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Outer membrane vesicles harboring modified lipid A moiety augment the efficacy of an influenza vaccine exhibiting reduced endotoxicity in a mouse model

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ABSTRACT

Influenza is an acute respiratory disease and a major health problem worldwide. Since mucosal immunity plays a critical role in protection against influenza virus infection, mucosal immunization is considered a promising vaccination route. However, except for live-attenuated vaccines, there are no effective killed or recombinant mucosal influenza vaccines to date. Outer membrane vesicles (OMVs) are nano-sized vesicles produced by gram-negative bacteria, and contain various bacterial components capable of stimulating the immune system of the host. We generated an OMV with low endotoxicity (fmOMV) by modifying the structure of the lipid A moiety of lipopolysaccharide and investigated its effect as an intranasal vaccine adjuvant in an influenza vaccine model. In this model, fmOMV exhibited reduced toll-like receptor 4-stimulating activity and attenuated endotoxicity compared to that of native OMV. Intranasal injection of the vaccine antigen with fmOMV significantly increased systemic antibody and T cell responses, mucosal IgA levels, and the frequency of lung-resident influenza-specific T cells. In addition, the number of antigen-bearing CD103$^+$ dendritic cells in the mediastinal lymph nodes was significantly increased after fmOMV co-administration. Notably, the mice co-immunized with fmOMV showed a significantly higher protection rate against challenge with a lethal dose of homologous or heterologous influenza viruses without adverse effects. These results show the potential of fmOMV as an effective mucosal adjuvant for intranasal vaccines.

1. Introduction

Influenza is an acute respiratory disease caused mainly by influenza A and B viruses and has been a major health problem, worldwide. Due to the segmented RNA genome structure, the viruses frequently and constantly alter their antigenic characteristics, and consequently change their infectivity and pathogenicity. To cope with these diverse strains or subtypes, influenza vaccines need to induce cross-reactive immune responses capable of covering a wide range of subtypes. The use of adjuvants, such as alum and MF59, improves the potency of the vaccine in terms of breadth and the magnitude of immune responses to the vaccine antigens [1,2]. Therefore, efficacious adjuvants could be a breakthrough for the development of a 'universal' influenza vaccine.

Mucosal immunization has been considered a promising route of the vaccine delivery because it efficiently induces strong mucosal immunity, resulting in a more efficient defense against mucosal infections compared to a systemic immune response [3,4]. Among diverse mucosal routes, intranasal delivery is particularly advantageous in eliciting the strongest respiratory immune response, which plays a critical role in the protection against respiratory infections such as influenza [5]. Two intranasal influenza vaccines, FluMist and NASOVAC, are currently available and both consist of attenuated-live viruses. In addition to these licensed vaccines, many studies have revealed the possibility of protein-based intranasal vaccination against respiratory pathogens such as *Streptococcus pneumoniae* and respiratory syncytial virus [6,7]. However, no

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approved intranasal adjuvant, capable of enhancing the immunogenicity of protein-based or killed-virus vaccine antigens, has been developed to date.

Outer membrane vesicles (OMVs), which are naturally produced nano-sized vesicles from Gram-negative bacteria, contain various bacterial components such as lipopolysaccharide (LPS), lipoproteins, flagellin monomers, and bacterial DNA fragments [8]. Due to the nature of these components, OMVs can stimulate the host immune system through innate immune receptors, including toll-like receptors (TLRs) and NOD-like receptors (NLRs) [9]. In recent studies, intramuscular injection of OMV with irrelevant antigens enhanced antigen-specific humoral and cellular immune responses, and increased the protection rate against tumor and virus challenges [10,11]. However, in order to use OMVs as vaccine adjuvants or delivery vehicles, the safety of this system must be addressed because LPS in OMVs may excessively provoke innate immune responses and lead to endotoxicity.

In this study, we generated a novel OMV with attenuated endotoxicity (fmOMV) by modifying the structure of the lipid A moiety of LPS and investigated the safety and efficacy of fmOMV as a mucosal vaccine adjuvant using an influenza vaccine model. fmOMV exhibited attenuated endotoxicity compared with native OMV (nOMV), and intranasal injection of vaccine antigens with fmOMV significantly enhanced both systemic and mucosal immune responses. Furthermore, co-administration of fmOMV provided protective immunity against homologous and heterologous virus challenge, suggesting the potential of fmOMV as an effective mucosal adjuvant for intranasal vaccines.

2. Methods

2.1. Modification and purification of OMVs

fmOMV was purified as described previously with slight modifications [12]. Briefly, the *Escherichia coli* W3110 Δ*msbB*/Δ*pagP* strain [13] was transformed with pWSK29-LpxF plasmid, which encodes lipid A 4′-phosphatase, and cultured in LB broth at 37 °C. The culture broth was filtered using a 0.22-μm pore-sized filter (Merck, NJ) and precipitated in a 390 g/l ammonium sulfate solution. After resuspending the pellets, the suspension was centrifuged again at 16,000*g*. The crude fraction was further purified by performing sucrose-gradient ultracentrifugation. nOMV was similarly prepared except the transformation procedure.

2.2. Analysis of lipid A

The composition of lipid A on fmOMV was analyzed as described previously [14]. Briefly, cultured *E. coli* cells were incubated in the presence of 5 μCi/ml of ^{32}Pi at 37 °C for 3 h. After collecting and washing the cells by centrifugation, the pellet was dissolved in a chloroform/methanol/water (1:2:0.8, v/v) solution. The insoluble fraction was collected and hydrolyzed in 12.5 mM sodium acetate (pH 4.5) containing 1% SDS at 100 °C for 30 min. A mixture of methanol and chloroform was added to make the ratio of chloroform/methanol/water 2:2:1.8 (v/v). The lower phase was dried and then 1000 cpm of the sample was run on a Silica Gel 60 TLC plate. The plate was visualized using an FLA-7000 image analyzer (Fujifilm, Tokyo, Japan).

2.3. TLR signaling assay

HEK-Blue™ cell lines expressing mouse TLR2, TLR4, or TLR5 (InvivoGen, San Diego, CA, USA) were cultured in RPMI1640 media (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; GE Healthcare, Little Chalfont, UK) and 1X antibiotics (Life Technologies). After resuspending 5×10^4 cells in HEK-BlueTM Detection media (Life Technologies), each cell line was treated with nOMV, fmOMV, or control reagents; Pam3Cys-Ser-(Lys)4 (Pam3; Merck Millipore, Billerica, MA, USA), LPS (InvivoGen), or flagellin (InvivoGen). After 24-h incubation, the activity of secreted alkaline phosphatase was determined.

2.4. Mice

Six- to eight-week-old C57BL/6 female mice were purchased from KOATECH (Korea) and kept in a specific pathogen-free, biosafety level-2 facility at Korea Research Institute of Bioscience and Biotechnology (KRIBB). All animals were treated in accordance with the guidelines established by the Institutional Animal Use and Care Committee of KRIBB.

2.5. Viruses

Influenza A/California/04/2009 (pandemic H1N1, pH1N1), influenza A/Puerto Rico/8/1934 (H1N1, PR8) and influenza A/aquatic bird/Korea/CN2-MA/2009 (H5N2) viruses were cultivated in the allantoic cavities of embryonated chicken eggs. Viruses were titrated by calculating the 50% egg infectious dose (EID_{50}) and stored at −80 °C until use.

2.6. Immunization and challenge

Mice were immunized intranasally with the trivalent split influenza vaccine antigen containing A/California/7/2009 (H1N1), A/Victoria/361/2011 (H3N2), and B/Massachusetts/2/2012 (0.8 μg of each subtype HA/mouse, Green Cross, Korea) twice at a two-week interval. Purified fmOMV (1 to 10 μg/head) or cholera toxin (CT; List Biological Laboratories, CA) was mixed with the vaccine antigen immediately before injection. The total injection volume was adjusted to 30 μl/mouse by using PBS. Two weeks after the second injection, the mice were challenged with a 10 LD_{50} of pH1N1, PR8, or H5N2 influenza virus. The body weight and mortality rates were monitored for two weeks. A humane endpoint of 20% weight loss was used for this challenge study.

2.7. Antigen uptake and flow cytometry

DQ™ Ovalbumin (DQ-OVA) (40 μg/head; ThermoFisher Scientific, MA) was delivered intranasally into the lungs in the presence or absence of fmOMV (3 μg/head). After 24 h, mediastinal lymph node (mLN) cells were resuspended in FACS buffer (PBS containing 0.1% bovine serum albumin and 0.01% sodium azide) and incubated with Fc-block (anti-CD16/CD32; eBioscience, CA). After washing, the cells were stained with fluorescence dye-conjugated anti-CD11b, CD11c, Gr-1, CD80, and CD103 antibodies (eBioscience). Samples were acquired on Gallios™ (Beckman Coulter, CA) and data were analyzed using FlowJo software (Tree Star, OH).

2.8. Enzyme linked-immunosorbent assay (ELISA)

Two and four weeks after the first immunization, serum and bronchoalveolar lavage fluid (BALF) samples were analyzed for antigen (Ag)-specific IgG and IgA by enzyme linked-immunosorbent assay. ELISA plates (ThermoFisher Scientific) were coated with vaccine antigen (200 ng/well) and then incubated with the samples. After sequential incubation with peroxidase goat anti-mouse total IgG and IgA (Cell Signaling Technology, MA), 3,3′,5,5′-tetramethylbenzidine substrate (BD Bioscience, CA) was added to each well. The optical density was measured at a 450 nm wavelength by using VICTOR3™ (PerkinElmer, MA). To determine the HA stalk-specific Abs, $HA_{419-473}$ from PR8 and $HA_{379-473}$ from

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