Bacterial Outer Membrane Vesicles Provide Broad-Spectrum Protection against Influenza Virus Infection via Recruitment and Activation of Macrophages

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Keywords

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Abstract

Influenza A virus (IAV) poses a constant worldwide threat to human health. Although conventional vaccines are available, their protective efficacy is type or strain specific, and their production is time-consuming. For the control of an influenza pandemic in particular, agents that are immediately effective against a wide range of virus variants should be developed. Although pretreatment of various Toll-like receptor (TLR) ligands have already been reported to be effective in the defense against subsequent IAV infection, the efficacy was limited to specific subtypes, and safety concerns were

also raised. In this study, we investigated the protective effect of an attenuated bacterial outer membrane vesicle harboring modified lipid A moiety of lipopolysaccharide (fmOMV) against IAV infection and the underlying mechanisms. Administration of fmOMV conferred significant protection against a lethal dose of pandemic H1N1, PR8, H5N2, and highly pathogenic H5N1 viruses; this broad antiviral activity was dependent on macrophages but independent of neutrophils. fmOMV induced recruitment and activation of macrophages and elicited type I IFNs. Intriguingly, fmOMV showed a more significant protective effect than other TLR ligands tested in previous reports, without exhibiting any adverse effect. These results show the potential of fmOMV as a prophylactic agent for the defense against influenza virus infection.

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Introduction

Currently available influenza vaccines have effectively reduced disease incidence; however, 3–5 million cases of severe illness, with 250,000–500,000 deaths, are reported annually, worldwide [1]. One of the reasons for high incidence is constant genomic changes in the virus, termed as antigenic drift or shift, which allows the virus to evade the antibody response generated by vaccination [2]. These genetic changes elicit new, highly pathogenic strains, which have the potential to cause a pandemic [3]. For these, new seasonal vaccines need to be formulated annually, which is a time-consuming process. Therefore, there is a need for the development of a broad-spectrum antiviral agent that is effective against a large variety of viral strains and elicits antiviral effect immediately.

Innate immunity can confer broad-spectrum and immediate defense against various pathogens including influenza virus. Innate immune responses are initiated mainly by recognition of pathogen-associated molecular patterns (PAMPs) in the pathogen via pattern recognition receptors such as Toll-like receptors (TLRs) [4–6], and TLR-stimulated innate immune cells protect the host against the pathogen's first attack as well as subsequent reinfections, which is termed "trained immunity" [7, 8]. Based on these findings, various PAMPs such as lipopolysaccharide (LPS), palmitoylated peptides, and the unmethylated CpG oligodeoxynucleotide have been utilized to induce anti-influenza innate immunity [9–14]. However, the effects achieved so far are insufficient to protect against infection by a wide spectrum of viral strains. For example, LPS protects the host against a lethal dose of PR8 virus, but the effect was significantly reduced in the SpHA/WSN virus infection model [11, 15], and the stimulation of TLR2 also required supplement of a TLR9 ligand for the significant protection [12]. Another study reported that stimulation of TLR3 by synthetic liposomal polyriboinosinic-polyribocytidylic acid resulted in moderate survival rates (70 and 63% after infection by PR8 and H5N1, respectively) [13]. These previous studies indicate the requirement for a sophisticated strategy or novel agent to achieve broad-spectrum anti-influenza efficacy involving activation of innate immunity.

Outer membrane vesicles (OMVs) are phospholipid bilayer vesicles produced naturally by Gram-negative bacteria. Since OMVs contain various bacterial antigens [16], they have been studied as a vaccine candidate against pathogenic bacteria such as *Vibrio cholerae* and *Borrelia burgdorferi* [17, 18]. Notably, the OMV from *Neisseria meningitidis* was the first to be licensed for humans as a

vaccine component [19]. In addition to bacterial proteins, these vesicles also contain TLR ligands such as LPS, lipoproteins, and flagellin [16, 20], and in this regard, OMVs augmented adaptive immune response to the coadministered antigens via activation of innate immunity [5, 6]. Previously, we generated fmOMV, which stands for further-modified OMV, of which lipid A is a dephosphorylated and underacylated species that resulted from both expression of the LpxF 4'-phosphatase and deletion of the *msbB/pagP* lipid A myristoyl/palmitoyltransferase genes. This OMV showed a significant adjuvant effect on a seasonal influenza vaccine exhibiting much attenuated TLR4-stimulating activity in vitro and endotoxicity in vivo [21, 22]. Although the adjuvant effect of OMVs has been reported in various antigen models, the antiviral effects via activation of innate immunity have not yet been addressed.

In this study, we showed that fmOMV elicits host innate immune response against influenza virus infection. Intranasal administration of fmOMV resulted in the recruitment and activation of macrophages in the lung tissue, and these macrophages sufficiently protect infection from different influenza viruses; pH1N1, PR8, H5N2, and highly pathogenic H5N1. These findings suggest the potential of fmOMV as a novel antiviral agent against infection from broad-spectrum influenza viruses.

Materials and Methods

Purification of fmOMV

fmOMV was produced by transforming *E. coli* W3110 $\Delta msbB/\Delta pagP$ mutant strain with pWSK29-LpxF plasmid as previously reported [23]. After removing the bacteria by centrifugation (11,000 g), the supernatant was filtered using a 0.22- μ m pore filter (Merck, NJ, USA). fmOMV in the filtrate were precipitated in 390 g/L ammonium sulphate solution, and the collected pellets were centrifuged again at 16,000 g for 15 min. The pellets were ultracentrifuged in a sucrose gradient solution for further purification.

Fig. 1. Modified outer membrane vesicle (fmOMV) stimulates diverse Toll-like receptors (TLRs). **a** Structure of lipid A moiety on fmOMV. **b** HEK293 cells expressing mouse TLR2, TLR4, TLR5, or TLR9 were cultured with indicated amounts of fmOMV or corresponding ligand (PAM3 100 ng/mL; LPS 100 ng/mL; flagellin 100 ng/mL; or CPG 100 ng/mL) for 24 h. The extent of fmOMV- or TLR ligand-induced TLR stimulation was determined by measuring secreted embryonic alkaline phosphatase activity using QUANTI-Blue colorimetric enzyme assay. Data are presented as mean $\pm$ standard deviation from triplicate culture wells, at optical density (OD) of 570 nm. *** $p < 0.001$, ** $p < 0.01$.

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a Lipid A moiety on native OMV Lipid A moiety on fmOMV

Fig. 2. Antiviral activity of fmOMV is independent of the adaptive immune response. Mice ($n = 10$) were intranasally immunized with the trivalent split influenza vaccine mixed with fmOMV or cholera toxin, and then challenged with pH1N1 virus at 5 and 14 days after the injection. **a** After challenge, the mice were monitored for changes in body weight and survival rate for 14 days. **b** Sera were collected before virus challenge, and the virus-specific antibody response was determined by ELISA. **c–f** Mice ($n = 8$) were injected with fmOMV alone. At 3, 7, or 14 days after the injection, the mice were infected with pH1N1 virus. The vaccinated group was immunized intramuscularly with pH1N1 split vaccine fol-

lowed by a booster injection after 2 weeks ($n = 8$). Two weeks after the second injection, the mice were challenged with the pH1N1 virus. **c** The survival rates were monitored for 2 weeks after the pH1N1 viral challenge. **d** The lung tissues were collected at 7 days after the viral challenge and virus titers were determined. **e** Influenza-specific IgG antibodies were measured by ELISA using sera collected on the day of virus challenge. **f** IFN- γ -secreting T cells were estimated by enzyme-linked immunospot assay, using splenocytes harvested on the day of virus challenge. Data are presented as mean $\pm$ standard error of mean. *** $p < 0.001$, * $p < 0.05$.

(Figure continued on next page.)

TLR Signaling Assay

HEK-Blue™ mTLR2, mTLR3, mTLR4, mTLR5, or mTLR9 (Invivogen, CA, USA) cell lines were maintained in RPMI1640 media (Life technologies, MA) supplemented with 10% fetal bovine serum (FBS; GE Healthcare, UK) and 1 $\times$ antibiotics (Life Technologies). During activation, each cell line was resuspended in HEK-Blue™ Detection media (Life Technologies), seeded at 5×10^4 cells/well in 96-well plates, and treated with fmOMV or control reagents; Pam₃Cys-Ser-(Lys)₄ (Pam3, 1.0 μ g/mL; Merck Millipore, Germany), poly I:C (10 μ g/mL), LPS (100 ng/mL), flagellin (1.0 μ g/mL), or CpG ODN 1826 (CpG, 1.0 μ g/mL) (Invivogen). After 24-h incubation, the secreted alkaline phosphatase activity was measured at 630 nm.

Viruses

Influenza A/California/04/2009 (pandemic H1N1, pH1N1), influenza A/Puerto Rico/8/1934 (PR8), influenza A/aquatic bird/Korea/CN2-MA/2009 (H5N2), and influenza A/Environment/Korea/W149/2006 (H5N1) viruses were cultivated in the allantoic cavities of embryonated chicken eggs. Viruses were titrated by calculating the 50% egg infectious dose (EID₅₀) and stored at -80°C until use.

Animals and Experimental Schedule

Seven-week-old, female C57BL/6 mice were kept in pathogen-free, biosafety level-2 or -3 facilities at Korea Research Institute of Bioscience and Biotechnology (KRIBB) or Chungbuk National

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University, respectively. Mice were injected intranasally with fmOMV (10 µg/mouse) once. Three, 7, or 14 days after fmOMV injection, mice were challenged with a 10 lethal dose 50 (LD₅₀) of PR8, pH1N1, H5N2, or highly pathogenic H5N1 viruses and their mortality rates were monitored for 2 weeks. The trivalent split influenza vaccine containing A/California/7/2009 (H1N1), A/Victoria/361/2011 (H3N2), and B/Massachusetts/2/2012 (1.0 µg of HA/mouse, Green Cross, Korea) was immunized either intranasally (Fig. 2) or intramuscularly (Fig. 5) twice at a 2-week interval. The vaccine-immunized groups were challenged 2 weeks after the booster injection. For intranasal injection, the total volume was adjusted to 30 µL/mouse by using phosphate-buffered saline (PBS). A humane endpoint of 25% weight loss was used for this challenge study.

Enzyme-Linked Immunosorbent Assay

ELISA plates (ThermoFisher Scientific, MA, USA) were coated with vaccine antigen (200 ng/well), and then incubated with the serum samples. Bound antibodies were detected by sequential incubation with peroxidase goat anti-mouse total IgG (Cell Signaling

Technology, MA, USA) and 3,3',5,5'-tetramethylbenzidine substrate (BD Bioscience, CA, USA). Optical density was measured at 450 nm wavelength using VICTOR3™ (PerkinElmer, MA, USA).

Enzyme-Linked Immunospot (ELISPOT) Assay

Influenza-specific IFN-γ-producing cells were quantified using a mouse IFN-γ enzyme-linked immunospot set (BD Biosciences) on the day of virus challenge. Briefly, splenocytes (5 × 10⁵ cells/well) were incubated with inactivated pH1N1 virus on ELISPOT plates coated with IFN-γ capture antibody. After 40-h incubation, the plates were further incubated with biotinylated IFN-γ detection antibody and then horseradish peroxidase-conjugated streptavidin. Spots were visualized by adding 3-amino-9-ethyl-carbazole substrate solution and counted using the BioSpot analyzer (Cellular Technology, OH, USA).

Virus Titration

Total lung homogenate samples were obtained at indicated time points, and then added to Madin-Darby canine kidney cells

with 10-fold serial dilution. Three days after infection, virus titer was determined with a hemagglutinin test and calculated by the method of Reed and Muench, as previously described [24]. Virus titer was expressed as log₁₀ of the 50% tissue culture-infective dose (TCID₅₀) per milliliter.

Fluorescent Labeling of fmOMV

fmOMV was labeled with Alexa Fluor 488 fluorescent dye (Alexa488) according to the manufacturer's instructions (Thermo-Fisher Scientific). In brief, 1 M sodium bicarbonate buffer was added to fmOMV diluted in PBS, and then Alexa488 tetrafluorophenyl ester was added to the mixture. After incubation for 15 min at room temperature, unreacted dye was removed using a spin filter containing Bio-Gel P-6 fine resin.

Flow Cytometry

Samples were resuspended in fluorescence-activated cell sorting buffer (PBS containing 2.5% FBS and 0.1% sodium azide) and incubated with Fc-block (anti-CD16/CD32; eBioscience, CA, USA). After washing, the cells were stained for CD11b, CD11c, SiglectF, CD40, CD45, CD80, CD86, MHC class II, F4/80, and Ly6G (eBioscience). Samples were acquired on Gallios™ (Beckman Coulter, CA, USA) and analyzed using FlowJo software (Tree Star, OH, USA).

Depletion of Neutrophils and Macrophages

Neutrophils were depleted by intraperitoneal injection of anti-mouse Ly6G antibody (500 µg/mouse, 1A8 clone; Biolegend, CA, USA) 24 h before fmOMV treatment. For macrophage depletion, empty or clodronate-encapsulating liposomes (FormuMax, CA, USA) were administered via intravenous routes (1.0 mg/mouse) at 2 days before fmOMV administration.

Quantitative RT-PCR

RNA was isolated from the lung tissue using TRIzol (Invitrogen) and was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase and oligo-d(T) according to the manufacturer's instructions. Quantitative RT-PCR was performed using specific primers (IFN-α: sense, 5'-ATGGCTAGGCTCTGTGCTTTCCT-3', antisense, 5'-AGGGCTCTC CAGACTTCTGCTCTG-3'; IFN-β: sense, 5'-CCCTATGGAGATGACGGAGA-3', antisense, 5'-TCCCACGTCAATCTT TCCTC-3'; HPRT: sense, 5'-CAGACTGAAGAG CTACTGTAATGATCA-3', antisense, 5'-TCA ACA ATCAAGACATTCTTTCCA-3') and SYBR Premix Ex Taq (Takara Bio) on a Dice TP800 Thermal Cycler (Takara Bio). The mRNA levels were normalized relative to the expression of Hprt mRNA.

Multiplex Cytokine Immunoassay

Bronchoalveolar lavage fluid (BALF) samples were harvested 3 days after fmOMV treatment. The cytokine levels were measured using a multiplex cytokine immunoassay system (Bio-Rad Laboratories, CA, USA) according to the manufacturer's instructions.

Statistical Analysis

Statistical differences among groups were assessed using a 2-tailed Student's *t* test or a log-rank test with GraphPad Prism software. *p* values of <0.05 were considered statistically significant.

Results

fmOMV Stimulates Diverse TLRs

Activation of various TLR signaling has been reported to initiate antiviral innate immunity [4]. Based on the fact that OMVs contain diverse bacterial components which can stimulate TLRs [23, 25, 26], we previously generated a modified OMV harboring less endotoxic LPS (fmOMV, Fig. 1a) and investigated its safety and adjuvant effect using an influenza vaccine model [21]. In this study, we tested whether fmOMV initiates TLR 2, 3, 4, 5, and 9 signaling simultaneously, using HEK-Blue cells expressing individual TLRs. Treatment with fmOMV induced TLR2, TLR3, TLR4, TLR5, and TLR9 signaling in a dose-dependent manner (Fig. 1b). These data suggest that fmOMV treatment elicits innate immunity required to generate antiviral host immunity via simultaneous triggering of various TLR signaling.

Antiviral Activity of fmOMV Is Independent of the Adaptive Immune Response

Previously, we reported that OMV injection with a vaccine antigen increases vaccine-induced antibody responses using ovalbumin and influenza vaccine antigens [21, 23]. Since rapid induction of protective immunity in particular is required for vaccines against highly transmissible infectious diseases such as influenza, we first tested whether fmOMV could induce protective efficacy of an influenza vaccine at early time points after the injection. When mice were challenged both 5 and 14 days after immunization, the mice coimmunized with fmOMV exhibited reduced body weight loss and an increased survival rate, which were significantly higher than those achieved by vaccination without fmOMV (Fig. 2a). Intriguingly, the antigen-specific antibody was not detected in sera 5 days after immunization (Fig. 2b). This discrepancy between the protection rate and antibody response at day 5 after vaccination led to the possibility that fmOMV induces a protective response before the vaccine-induced antibody response is generated. To verify this hypothesis, mice were inoculated intranasally with fmOMV alone and then challenged with pH1N1 influenza virus at 3, 7, or 14 days after fmOMV administration. All fmOMV-injected mice survived at 3 days after fmOMV injection (Fig. 2c); however, survival rates decreased with an increase in the interval between fmOMV injection and pH1N1 virus challenge (day 7: 80%, day 14: 20%). Additionally, the viral titer in the lungs was the lowest at 3 days after fmOMV injection (Fig. 2d). To investigate whether the protective effect of fmOMV was associated with adaptive immunity,

Gated on CD45⁺SiglecF^{hi}CD11b^{lo}CD11c^{hi}

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we measured antigen-specific antibody and T cell responses on the day of virus challenge. As expected, neither the antibody response nor the interferon (IFN)- γ -secreting T cell response specific for the vaccine were detected at 3, 7, or 14 days after fmOMV injection (Fig. 2e, f), indicating that the fmOMV-induced antiviral effect is not associated with adaptive immunity.

Macrophages but Not Neutrophils Are Associated with Anti-Influenza Effect by fmOMV

Given that the fmOMV-induced antiviral effect was independent of adaptive immunity, we hypothesized that the antiviral activity is associated with innate immunity. Since previous reports showed that macrophages and neutrophils comprise the majority of innate immune cells responsible for the antiviral response in an influenza infection model [27], we measured the change in counts of macrophages and neutrophils in the lungs after fmOMV injection. Both cell counts significantly increased after fmOMV injection (Fig. 3a). Additional analysis of the change in macrophage phenotypes showed that major population of F4/80⁺ cells in the lungs was CD11b^{hi}CD11c^{lo-int} (Fig. 3b) [28].

To identify which innate immune cells mainly contributed to the antiviral activity by fmOMV, we monitored the survival rate of fmOMV-injected mice under neutrophil- or macrophage-depleted conditions. Depletion of macrophages by clodronate-liposome injection completely abrogated the antiviral effect by fmOMV administration (Fig. 3c), indicating that macrophages play an important role in the antiviral effect by fmOMV against influenza virus infection (Fig. 3c, left). After neutrophil depletion by anti-Ly6G antibody injection, the antiviral

effect by fmOMV was not affected, showing that neutrophils are not associated with antiviral effect by fmOMV (Fig. 3c, right). These data suggest that the antiviral effect by fmOMV injection is associated with an increase in macrophages in the lungs.

fmOMV Activates Macrophages and Induces Antiviral Cytokines in the Lung Tissue

It has been known that alveolar macrophages (AMs) are the major population that firstly recognizes foreign materials and induces subsequent immune activation in the lungs. To investigate whether fmOMV-induced antiviral effect was mediated by direct interaction of fmOMV with AMs, we injected Alexa488-labelled fmOMV (fmOMV-Alexa488) and quantified the fluorescence signal in AMs. At 3 h after fmOMV-Alexa488 injection, 94.2% of CD45⁺SiglecF^{hi}CD11b^{lo}CD11c^{hi}F4/80⁺ cells were Alexa488-positive (Fig. 4a), showing direct interaction of fmOMV with AMs.

Upon activation, AMs secrete proinflammatory chemokines which in turn lead to recruitment of diverse immune cells ([29]), as shown in Figure 3a, b. Given that macrophages are indispensable for the fmOMV-mediated antiviral effect (Fig. 3c, left), we next investigated the characteristics of infiltrated macrophages in lung tissue 3 days after fmOMV injection. The activation markers, such as CD40, CD80, CD86, and MHC class II on macrophages, were upregulated after fmOMV injection (Fig. 4b). We also analyzed the levels of antiviral cytokines and chemokines after fmOMV injection. The level of IFN- α and β , IL-1 β , and C-C motif chemokine ligand 2 (CCL2, MCP-1) in the lung tissue was increased by fmOMV injection (Fig. 4c, d), indicating that fmOMV activated the innate immune system leading to antiviral soluble factors. Given that fmOMV induces significant production of type I IFNs, we further investigated whether type I IFNs contributed to fmOMV-mediated protection using mice lacking a type I IFN receptor (IFNAR1KO). When IFNAR1KO mice were treated with fmOMV and then challenged with a lethal dose of PR8 virus, all mice lost weight and eventually died (Fig. 4e), indicating that type I IFNs play an important role in the fmOMV-mediated antiviral effect.

fmOMV Provide Broad and Potent Protection against Diverse Influenza A Viruses

To further examine whether fmOMV protects against a broad spectrum of influenza virus subtypes, we additionally challenged fmOMV-injected mice with PR8, H5N2, and highly pathogenic H5N1 viruses. Eighty to

Fig. 4. fmOMV activates macrophages and induces antiviral cytokines in the lung tissue. **a** Mice were intranasally administered with 10 μ g of Alexa Fluor 488 (Alexa488)-labeled fmOMV. After 3 h, the population of Alexa488 and F4/80-positive cells were determined by flow cytometry, gated on CD45⁺SiglecF^{hi}CD11b^{lo}CD11c^{hi} bronchoalveolar lavage cells. **b** The expression of activation markers on macrophages (upper panels) and neutrophils (lower panels) in the lungs were analyzed at 3 days after fmOMV injection. **c, d** The levels of type I interferons, IL-1 β , and C-C motif chemokine ligand 2 (CCL2) in the lungs were determined by RT-PCR and multiplex cytokine immunoassay, respectively. **e** Type I IFN receptor 1 knockout (IFNAR1KO) mice ($n = 4$ or 6) were injected with fmOMV. Three days after the injection, the mice were infected with PR8 virus, and the survival rates were monitored for 2 weeks after the viral challenge. Data are presented as mean $\pm$ SEM and representative of at least three independent experiments. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

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one hundred percent of the fmOMV-injected mice survived the PR8, H5N2, and H5N1 viral challenge at 3 days after fmOMV injection (Fig. 5a). The protective effect of fmOMV was compared with other TLR ligands: Pam3, LPS, flagellin, and CpG, which have been reported to protect against influenza viruses [11, 12, 30]. Interestingly, while the TLR ligands except CpG were ineffective, fmOMV (3 and 10 μ g) completely protected against lethal influenza virus challenge (Fig. 5b). Although CpG completely protected the viral challenge in terms of the survival rate (Fig. 5b, right), the body weight of mice administered CpG significantly declined upon viral challenge compared to that of mice administered fmOMV (Fig. 5b, left). Consistent with a previous report [21], the mobility, food intake, and body weight of the mice were normal, and inflammation in the lung tissue was not observed when fmOMV was intranasally injected (data not shown). These data indicate that fmOMV provide broad and potent protection against diverse influenza A viruses (IAVs) without endotoxic inflammation.

Discussion

Emerging infectious diseases caused by yet unidentified viruses or new variants of known viruses are a constant threat to human health, as exemplified by Middle East respiratory syndrome coronavirus and influenza viruses. These pandemic or epidemic outbreaks necessitate the availability of therapeutics and vaccines. However, factors such as lack of information regarding the virus and the time-consuming developmental process have resulted in an urgent need for an effective antiviral agent that protects against a broad spectrum of viruses immediately. In this study, we demonstrated that intranasal injection of fmOMV not only protects against infection by a broad spectrum of influenza viral strains, but also elicits

this antiviral effect as early as 3 days after administration. Cell depletion and subtype analysis studies revealed that an increase and activation of macrophages by fmOMV injection provided broad-spectrum antiviral effects.

Previous reports showed that AMs play an indispensable role in the protection against airway infection with influenza viruses [31–34]. Upon infection with influenza viruses, AMs primarily produce type I IFNs that play an important role in the protection against influenza viruses by both inhibiting viral replication and suppressing excessive tissue inflammation [35, 36]. In this study, we observed activation of TLR3 and 4, which are known to induce type I IFNs via interferon regulatory factor 3, *in vitro* (Fig. 1b), and significant increase in type I IFNs *in vivo* after fmOMV treatment (Fig. 4c). In addition to LPS, OMVs contain bacteria-derived RNA molecules and activate TLR3, which coincides well with our observation [25, 37]. It is possible that type I IFNs induced by fmOMV promote antiviral molecular milieu in lung epithelial cells, and these “pre-armed” epithelial cells effectively suppressed influenza virus replication regardless of the evading mechanism of influenza virus in this study.

We observed that F4/80⁺CD11b^{hi}CD11c^{lo-int} cells massively infiltrated into the lungs 3 days after fmOMV injection (Fig. 3b) and that these macrophages are indispensable for the antiviral effect of fmOMV (Fig. 3c, left). When AMs are activated, they produce CCL2, resulting in the recruitment of bone marrow-derived macrophages to the lungs [29]. In this study, we showed that fmOMV increased the level of CCL2 in BALF (Fig. 4d, right), suggesting that massive infiltration of macrophages was induced by AM-derived chemokines. Inflammatory macrophages suppress viral replication after influenza virus infection [38]. In particular, stimulation of various TLRs using LPS, imiquimod, peptidoglycan, or β -glucan is known to transform macrophages to a state called “trained immunity” that is hyperresponsive to pathogenic re-infection [8, 39]. Specifically, TLR stimulation induces a decrease in repressive histone H3K9me2 marks via phosphorylation of ATF7. This epigenetic change causes an increase in basal expression of genes required for protection against pathogens by macrophages. Considering that fmOMV can stimulate various TLRs (Fig. 1b), fmOMV injection may transform recruited macrophages to the hyperresponsive state presumably via epigenetic changes.

Injection of fmOMV induced IL-1 β secretion in the lung tissue (Fig. 4d, left). In contrast to the case that LPS or flagellin located outside of the cells can only activate corresponding TLRs, the ligands introduced into the intracellular compartment activate NLRP3 and the NL-

Fig. 5. fmOMV provide broad and potent protection against diverse influenza A viruses. **a** Mice ($n = 8$ –9) were injected with fmOMV and challenged with 10 LD₅₀ of PR8, H5N2, and highly pathogenic H5N1 influenza viruses at the indicated time points. The vaccinated group was immunized intramuscularly with the trivalent split influenza vaccine containing pH1N1 antigen twice at a 2-week interval ($n = 8$). Two weeks after the second injection, the mice were challenged with each virus. **b** Mice ($n = 8$) were intranasally injected with each indicated TLR ligand (3 μ g) and challenged with 10 LD₅₀ of the pH1N1 virus. **a, b** The survival rates were monitored for 2 weeks after the viral challenge. These data are representative of two or three independent experiments. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

RC4-dependent inflammasome, respectively [40, 41]. Detection of IL-1 β in BALF indicates that fmOMV enters the target cells, leading to a sequential activation of the inflammasome and proteolytic cleavage of IL-1 β precursor [42]. Consistent with our interpretation, it was recently reported that OMVs mediate cytosolic localization of LPS and activate caspase-11 [43].

Neutrophils have a protective role in influenza virus infection [44, 45]. In this study, however, depletion of neutrophils did not decrease survival rate in fmOMV-injected and influenza-challenged mice, suggesting that neutrophils did not participate in antiviral activities provided by fmOMV in our experimental model (Fig. 3c, right). Antiviral activities of neutrophils are mainly associated with secretion of antiviral molecules such as long-chain pentraxin (PTX3), defensin, and formation of neutrophil extracellular traps after influenza virus infection [46–48]. These molecules are secreted from neutrophils after activation via interaction directly with influenza virus or virus-induced molecular inflammatory milieu. It is speculated that in fmOMV-injected mice, infiltrated macrophages have sufficiently suppressed disease severity after influenza virus infection. Therefore, viral load and inflammatory cytokines may have been insufficient to activate neutrophils to produce antiviral molecules after influenza virus infection in fmOMV-injected mice. Meanwhile, neutrophil activation is known to intensify disease severity during influenza infection [49]; however, this was not observed in the present study, in terms of change in body weight and survival rates of fmOMV-injected mice (Fig. 2c, 3c, and 5a). This suggests that there was nil or relatively low activation of neutrophils upon influenza virus infection in fmOMV-injected mice, coinciding well with our observation (Fig. 3c, right).

In our study, fmOMV pretreatment successfully protected the mice against diverse IAVs; PR8, pH1N1, H5N2, and highly pathogenic H5N1 (Fig. 2a and 5a). It has been reported that pretreatment with a single TLR ligand provided differential protective efficacy depending on the challenging influenza subtypes or strains [9, 11, 14, 30]. TLR4 activation alone was sufficient to protect H5 subtype, whereas simultaneous activation of TLR2 and 4 was required for the protection against PR8 (H1) subtype [11]. Pam2Cys, a TLR2 ligand, has been reported to elicit 80% survival rate after PR8 virus infection [9, 30]. In addition, costimulation of TLR2 and TLR9 protects the host against H3N2 virus infection completely, but the effect was reduced up to 50% in an H1N1 virus infection model [12]. Since fmOMV used in this study is a complex of various TLR ligands and presumably capable of simulta-

neously activating TLRs and cytosolic pattern recognition receptors, it is probable that fmOMV leads to the higher protective efficacy against diverse IAVs. Further investigation is necessary to determine the underlying mechanisms for differential protective efficacy of each TLR signal against different virus strains.

In conclusion, we showed that fmOMV injection protected against infection by a wide spectrum of IAV strains and this antiviral effect was mediated by infiltrated macrophages. Our findings show potential for development of an efficient and broad-spectrum antiviral agent against influenza as well as emerging infectious diseases.

Statement of Ethics

All animal experiments were approved by the Institutional Animal Use and Care Committee of KRIBB and performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Disclosure Statement

The authors declare that they have no competing interests.

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