multi-drug resistant, causing more dramatic forms of diseases that could be life threatening. Notably, recent research has demonstrated that *S. aureus* epidemics occur in the wave of antibiotic resistance [29] and that high antibiotic consumption rates could lead to increased MRSA infection rates over time [30].

Cationic peptides with antimicrobial activity constitute a formidable innate immunity defense barrier against infections [31]. Recent work has indicated that LL-37, as a modulator of innate immunity, modulates the expression of hundreds of genes in monocytes, epithelial cells and others; has the ability to directly recruiting immune cells; induces chemokines; resolves infections [32] and protects the mice from lethal endotoxin shock [33]. Notably, IDR-1 was originally identified as a synthetic peptide that selectively stimulates the innate immunity. IDR-1 reportedly stimulates innate defense activity mediated by T-lymphocytes and macrophages [34]. Our results indicated that both LL-37 and IDR-1 could contribute to innate immunity by the recruitment of immune cells to the site of infections. From the lung tissue pathology sections and the cell counting in the BALF (data not shown), we can tell that IDR-1 may recruit a greater number of immune cells, especially lymphocytes, to the site of infection *in vivo* at the same dosage. However, the function and mechanism of peptides LL-37 and IDR-1 in MRSA-induced pneumonia have never been reported.

The potent pro-inflammatory actions of IL-6 and TNF- α cytokines have been implicated in numerous inflammatory disorders [35]. Promoters of inflammatory genes, such as IL-6, TNF- α and a plethora of other genes, were upregulated following TLR activation [30]. There are 11 human and 13 mouse Toll-like receptors (TLRs). TLR2 and TLR4 are localized to the plasma membrane and predominately recognize components of the extracellular matrix of bacteria and viruses [36]. The most potent role of TLRs is the regulation of the host defense, specifically the innate and adaptive responses to eliminate the invading pathogens at mucosal sites such as the respiratory system and skin. Subsequently, a series of complicated reactions lead to the activation of the MAPKs and PI3K combined with the release of inflammatory cytokines IL-6 and TNF- α .

Our results demonstrated that MRSA could upregulate pro-inflammatory IL-6 and TNF- α levels and activate the phosphorylation of JNK and Akt while with certain dosages, both LL-37 and IDR-1 could downregulate IL-6 and TNF- α content and inhibit the activation of JNK and Akt. Nevertheless, higher dosages of LL-37 and IDR-1 appeared to have no protective effects and seemed to be toxic to the mouse. Firstly, from the pulmonary tissue pathology, we could observe that higher dosages have no protective effects. Secondly, in our experiments we found that the survival time in higher dosages peptides plus MRSA double treated group was shorter than the only MRSA treated group. A point of discussion is the relatively low specificity of LL-37 to distinguish between bacteria and eukaryotic cells. Peptides favorably interact with bacterial membranes at relatively low concentrations while with a high concentration, they may interact with erythrocytes, which contain sialic acid and cause hemolysis [10]. The reason for this high dose-toxicity of IDR-1 is unknown and will need further study to elucidate the exact mechanism involved. Therefore, a moderate dosage of LL-37 and IDR-1 should be used for treatment, and higher dosages should be used with acknowledgement of their toxic effects.

The majority of the literature proposes that LL-37 activates formyl peptide receptor 2 (FPR2) and reduces downstream pathways, resulting in chemotaxis [37], while IDR-1 stimulates neutrophil chemotaxis via a formyl peptide receptor [38]. In our present study, we investigate the function and signal transduction of JNK and Akt, both JNK and Akt play highly important roles in the process of inflammation. We found that the JNK and Akt pathways are activated in the MRSA-treated group compared with the control group, while in the LL-37

or IDR plus MRSA-treated groups, the phosphorylation of JNK and Akt is greatly inhibited. These findings indicate that LL-37 and IDR-1 may ameliorate MRSA-induced pneumonia via the inhibition of the JNK and Akt signal pathways *in vivo*. Therefore, the signal pathways of JNK and Akt could be important targets for inflammation treatment.

The mammalian immune system has evolved complex defense mechanisms to counteract myriad insults such as bacterial, viral, and parasitic infections and tissue injury. The inflammatory response, governed by both innate and adaptive immune systems, is the primary weapon in the host's arsenal to rapidly respond to such microbial and non-microbial events and restore tissue homeostasis [35]. In conclusion, our data proved that both LL-37 and IDR-1 are capable of exerting an anti-inflammatory effect and suppressing the release of pro-inflammatory cytokines, thus supporting the hypothesis that both the innate peptide LL-37 and innate defense regulator peptide IDR-1 could protect against MRSA-induced pneumonia *in vivo*. These results and recent findings have shown an association between cationic peptides and lung diseases, suggesting that peptides may serve as a target for novel antimicrobial and anti-infective treatment strategies for pulmonary diseases.

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