Experimental Cell Research xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Experimental Cell Research

journal homepage: www.elsevier.com/locate/yexcr

The antimicrobial cathelicidin peptide hlF(1-11) attenuates alveolar macrophage pyroptosis induced by *Acinetobacter baumannii* in vivo

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ARTICLE INFO

Keywords:
Acinetobacter baumannii
hLF(1-11)
Caspase-1
Pyroptosis

ABSTRACT

Acinetobacter baumannii is a Gram-negative coccobacillus found primarily in hospital settings that has recently emerged as a source of hospital-acquired infections, including bacterial pneumonia. The hLF(1-11) peptide comprising the first 11 N-terminal residues of human lactoferrin exerts antimicrobial activity in vivo and was highly effective against multidrug-resistant *A. baumannii* strains in vitro and in vivo. Pyroptosis is a caspase-1-dependent inflammatory cell death process and is induced by various microbial infections. In the present study, we investigated the molecular mechanisms that regulate pyroptosis induced by *A. baumannii* in macrophages. Our results revealed that *A. baumannii* induced pyroptosis through caspase-1 activation and IL-1β production. We also found that caspase-1 activation and IL-1β maturation in *A. baumannii*-triggered pyroptotic cell death were reduced by hLF(1-11) treatment. Moreover, hLF(1-11) inhibited the *A. baumannii*-induced caspase-1 activation and pyroptosis of pulmonary alveolar macrophages in vivo.

1. Introduction

Acinetobacter baumannii has emerged as an important nosocomial and opportunistic pathogen worldwide, especially in intensive care units. Infections caused by *A. baumannii* that lead to pneumonia, septicemia, urinary tract infections, and meningitis are very difficult to treat due to the emergence of multi-drug- or even pan-drug-resistant strains [1–3]. Recently, *A. baumannii* has become increasingly important because of its broad resistance to all clinical antibiotics and because of the mortality rates of *A. baumannii* infections, which can reach 35% [4,5]. Higher mortality rates were found in patients with drug-resistant strains compared to those with drug-susceptible strains due to the greater severity of illness and inappropriate empirical antibiotic treatment [6]. The continuously increasing mortality caused by multi- and pan-drug-resistant strains of *A. baumannii* indicates the importance of identification of new treatment strategies against this infectious threat. *A. baumannii* is responsible for numerous types of infections, among which ventilator-associated pneumonia is most common [3]. Bacterial pneumonia is a major nosocomial infection that is associated with rising morbidity and mortality [7,8]. The innate immune mechanisms that defend the organism against inhaled bacteria are incompletely identified. After precise regulation of signaling events, the optimal result for organisms is pathogen clearance without injury to the lung tissue. When the upper airway is exposed to ubiquitous microorganisms in the environment, both the innate and adaptive immune systems efficiently defend against this potential pathogen. However, when high levels of bacteria, such as those involved in hospital-associated infection, are inhaled through the airway, severe pneumonia associated with significant mortality often occurs [9]. Although the exact mechanisms of immune activation in *A. baumannii*-induced pneumonia are unclear, several recent studies have shown that *A. baumannii* could attach to and invade several mammalian cell lines and that it could also survive in infected host cells [10,11]. Pyroptosis in macrophages exposed to gram-negative bacteria is often induced by lipopolysaccharide via TLR4 receptor binding to the membrane [12]. Alveolar macrophages (AMs), one of the major phagocytes in the lung, express a large array of pathogen pattern recognition molecules that recognize and phagocytose invasive pathogens and activate a series of inflammatory responses [11,13,14]. Pyroptosis is an inflammatory form of programmed cell death that is observed in various types of microbial infections and is primarily associated with macrophages and dendritic cells; this process is characterized by rapid plasma membrane pore formation, chromatin condensation, and production of pro-inflammatory cytokines [15,16]. Recently, several cases have

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https://doi.org/10.1016/j.yexcr.2018.01.035
Received 13 October 2017; Received in revised form 22 January 2018; Accepted 24 January 2018

demonstrated that excessive activated inflammasomes due to cell pyroptosis are harmful to the host. Excessive caspase-1 activation and pyroptosis can damage innate immunity in response to infection by *Pseudomonas aeruginosa* [17,18] and inhibit the protection of CD8+ T cell-mediated immunity to *Listeria monocytogenes* [19]. Interestingly, caspase-1/11 knockout provided pulmonary protection in mice infected by *A. baumannii,* as shown by the decreased IL-1β and IL-6 levels in the BALF and the attenuated lung pathology. Thus, pyroptosis may play an important role in the pathogenesis of *A. baumannii*-induced pneumonia.

Antimicrobial peptides (AMPs) act on the defense systems and have relatively potent and short-lived effects against many microorganisms, including multidrug-resistant (MDR) pathogens [20,21]. To date, most of the studies related to AMPs have focused on the mechanisms of these peptides, including the capacity to kill pathogens directly and immunomodulatory effects [22–24]. For therapeutic potency of AMPs, it is important to not only assess the microbicidal effects of AMPs but also the interactions between these peptides and host immune cells. Several studies have suggested that the lactoferrin-derived peptide hLF(1-11) is a promising candidate for the treatment of MDR bacteria, including MDR *Staphylococcus aureus* [25] and MDR *A. baumannii* [26,27], in animal models. HLF(1-11) consists of 11 amino acids and is a synthetic N-terminal fragment of human lactoferrin. Given its origin from a natural human protein, hLF(1-11) has few side effects and was tolerable in autologous HSCT recipients [28]. Previous investigations reported that incubation of monocytes with hLF(1-11) modulated the GM-CSF-driven differentiation of these cells, resulting in a macrophage subset that showed enhanced recognition and clearance of pathogens [29].

HLF(1-11) is highly effective against experimental infections with MDR *A. baumannii*, which is associated with caspase-1/11 during infection in mice [26,27]. However, except for the direct bactericidal effect of this peptide, the protective host mechanisms of this antimicrobial molecule during *A. baumannii* infection are unclear. Therefore, we established an *A. baumannii*-infected pneumonia mouse model to confirm whether macrophage pyroptosis is involved in the process of *A. baumannii* infection and to explore whether hLF(1-11) could modulate macrophage pyroptosis during *A. baumannii* infection.

2. Materials and methods

2.1. HLF(1-11) synthesis and characterization

Antimicrobial cathelicidin peptide hLF(1-11) (GRRRRSVQWCA, C-terminal amide) and the control peptide (GAARRAVQWAA, N-terminal biotinylated hLF [1–11]) lacking in vitro and in vivo antimicrobial activity were produced by solid-phase peptide synthesis using Fmoc (9-fluorenyl-methoxycarbonyl) and analyzed for purity by Shanghai Sangon Biotech (Shanghai, China) [30,31]. The peptides exceeded 98% purity as determined by reverse-phase high-performance liquid chromatography and were free of endotoxin. The peptides were stored at −20 °C at a concentration of 10 mM in 0.01% acetic acid (pH 3.7) and diluted in phosphate buffered saline (PBS; pH 7.4; Department of Pharmacy, Xiangya Hospital Central South University) before use.

2.2. Mice

Specific pathogen-free male C57BL/6 at 6–8 weeks of age were purchased from SJA Lab Animal (China) and maintained under controlled conditions for 2 weeks before the experiment. The animals were acclimatized in cages with the desired temperature, a laminar-flow atmosphere, light and humidity under controlled conditions and received sterilized water and food ad libitum. Each experimental group consisted of ten mice, and the animal experiment was conducted once. All protocols for this study were approved by the local area animal Health Service and performed in compliance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

2.3. Bacterial strains

An *A. baumannii* clinical strain (ST191) susceptible to amikacin was used, and isolates of a major carbapenem-resistant *A. baumannii* strain that causes nosocomial pneumonia in Respiratory Intensive Care Units (RICU) in Xiangya Hospital were collected from the airways of RICU patients who were diagnosed with nosocomial pneumonia [32].

2.4. Model establishment and grouping

A well-characterized mouse model of pneumonia was used with some modification [33]. Briefly, the mice were anesthetized by an intraperitoneal (i.p.) injection of 1.2 ml/100 g 2% chloral hydrate (obtained from the pharmacy of the Outpatient Department). The trachea of each mouse was inoculated with a blunt-tipped metal needle of a 20 G syringe in a vertical position. Then, 0.05 ml (9 log CFU/ml) of bacterial suspension, which had been grown for 24 h in LB broth at 37 °C, washed twice, centrifuged at 4000 × *g* for 5 min, and resuspended in sterile 0.9% NaCl solution, was placed in the trachea by the microliter syringe (Hamilton, Reno, NV). After they were suspended vertically for 3 min, the mice were changed to a 30° position until they awakened.

Experimental animals were randomly divided into six groups: (1) the sham group (intratracheal injection with 0.05 ml 0.9% saline solution), (2) the pneumonia group (intratracheal injection with 0.05 ml [9 log CFU/ml] of bacterial suspension in the same volume as the saline, n = 20), (3) the caspase-1 inhibitor group (i.p. injection with Ac-YVAD-CMK [in the same volume of PBS containing 1% DMSO] at a dose of 6.5 mg/kg 1 h before administration of bacterial suspension, n = 20), which was treated with a caspase-1 inhibitor to explore the function of pyroptosis in severe pneumonia induced by *A. baumannii* compared with the pneumonia group, (4) the hLF(1-11) group (i.p. injection with hLF [1–11] diluted by PBS at a dose of 400 μg/kg 20 h after administration of bacteria, n = 20), (5) the control peptide group (the same operation as the hLF [1–11] group) and (6) the amikacin group (i.p. injection with amikacin diluted by PBS at 7.5 mg/kg 12 h after administration of the bacterial suspension, n = 20), which had no significant difference in the concentration of bacteria in the lung and BALF compared to the hLF(1-11) group and was used to eliminate the effect of reducing the number of bacteria on pyroptosis. The survival rates of the experimental groups were observed for 7 days. In other experiments, blood samples to detect cytokine levels were obtained by cardiac puncture, and the lungs were aseptically excised via thoracotomies following sacrifice of the mice 24 h post-intratracheal injection. The left lung was homogenized for quantitative culture and western blot analyses. The right one was removed and fixed in 4% paraformaldehyde for histological assessment and immunohistochemical (IHC) detection. Lungs from the sacrificed mice were immediately instilled with 0.5 ml PBS three times through an intratracheal catheter to harvest the BALF. Both BALF and serum were used for measuring the cytokine levels (IL-1β, TNF-α and IL-6) with appropriate ELISA kits.

2.5. Isolation of AMs from BALF

The grouping was performed as described above. AMs were harvested from the mouse lungs by lavage with 1 ml of EDTA-treated sterile saline through an intratracheal catheter, and a total of 10 ml of BALF was withdrawn from each mouse. Then, the BALF was centrifuged at 200 × g for 10 min at 4 °C to pellet the AMs. AM were separated by magnetic bead sorting as described previously [34]. Briefly, the immunomagnetic separation system (BD Biosciences Pharmingen, San Diego, CA, USA) was employed to isolate AMs from BALF. Magnetic nanoparticle-conjugated antibodies, including anti-mouse Gr-1, anti-CD4, anti-CD8, and anti-CD45R/B220 antibodies (BD Biosciences Pharmingen, San Diego, CA, USA), were used to label AMs. The purity