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Adjuvant Efficacy of mOMV against Avian Influenza Virus Infection in Mice

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Highly pathogenic avian influenza H5N1 viruses are found chiefly in birds and have caused severe disease and death in infected humans. Development of influenza vaccines capable of inducing heterosubtypic immunity against a broad range of influenza viruses is the best option for the preparedness, since vaccination remains the principal method in controlling influenza viral infections. Here, a mOMV-adjuvanted recombinant H5N2 (rH5N2) whole virus antigen vaccine with A/Environment/Korea/W149/06(H5N1)-derived H5 HA and A/Chicken/Korea/ma116/04(H9N2)-derived N2 NA in the backbone of A/Puerto Rico/8/34(H1N1) was prepared and generated by reverse genetics. Groups of mice were vaccinated by a prime-boost regime with the rH5N2 vaccine (1.75 μg of HA with/without 10 μg mOMV or aluminum hydroxide adjuvant for comparison). At two weeks post-immunizations, vaccinated mice were challenged with lethal doses of $10^{3.5}$ EID_{50}/ml of H5N1 or H9N2 avian influenza viruses, and were monitored for 15 days. Both mOMV- and alum-adjuvant vaccine groups had high survival rates after H5N1 infection and low levels of body weight changes compared to control groups. Interestingly, the mOMV-adjuvanted group induced better cross-reactive antibody responses serologically and promoted cross-protectivity against H5N1 and H9N2 virus challenges. Our results suggest that mOMV could be used as a vaccine adjuvant in the development of effective vaccines used to control influenza A virus transmission.

***Keywords*:** avian influenza A virus, H5N2 vaccine, mOMV adjuvant

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Introduction

Avian influenza A virus infections can cause a wide range of respiratory illnesses in animals and humans, which create concerns in both the veterinary and human medical fields (Atmar and Keitel, 2009). Highly pathogenic avian influenza (HPAI) H5N1 viruses have been constantly found in many countries including Vietnam, Indonesia, Egypt, Thailand, China, Bangladesh, Turkey, and South Korea (Yuen *et al.*, 1998; Chokephaibulkit *et al.*, 2005; Chotpitayasunondh *et al.*, 2005; de Jong *et al.*, 2005; Buchy *et al.*, 2007; Kandun *et al.*, 2008; Taylor *et al.*, 2008; Wang *et al.*, 2008; Brooks *et al.*, 2009; Earhart *et al.*, 2009; Hien *et al.*, 2009; Adisasmito *et al.*, 2010; Kim *et al.*, 2012; Kwon *et al.*, 2012; Choi *et al.*, 2013). Outbreaks of HPAI H5N1viruses in birds have been increasing in Europe and Africa, elevating concerns that the virus might eventually spread to human populations (Sellwood *et al.*, 2007). Preparing for potential pandemics caused by the HPAI H5N1 virus has remained a top priority worldwide due to its associated high mortality rates (Guan and Smith, 2013; Perovic *et al.*, 2013).

To induce broadly cross-reactive antibody and T cell immune responses upon influenza vaccination, the AI vaccine should include an immune modulation component referred to as the adjuvant. An optimal adjuvant, which would increase vaccine efficacy leading to heterosubtypic immunity, should have the ability to control antigen release and simultaneously generate sufficient immune stimulatory signals via Toll-like receptor (TLR) activation (Atmar *et al.*, 2009; Schwarz *et al.*, 2009). Currently available influenza vaccines for humans contain either alum (aluminum hydroxide; approved for use in human vaccine in the United State) or MF59 adjuvant (Trilla *et al.*, 2008; Baz *et al.*, 2013). Results with MF59 (Novartis Vaccines and Diagnostics) during the 2009 pandemic H1N1 vaccine campaign indicate that it is relatively safe, exhibits good antigen-sparing effects, and the capability, to some extent, to generate cross-reactive antibodies against heterologous influenza viruses (O'Hagan *et al.*, 2011). Thus, MF59 has been a stand-alone influenza vaccine adjuvant thus far; however, it is quite expensive for general applications with veterinary vaccine products including influenza vaccines for farm and companion animals. In this respect, there is an elevated need for new adjuvants to combine with animal AI vaccines that are cheaper and more stable outside the cold-chain supply.

Modified outer membrane vesicles (mOMV) produced from the *E. coli* W3110 Δ*msbB*/*pagP* mutant are characterized as bacterially-derived, non-replicating nanoparticles possessing adjuvant effects (Lee *et al.*, 2011). The mOMV is composed of a penta-acylated lipid A moiety of lipopoly-

saccharide (LPS), which is much less-endotoxic than the normal hexa-acylated moiety (Kim *et al*., 2009). A recent study indicated that mOMV is a safe vaccine delivery vehicle that includes adjuvant properties that stimulate T cell immunity in a TLR4-dependent manner (Lee *et al*., 2011). Therefore, in this study, mOMV was used to evaluate its adjuvant efficacy with an experimental AI vaccine containing recombinant H5N2 virion composed of the HA of HPAI H5N1 (A/Environment/Korea/W149/06) and the NA of H9N2 (A/Chicken/Korea/ma116/04) in the internal backbone of A/Puerto Rico/8/34(H1N1) virus. In the present paper, efficacy of the mOMV-adjuvanted vaccine was evaluated by challenge experiments with HPAI H5N1, and cross-protection against heterosubtypic virus challenge was assessed following challenge with H9N2 virus. Results indicate that antigen containing the mOMV adjuvant improved the survival rates and achieved cross-protection against the HPAI H5N1 and H9N2 AI virus challenges.

Materials and Methods

Vaccine generation

Reverse genetics was performed to generate the recombinant reassortant H5N2 (rH5N2) virus carrying the HA gene of the HPAI A/Environment/Korea/W149/06 (H5N1) virus and the NA gene of the low pathogenic A/chicken/Korea/116/04 (H9N2) virus in the backbone of the laboratory strain A/Puerto Rico/8/34 (H1N1) as previously described. The vaccine virus (rH5N2) was propagated in 10 day-old embryonic eggs at 37°C for 48 h. The allantoic fluid containing the viruses was harvested and purified by sucrose density gradient centrifugation. Purified vaccine viruses were inactivated with 0.05% formalin for 3 days. The inactivated viruses were then inoculated into the embryonic eggs to confirm the absence of viral growth.

Vaccination and virus challenge

Four-week-old female BALB/c mice (*Mus musculus*) were purchased from Samtako (S. Korea). Twelve groups of mice (n=18) were assigned to the vaccination study including the antigen (1.75 μg HA of rH5N2) only group (no adjuvant). mOMVs were prepared as described previously (Kim *et al*., 2009). Two adjuvant-vaccine groups, rH5N2+mOMV or rH5N2+alum were vaccinated intramuscularly (i.m.) with 1.75 μg HA of rH5N2 virus plus 10 μg of mOMV adjuvant or the same HA dose mixed with 40 μl of alum (2% aluminum hydroxide gel) in 200 μl total volume via a two-dose (prime-boost) vaccination schedule with two-week intervals. The adjuvant control groups received only 10 μg of mOMV or 40 μl of alum, and non-vaccinated control group received PBS only. For survival rate, body weight loss and virus titration, mice were challenged intranasally (i.n.) with 10 times the 50% mouse lethal dose (10X MLD_{50}) of the HPAI H5N1 (A/Environment/Korea/W149/06) and mouse-adapted (ma) H9N2 (A/Chicken/Korea/ma116/04) viruses two weeks after the last immunization. The body weight changes and survival of the three vaccine groups were monitored for 15 days post-infection (n=12) and lung samples were harvested at 3, 5, and 7 dpi (2 heads /day). The research protocol for the use of mice in this study were conducted in strict accordance and adherence to policies regarding animal handling as mandated under the Guidelines for Animal Use and Care and was approved by the Laboratory Animal Research Center (LARC) (approval number CBNUA-567-13-01). All procedures for handling vaccine, viruses, and animals were conducted in a enhanced biosafety level 3 facility (BSL-3+) in accordance with relevant regulatory policies regarding animal handling mandated by the Guidelines for Animal Use and Care of the Korea Center for Disease Control.

Virus titration

Lungs were aseptically extracted and homogenized in minimal essential medium (MEM) with antibiotics. Ten-fold serial dilutions of samples were added in quadruplicate to a monolayer of MDCK cells seeded in 96-well cell culture plates 18 h before infection, and allowed to absorb virus for 1 h at 37°C. Virus inoculum was replenished with fresh medium containing 1 μg of TPCK-treated trypsin after removal of unattached viruses by PBS washing, and incubated for 48 h. Viral cytopathic effect (CPE) was observed daily and the viral titer was determined by the hemagglutination (HA) test. The virus titer was calculated by the Reed and Muench method and expressed as $\log_{10}$ $TCID_{50}$/g of lung tissue (Reed and Muench, 1938).

Serological assays

Hemagglutination inhibition (HI) assays were performed as described elsewhere (Lu *et al*., 2003). Briefly, receptor destroying enzyme (RDE, Denka Seiken, Japan) was added to mouse serum samples to inactivate non-specific HA inhibitors at a final serum dilution of 1:10. RDE-treated sera were serially diluted (2-fold) in 96-well plates and equal volumes of each virus (8 HA units/50 μl) were added to each well. The microplates were incubated at room temperature for 30 min, followed by the addition of 0.5% (v/v) chicken red blood cells (cRBCs). The plates were gently mixed and incubated at 37°C for 30 min. The HI titer was determined by the reciprocal of the last serum dilution that did not agglutinate cRBCs. The detection limit for the HI assay was set at <20 HI units.

Statistical analysis

The student's *t*-test was used to determine the significance of differences between two sets of values and the log rank test by Prism 5.0 program (GraphPad) was used to determine the *P*-value of survival tests.

Results

Serologic titers and mOMV-adjuvant vaccine efficacy

To investigate the adjuvant efficacy of mOMV in mice, sera (n=10) from all six groups of mice were analyzed by HI assays. The first immunizations with the rH5N2+mOMV and rH5N2+alum vaccine formulas elicited basally detectable mean HI titers (~20 HI unit) against H5N1 virus but

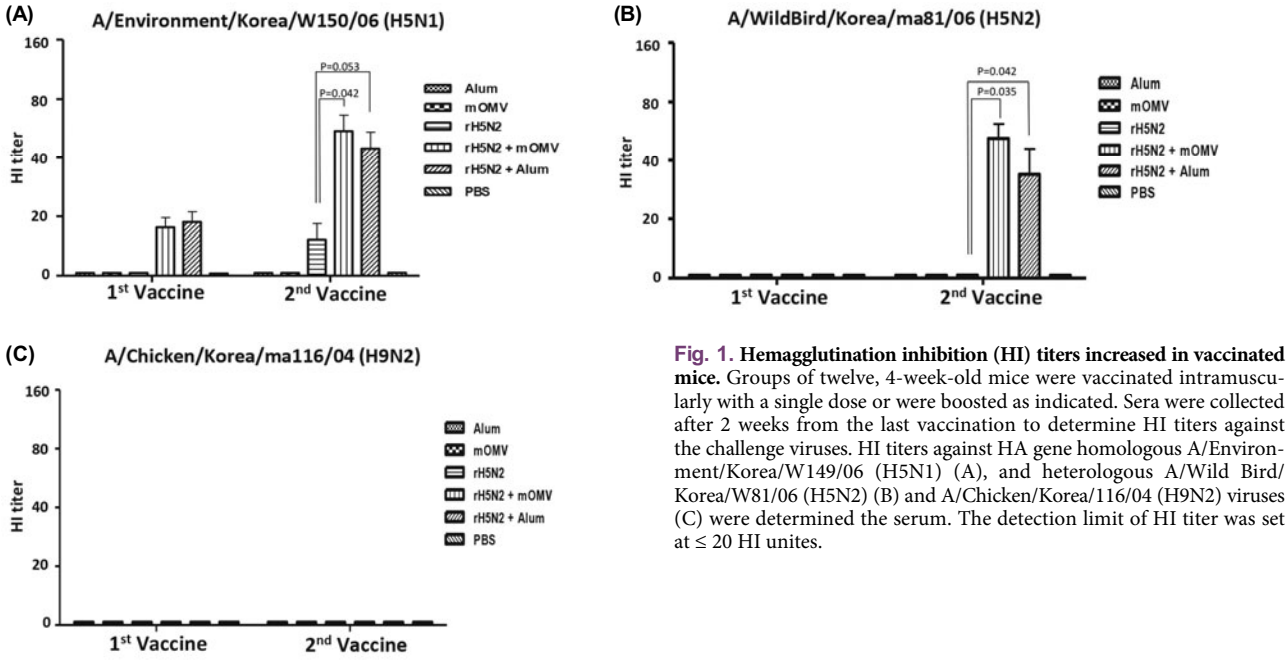

Fig. 1. Hemagglutination inhibition (HI) titers increased in vaccinated mice. Groups of twelve, 4-week-old mice were vaccinated intramuscularly with a single dose or were boosted as indicated. Sera were collected after 2 weeks from the last vaccination to determine HI titers against the challenge viruses. HI titers against HA gene homologous A/Environment/Korea/W149/06 (H5N1) (A), and heterologous A/Wild Bird/Korea/W81/06 (H5N2) (B) and A/Chicken/Korea/116/04 (H9N2) viruses (C) were determined the serum. The detection limit of HI titer was set at ≤ 20 HI unites.

no detectable HI titer was observed in the other groups (Alum only, mOMV only, and PBS control groups). Two weeks after the last (2nd vaccination) vaccination, the highest HI titer (60 HI, P=0.042) was detected in the rH5N2+mOMV group, while rH5N2 antigen-only group showed the lowest HI titers (~20 HI) against H5N1 virus (Fig. 1A). In addition, rH5N2+alum induced 40 HI titers (*P*=0.053) against the H5N1 virus. However, the boosted vaccine groups of rH5N2+mOMV and rH5N2+alum induced 53 (*P*=0.035) and 38 (*P*=0.042) HI titers against a heterologous low pathogenicity A/Ab/Korea/W81/06 (H5N2) strain, respectively, although single immunization could not induce any detect-

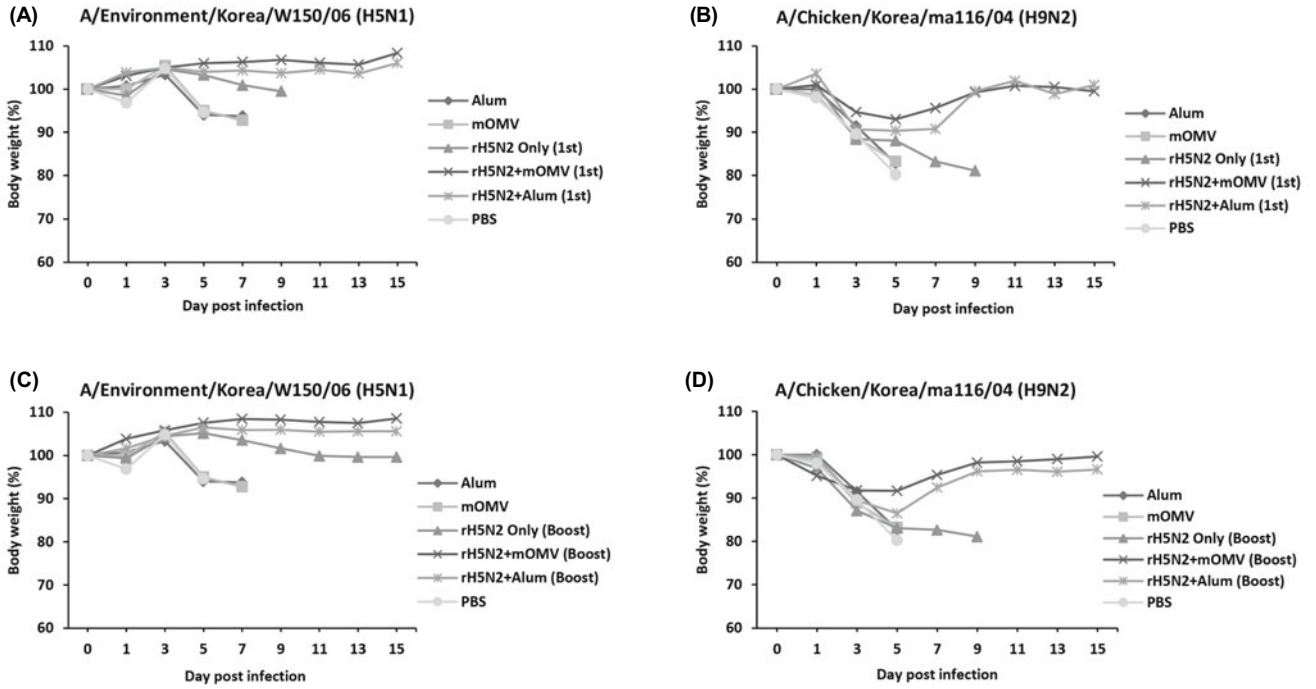

Fig. 2. Body weight change of vaccinated mice after challenge. The single-dose and boosted mice were challenged with two virulent viruses (H5N1 (A and C) and maH9N2 (B and D)) and body weight changes were recorded during experimental days at two day intervals; mice from the single dose group (A) and boosted group (C) were challenged with A/Environment/Korea/W149/06 (H5N1) virus. Mice from the single dose group (B), boosted group (D), and rH5N2+mOMV vaccinated mice resisted body weight loss following challenge with mouse-adapted A/Chicken/Korea/116/04 (maH9N2).

able HI titer (Fig. 1B). Mouse sera from all vaccine groups induced titers that remained below the detectable mean level (≤20 HI titer) against H9N2 virus even after the booster vaccination (Fig. 1C). These results demonstrate that the mOMV can induce strong immunogenicity (comparable to that of alum) with the rH5N2 vaccine, but might be a better adjuvant than alum for inducing immunogenic responses that extended to against a heterologous H5N2 avian virus strain.

Assessment of protection in vaccinated mice upon lethal virus challenge

Two weeks after the last vaccination, all mice were challenged i.n. with 10 MLD_{50} of a wild-type HPAI H5N1, or mouse-adapted variant of the A/chicken/Korea/116/04(H9N2) (maH9N2) virus to evaluate the protective efficacy of the rH5N2+mOMV vaccine in mice. All mock- or adjuvant-only vaccinated mice showed gradual body weight loss and exhibited severe clinical signs of infection including hunched back, ruffled fur, lethargy, and anorexia before succumbing to death within 7 dpi due to infection with HPAI H5N1 virus (Fig. 2A). The group given one dose of rH5N2 antigen-only also showed gradual body weight loss and 84% of mice succumbed at 11 dpi. However, the rH5N2+mOMV vaccine group showed the highest survival rate (83%) while the rH5N2+alum vaccine group showed only 50% survival rate (Figs. 2A and 3A). All mice from the boosted groups, except the mock- or adjuvant-only vaccine groups, survived the HPAI H5N1 virus challenge (100% survival rates) until the end of experiment with mild clinical signs (Figs. 2C and 3C).

Since the neuraminidase gene of rH5N2 virus was derived from an H9N2 virus, we attempted to test whether the rH5N2 vaccine could induce cross-protection from challenge with the maH9N2 virus. All mock- or adjuvant-only mouse groups showed rapid body weight loss and succumbed within 5 dpi of maH9N2 infection. In addition, the mice in the rH5N2 antigen-only group, regardless of the dosage received, exhibited a gradual decrease of body weight and died within 9 dpi (Figs. 2B and 2D). Interestingly, mice that received the rH5N2+mOMV vaccine showed the highest attenuation of body weight change with survival rates of 83% at a single dose and 100% for two doses (Figs. 3B and 3D). In contrast, rH5N2+alum groups had delayed recovery from the loss of body weight and showed only partial protection against H9N2 challenge, with survival rates of 67% and 83% for mice receiving a single dose and two doses, respectively (Figs. 3B and 3D).

mOMV promotes inhibition of viral growth in rH5N2-immunized mice

Since the mOMV-adjuvanted vaccines provided protection

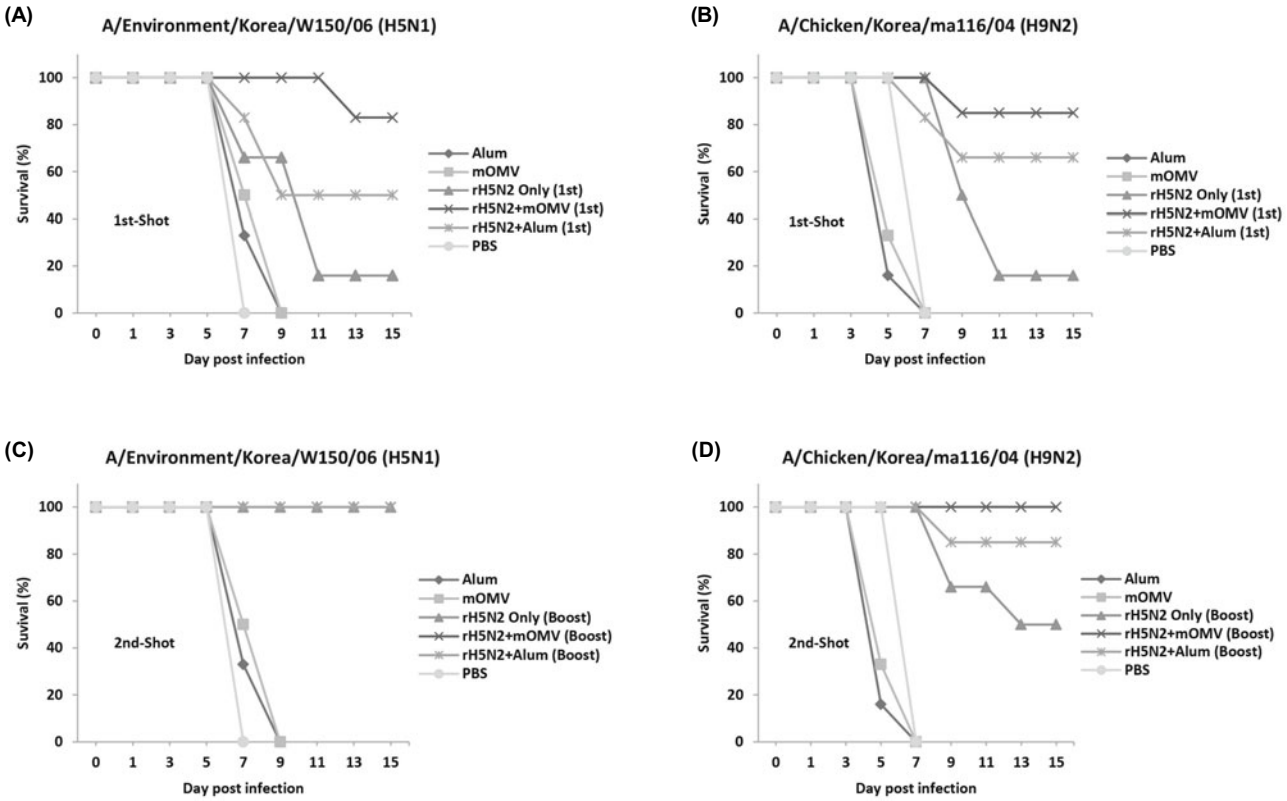

Fig. 3. Survival rate of the vaccinated mice after challenge. The survival rate for the vaccinated mice was monitored at two-day intervals up to 14 dpi following challenge with H5N1 or H9N2 viruses. Results from mouse groups are shown. (A) Survival of the single vaccinated mouse group challenged with A/Environment/Korea/W149/06 (H5N1) or (B) with A/Chicken/Korea/ma116/04 (H9N2). Survival of mice given two doses of vaccine (boosted) challenged with A/Environment/Korea/W149/06 (H5N1) (C) or A/Chicken/Korea/ma116/04 (H9N2) (D).

Table 1. Vaccine-induced $TCID_{50}$ titers and inhibition of virus replication

	H5N1			H9N2		
	3	5	7 (dpi)	3	5	7 (dpi)
Alum	3	4	4.5	4.5	4.5	3
mOMV	2.5	4	4	3.5	4.5	2.5
rH5N2 (1st)	2	2	3.5	4	4.5	2
rH5N2 (Boost)	ND	ND	ND	3.5	4.5	ND
rH5N2+mOMV(1st)	1.5	2	2.5	2.5	3.5	ND
rH5N2+mOMV(Boost)	ND	ND	ND	2.5	2.5	ND
rH5N2+Alum (1st)	1.5	2	2.5	3.5	3.5	1.5
rH5N2+Alum(Boost)	ND	ND	ND	3.5	3.5	ND
PBS	3	4.5	4.5	4.5	4.5	3

Dpi, Day of post infection
ND, Non Detection
H5N1, A/Environment/Korea/W150/06
H9N2, A/Chicken/Korea/ma116/04

from virus-induced mortality following lethal challenge with H5N1 and maH9N2 viruses, we further investigated viral titers in the lungs of each group of vaccinated mice at day 3, 5, and 7 dpi (Table 1). Consistently, the lungs of one-dose vaccinated mice receiving rH5N2+mOMV or rH5N2+alum showed comparatively high virus titers ranging from 1.5 to 2.0 $\log_{10}$ $TCID_{50}$/g after challenge with HPAI H5N1 virus. In addition, those of rH5N2-only group showed 2.0 to 3.5 $\log_{10}$ $TCID_{50}$/g lung viral titers. However, the virus was not detected in the lungs of the two-dose vaccination groups (rH5N2, rH5N2+mOMV, and rH5N2+alum). In comparison, the HPAI H5N1 virus replicated efficiently well in the lungs of the mock (PBS, 4.5 $\log_{10}$ $TCID_{50}$/g)-vaccinated groups, as well as both adjuvant-only groups.

Similar results were observed in lethal challenges with the maH9N2 virus. Lungs collected from mock-, mOMV-, and alum-only groups showed relatively high mean viral titers (more than 3.5 $TCID_{50}$/ml) at designated time points (Table 1). In contrast, receipt of the rH5N2+mOMV boosted (two-dose) group demonstrated low mean lung viral titer (2.5 $\log_{10}$ $TCID_{50}$/g) which was 10 times lower than those of the rH5N2+alum group (2.5 vs. 3.5 $\log_{10}$ $TCID_{50}$/g, P=0.0769) at both 3 and 5 dpi, respectively. These results showed that the rH5N2+mOMV vaccine could induce a stronger cross-protective immunity against lethal challenge with H5N1 and maH9N2 viruses than the rH5N2+alum vaccine.

Discussion

Development of an effective and affordable influenza vaccine for animals is an attractive approach to prevent or limit the spread of zoonotic influenza viruses to humans (Bernstein *et al*., 2008). However, current influenza vaccine approaches used in the human population would be prohibitively expensive for veterinary purposes. As a potentially new adjuvant applicable to animal AI vaccines, mOMV would be valued as cheaper and more stable (outside cold-chain supply) alternative, especially since mOMVs are natural, nano-sized particles possessing TLR ligands capable of immune-stimulating activity (Lee *et al*., 2011). Previously, use of mOMV was proven to be safe and effective in studies in Cuba, Norway, Brazil, and New Zealand against clonal serogroup B meningococcal outbreaks (Collins, 2011; Norheim *et al*., 2012). Furthermore, mOMVs are thought to be taken up easily by antigen presenting cells, and to exert self-adjuvanticity in particulate form (Collins, 2011; Lee *et al*., 2011; Roier *et al*., 2012). Therefore, we investigated the adjuvant efficacy of the bacteria-derived mOMV (Lee *et al*., 2011) as an influenza vaccine adjuvant.

To evaluate the adjuvant efficacy, we compared the immune response and cross protection rates of mOMV with a commercial alum adjuvant, widely used for human and animal vaccines, including a non-adjuvanted AI vaccine in mice. The rH5N2+mOMV vaccine induced high antibody titers, comparable to the rH5N2+alum vaccine, while inducing relatively higher HI titers against a heterologous H5N2 avian virus than the rH5N2+alum vaccine (Fig. 1). Apparently, boosted rH5N2+mOMV adjuvant vaccine group provided complete protection with inhibition of virus replication against the HPAI H5N1 virus challenge (Table 1 and Fig. 3). In another promising manner, mOMV-adjuvanted vaccine tested in this study further demonstrated that it produced better results in the cross-reactive antibody response and cross-protection than those of alum adjuvant vaccine against heterosubtypic H9N2 virus. Anti-NA antibodies could also play a significant role in neutralizing influenza viruses by steric inhibition of virus adsorption and by interfering with viral release (Webster and Laver, 1967; Webster *et al*., 1968). Moreover, it has been shown that NA-derived immunogenicity correlated to cross-protection against lethal H5N1 influenza in ferrets that were immunized with a human seasonal influenza H1N1 vaccine (Rockman *et al*., 2013). Therefore, it is likely that the H9N2-derived NA gene component of our rH5N2 vaccine strain likely promoted heterosubtypic H9N2 virus immunogenicity and cross-protectivity.

Given the unpredictable outbreaks of AI subtypes in humans (such as those with the H5N1 and recent H7N9 virus infections) especially in Asia, development of vaccines capable of inducing heterosubtypic immunity against a broad range of AI viruses is the best option for preparedness, since vaccination is considered to be the principal measure in controlling influenza viral infections (Girard *et al*., 2010a, 2010b; Rivers *et al*., 2013). Previous cross-reactivity studies suggested that mOMV contains a considerable number of other proteins, such as factor H binding protein (fHbp) or

iron-regulated membrane proteins, that may be relevant for improving cross-protection (Urwin *et al*., 2004; Wheeler *et al*., 2007; Zollinger *et al*., 2010, 2011). In addition, Lee *et al.* recently showed that mOMV could stimulate mouse bone marrow-derived dendritic cells to upregulate expression of costimulatory and antigen-presenting molecules, and Monophosphoryl lipid A (MPL®)-like Th1-mediated T cell immunity in a TLR4-dependent manner (Lee *et al*., 2011).

Overall, we have demonstrated that the mOMV-adjuvanted rH5N2 vaccine induced high HI titers and protected mice from mortality after challenge with HPAI H5N1 and maH9N2 viruses. Results were comparable or considerably better than the immunogenicity and cross-protectivity afforded by an alum-adjuvanted vaccine after challenge in a similar manner. Therefore, we hypothesize that mOMV has great potential for use as an effective adjuvant in the development of animal vaccines, especially in regards to control of influenza A viral spread from animals to humans. Furthermore, additional studies of mOMV-adjuvanted influenza vaccines in larger animal models (e.g., pigs and ferrets) are warranted for potentially more broad applications, due to its ability to induce strong heterosubtypic immunity.

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