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Immunization with *Pseudomonas aeruginosa* outer membrane vesicles stimulates protective immunity in mice

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ABSTRACT

Pseudomonas aeruginosa is an opportunistic pathogen responsible for a wide range of severe nosocomial and community acquired infections, these infections are major health problems for cystic fibrosis patients and immune-compromised individuals. The emergence of multidrug-resistant isolates highlights the need to develop alternative strategies for treatment of *P. aeruginosa* infections. Outer membrane vesicles (OMVs) are spherical nanometer-sized proteolipids that are secreted from numerous of pathogenic Gram-negative bacteria, and a number of studies have confirmed the protective efficacy for use of OMVs as candidate vaccines. In this study, OMVs from *P. aeruginosa* (PA_OMVs) were isolated, formulated with aluminum phosphate adjuvant and used as a vaccine in a mouse model of acute lung infection. The results confirmed that active immunization with PA_OMVs was able to reduce bacterial colonization, cytokine secretion and tissue damage in the lung tissue, thus protecting mice from lethal challenge of *P. aeruginosa*. Cytokines assay validated that immunization with PA_OMVs was efficient to induce a mixed cellular immune response in mice. Further, high level of specific antibodies was detected in mice immunized with PA_OMVs, and results from opsonophagocytic killing assay and passive immunization suggested that humoral immune response may be critical for PA_OMVs mediated protection. These findings demonstrated that PA_OMVs may be served as a novel candidate vaccine for the prevention of *P. aeruginosa* infection.

1. Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is a Gram-negative opportunistic pathogen that responsible for severe nosocomial and community acquired infections at various body systems, such as the urinary tract, surgical or burn wounds, and lower respiratory tract [1]. Two populations of patients with a weakened immune system, including cystic fibrosis (CF) and hospitalized patients, are extremely sensitive to *P. aeruginosa* infection [2]. Due to the emergence of multiple-drug resistant isolates and even pan-drug resistant isolates, it is become increasingly difficult to cure *P. aeruginosa* related infections, imposing a high and increasing burden on health care resources [3]. Therefore, the development of novel alternative treatment strategies is urgently required, and vaccine is the most cost-effective choice and may indeed be useful.

Outer membrane vesicles (OMVs) are spherical portions of the outer membrane that released by numerous pathogenic Gram-negative bacteria. Typically, they are 20–200 nm in diameter, and vesicle membrane is usually composed by lipopolysaccharide (LPS), glycerophospholipids and outer membrane proteins (OMPs), whereas the vesicle lumen contains periplasmic components, such as RNA, DNA and cytoplasmic proteins [4,5]. Since there are so many outer membrane components identified in OMVs, studies have focused on investigating the possibility to use them as candidate antigens for vaccine development. The results showed that OMVs from a number of Gram-negative bacteria, such an *Neisseria meningitides* [6], *Acinetobacter baumannii* [5], *Helicobacter pylori* [7] and *Klebsiella pneumonia* [8], exhibited strong immunogenicity and was able to provide protective immunity in animal models of infection.

A number of studies have observed the secretion of OMVs by *P. aeruginosa in vitro* [9,10]. Some virulence factors, including proelastase, hemolysin, phospholipase C, and alkaline phosphatase, as well as the quorum-sensing signaling molecule PQS and

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antimicrobial quinolones were identified in PA_OMVs, indicating that the vesicles played an important role in the pathogenesis of the bacterial [11]. Further, it has been confirmed that purified PA_OMVs was able to activate alveolar epithelial cells and induce the release of IL-8 *in vitro* [12,13]. Moreover, PA_OMVs was also able to induce inflammation reaction in the lung *via* TLR2 and TLR4 pathway *in vivo* independent of live bacterial [14,15]. These data revealed that PA_OMVs was capable of initiating the immune response of host cells.

Despite extensive studies have focused on the development of vaccine against *P. aeruginosa* infection, clinical trials have so far not resulted in a marketable product. Since the protective efficacy of OMVs was reported in several Gram-negative bacteria, we speculated that PA_OMVs could also be used as candidate antigen for vaccine development. Thus, in the current study, PA_OMVs were isolated, formulated with aluminum phosphate adjuvant, and its immunogenicity and protective efficacy were determined in an experimental mouse pneumonia model of *P. aeruginosa* acute infection.

2. Materials and methods

2.1. Ethics statement

All animal care and use protocols were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of People's Republic of China. All animal experiments were approved by the Animal Ethical and Experimental Committee of the Third Military Medical University (Chongqing, Permit No. 2011-04) in accordance with their rules and regulations.

2.2. Purification and characterization of OMVs

P. aeruginosa strain PAO1 was purchased from ATCC (Manassas, VA, USA) and was cultured in 1 L of LB culture medium at 37 °C shaking at 200 rpm to an optical density of 1.2. Bacterial cells were discarded by centrifugation at 10,000*g* for 20 min, and the supernatant was filtered through a 0.22 μm membrane (Millipore Corporation, Bedford, MA) and subjected to ultracentrifugation at 200,000*g* for 2 h at 4 °C by using a type 45 Ti rotor (Beckman Instruments, CA, USA). The vesicle pellet was resuspended in PBS, pH 7.4 and analyzed by SDS-PAGE. The concentration of proteins in OMVs was determined by BCA method according to the manufacturer's instructions. The prepared OMVs were quick-freezed in liquid nitrogen and then stored at -80 °C for further use. OMVs from *Klebsiella pneumonia* strain MGH78578 (ATCC 700721) was isolated by the same protocol.

2.3. Transmission electron microscopy

For OMVs visualization by electron microscopy, OMVs diluted by PBS to a concentration of 1 mg/ml were negatively stained with 1% phosphotungstic acid (pH 7.0) and applied directly to 0.5% formvarcoated 300-mesh copper grids. Samples were observed by using a TECNAI 10 transmission electron microscope (Philips), and micrographs were taken at an accelerating voltage of 80 kV.

2.4. Immunization and infection

Six to eight-week old female BALB/c mice were purchased from Beijing HFK Bioscience Limited Company (Beijing, China). Mice were matched for age and sex, and kept under specific pathogen-free (SPF) conditions during the experiment. For immunization, OMVs were diluted by PBS and formulated with the $AlPO_4$ adjuvant (Pierce) at a ratio of 1:1 (v:v), to a final concentration of 150 μg/ml. Mice were immunized intramuscularly with 100 μl of the mixture into each quadriceps muscle (30 μg OMVs per mouse) on days 0, and then boosted twice at day 14 and 21. To ensure the specificity of PA_OMVs mediated protection, mice immunized with an equal volume of 30 μg of KP_OMVs and recombinant protein FliC (prepared by our Lab) plus adjuvant were served as controls, and negative control mice were immunized with an equal volume of PBS or PBS plus adjuvant. For *P. aeruginosa* infection, one week after the last boost immunization(day 28), mice in each group were anesthetized with pentobarbital sodium followed by intracheal injection of a lethal dose of the parent PAO1 strain or 3 clinical strains of *P. aeruginosa* (XN-1, BJ-15 and KM-9) that reported previously[16], the lethal dose of which were determined to be 1.0×10^7, 6.0×10^6, 2.0×10^7 and 4.0×10^6 CFUs, respectively. The number of deaths in each group was recorded every day during a 10-day observation period post-challenge to calculate the survival rates. For bacterial burdens, histopathology, inflammatory cells and cytokines analysis, mice were infected by intracheal injection with 5.0×10^6 CFUs of PAO1.

2.5. Detection of antigen specific antibodies

One day before each immunizations (day -1, day 13 and day 20), and one week after the last immunization (day 28), mice were exsanguinated, and serum samples were collected for the enzyme-linked immunesorbent assay (ELISA) of antigen specific antibody. Wells of microtiter plates (Thermo Labsystems) were coated with PA_OMVs (0.4 μg per well), FliC (0.2 μg per well) or *P. aeruginosa* strain PAO1 lysate (2 μg per well) in 50 mM carbonate buffer (pH 9.5) overnight at 4 °C. Serum samples were 1000-fold diluted by PBS and used as the primary antibodies. The secondary antibodies were HRP-conjugated goat anti-mouse IgG, anti-IgG1 or anti-IgG2a (Sigma). Absorbance was read at 450 nm (OD450). Serum form PBS immunized mice was used as negative control, all samples were tested in triplicate.

2.6. Bacterial burden and histological analysis

The lungs and spleens from immunized mice were harvested and assessed for bacterial colonization 8 and 24 h after infection. To calculate the bacterial number in each of the organs, organ homogenates were prepared in PBS and plated at 10-fold serial dilutions on LB agar. The number of colonies was quantified after 24 h of incubation at 37 °C. For histological analysis, lung tissues were collected 24 h post infection, fixed in neutral 10% formalin, embedded with paraffin, sectioned, and stained with hematoxylin and eosin (HE). The sections were then viewed at 200× magnifications. Each lung section was given a score of 0–4 (no abnormality to most severe) according to established criteria based on hyperemia, edema, hemorrhage, and neutrophil infiltration [16].

2.7. Cytokine assay

To quantify pro-inflammatory responses, BALF from immunized mice were collected 24 h after the last immunization or 24 h after infection with PAO1 as described previously [17]. The concentrations of pro-inflammatory cytokines including TNF-α, IL-1β and IL-6 in BALF were determined using Mouse Quantikine ELISA kit for TNF-α, IL-1β and IL-6 (R&D Systems, USA), respectively, according to the manufacturer's instructions. The levels of chemokines, including CXCL1 and CCL2, from BALF collected after infection were also determined using Mouse Quantikine ELISA kit (R&D Systems, USA). Cytokine assays were performed as described previously [18]. In brief, mice were immunized with PBS, FliC and PA_OMVs, respectively. Two weeks after the final immunization,