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Enhanced Influenza VLP vaccines comprising matrix-2 ectodomain and nucleoprotein epitopes protects mice from lethal challenge

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ABSTRACT

The matrix protein 2ectodomain (M2e) of the influenza A virus is a rational target antigen candidate for the development of a universal influenza virus-like particle (VLP) vaccine. In this study, a recombinant M2 protein with three tandem copies of M2e (3M2e), nucleoprotein (NP) epitopes and hepatitis B virus core (HBc), were expressed in *Escherichia coli* and purified by column chromatography. Mice immunized with 3M2e-NP-HBc in combination with an oil-in-water SP01 adjuvant produced robust M2e specific antibodies and cellular immune responses. Most importantly, the 3M2e-NP-HBc VLP vaccine provided enhanced protection against a lethal challenge with pandemic 2009 H1N1 and HPAI H5N1 virus through increased survival rates, a significant decrease in viral replication, and obvious alleviation of histopathological lung changes in challenged mice. Our results imply that a cellular immune response to NP is a plausible mechanism mediating this enhanced protection. These findings suggest that 3M2e-NP-HBc VLP has great potential as the basis development of a broadly protective influenza vaccine.

1. Introduction

Influenza virus typically infects 10–20% of the global population during seasonal epidemics, resulting in 3–5 million cases of severe illness and 250,000–500,000 deaths per year (Palese, 2004; Tumpey et al., 2004). An error-prone RNA-dependent RNA polymerase and the presence of a segmented genome permit influenza viruses to frequently mutate and recombine, causing antigenic drift and antigenic shift, respectively, which enable them to escape detection by the immune system (Gerhard et al., 2006; Shim et al., 2011). A vaccine is the best way to prevent influenza. Current influenza vaccines are derived from prototype viruses by inducing antibodies against two major viral surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). However, current influenza vaccines do not meet the large vaccine demand during an influenza outbreak and the development of influenza vaccines capable of inducing cross-protection against different influenza variants or strains are urgently needed.

The M2 protein is a type III integral membrane protein of the influenza virus. The extracellular N-terminal domain of M2 protein (M2e) is highly conserved across human influenza A subtypes and provides a potential antigen target for developing a universal influenza vaccine with broad-spectrum prevention (Du et al., 2010; Fiers et al., 2004; Zhao et al., 2010b). However, the M2e antigen is a non-immunogenic 23 amino acid peptide, which means that a highly immunogenic antigen is needed. Strategies for the development of such an antigen have included increasing peptide density, fusing M2e with carrier molecules such as glutathione S-transferase, the hepatitis B virus core (HBc), keyhole limpet hemocyanin (KLH), and *Neisseria meningitides* outer membrane protein complex (OMPC), or co-administration with adjuvants such as flagellin and cholera toxin (Ebrahimi and Tebianian, 2011; Fu et al., 2009; Shim et al., 2011; Zhou et al., 2012). When the M2e peptide is inserted into the C-terminally truncated HBc protein, it is highly immunogenic, induces an anti-M2e antibody titer higher than the anti-HBc antibody titer, and provides effective protection against a lethal viral challenge to mice (De Filette et al., 2005; Zhao et al., 2010b). Moreover, the HBc viral-like particle is efficiently produced in *Escherichia coli* and can be easily purified (Leslie et al., 2008).

Internal influenza nucleoprotein (NP) antigens are also relatively conserved, making them another attractive candidate for a universal flu vaccine. Influenza vaccines based on M2e, NP, or both

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have been tested extensively in animal models. However, in clinical trials, promising efficacy has only been shown for licensed vaccines. Choosing suitable epitopes of NP may decrease vaccine risk and facilitate fusion of the HBc protein to form chimeric virus like particles (VLPs). The cytotoxic T lymphocyte (CTL) epitope at amino acids 418–426 of the influenza virus NP ($NP_{418–426}$), which can be cross-recognized by human influenza CTL (Kreijtz et al., 2008), may be such an epitope. The $NP_{418–426}$ epitope could potentially complement an M2e-based influenza vaccine to produce a more efficient vaccine capable of preventing a future influenza outbreak.

Here, we generated a VLP vaccine candidate containing a fusion of 3M2e-NP-HBc. We investigated whether the fused $NP_{418–426}$ epitope could produce an M2e-based influenza VLP vaccine that in combination with an oil-in-water SP01 adjuvant provides cross-protection from a lethal challenge with the pandemic 2009 H1N1 and HPAI H5N1 viruses.

2. Materials and methods

2.1. Mice

Six- to eight-week-old female BALB/c mice were purchased from the Laboratory Animal Center of Academy of Military Medical Sciences (Beijing, China). All mouse studies were performed according to the Guidelines for Animal Experiments published by Academy of Military and Medical Sciences (AMMS) and approved by the Institute Animal Care and Use Committee of the AMMS (Beijing, China). Mice were maintained in a specific pathogen-free environment according to guidelines set forth by the Laboratory Animal Center of the AMMS.

2.2. Influenza virus

The highly pathogenic avian influenza H5N1 virus used in the study was A/Ostrich/SuZhou/097/2003 (H5N1), isolated on a farm at Yangzhou University (Yang et al., 2010a). The pandemic 2009 H1N1 virus used in this study was A/Beijing/501/2009isolated from a confirmed H1N1 case in China (Li et al., 2012).Viruses were propagated in 10-day-old embryonated chicken eggs and stored at −80 °C until use. The median lethal dose (LD_{50}) of each stain was determined as described previously (Yang et al., 2010b).All experiments related to live viruses were performed in biosafety level 3 laboratory facilities.

2.3. Construction, expression and purification of 3M2e-NP-HBc or 3M2e-HBc

The 3M2e-NP-HBc particle was produced by fusing the$NP_{418–426}$ epitope to the C-terminus of three tandem copies of M2e and inserting the chimeric peptide antigen into the major immunodominant region (MIR) of C-terminally truncated HBc (HBc-N149). Insertion of the same three tandem copies ofM2e into the MIR of HBc-N149wasdesignated as 3M2e-HBc. A stabilized, truncated HBc particle was used as a control.

DNA fragments encoding 3M2e-NP-HBc and 3M2e-HBc were chemically synthesized by Sangon (Shanghai, China) and cloned into pET21a (Novagen) by digesting with BamHI and HindIII to create pET21a-3M2e-NP-HBc and pET21a-3M2e-HBc. A 6X-His epitope tag at the C-terminus of recombinant proteins was added by the plasmid vector pET21a to facilitate purification of recombinant proteins. Plasmids were propagated by transformation into BL21 (DE3) competent cells. The positive clones were inoculated in LB broth containing 100 mg/L ampicillin and cultured until the OD_{600} reached 0.6. Expression of the recombinant proteins was induced by addition of 0.5 mM of isopropy-beta-d-thiogalactopyranoside (TaKaRa) and incubation for 4 h at 37 °C. The bacteria were collected by centrifugation at 10,000 rpm for 15 min at 4 °C. Target proteins were purified using the AKTA Purifier (GE) with a Ni-chromatography column and analyzed by sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS–PAGE). The recombinant fusion proteins were concentrated by dialysis to PBS for microscopy and immunization.

2.4. Western blot

Electrophoresis was performed using 15% SDS–PAGE and the recombinant proteins detected by Western blotting using influenza A virus M2 protein monoclonal antibody (Abcam 14C2). Briefly, the purified proteins were transferred to nitrocellulose membranes (Amersham Biosciences) and blocked with 5% (w/v) skim milk in PBS containing 0.05% (v/v) Tween 20 (PBST) at 4 °C overnight. Membranes were subsequently incubated with a 1:2000 dilution of 14C2 in 5% (w/v) skim milk/PBST for 1 h at 37 °C. The blots were then washed five times with PBST and incubated with a 1:6000 dilution of HRP-conjugated anti-mouse IgG (BETHYL) for 1.5 h at room temperature. After washing membranes five times with PBST, immunoreactivity was visualized by chemiluminescence with an ECL kit (Thermo).

2.5. Electron microscopy

Purified HBc-N149, 3M2e-NP-HBc and 3M2e-HBcwere observed by transmission electron microscopy. Briefly, proteins were adsorbed to a carbon membrane on top of a copper screen and samples stained with 2% phosphotungstic acid. Specimens were evaluated using a Philips Tecnai 10 electron microscope (Philips, Inc.).

2.6. Immunization and virus challenge

Mice were vaccinated 2 weeks apart with an intraperitoneal (i.p.) injection of 10 μg 3M2e-NP-HBc, 3M2e-HBc or HBc-N149 in an oil-in-water SP01 adjuvant. The oil-in-water emulsion adjuvant SP01 containing squalene, polyether and castor oil was added by mixing 1:1 v/v with the antigen as previously described (Yang et al., 2012; Yu et al., 2012). Sera and mucosal samples were collected 2 weeks after the last immunization, and the spleens of vaccinated mice were harvested for detection of cell immune responses. Two weeks after the last immunization, mice were intranasally (i.n.) challenged with a lethal dose of influenza virus A/Beijing/501/2009 (H1N1) ($50LD_{50}$) or A/Ostrich/SuZhou/097/2003 (H5N1) ($10LD_{50}$). Challenged mice were observed and weighed daily for 2 weeks. Lung tissues were collected 4 days post-challenge for analysis of lung viral titers and histopathological changes.

2.7. Elisa

ELISA was used to measure IgG, IgG1 and IgG2a antibody titers in sera. We coated 96-well ELISA plates with 10 μg/ml synthetic M2e peptide (Sangon, China), 3M2e-NP-HBc, 3M2e-HBc or HBc-N149 dissolved in pre-coating buffer (50 mM Na_2CO_3–$NaHCO_3$, pH 9.6) overnight. Coated plates were washed with PBS containing PBST five times and blocked with 2% (w/v) bovine serum albumin (BSA) in PBS at 4 °C overnight. Serial 2-fold dilutions of mouse sera were added to the plates and incubated at 37 °C for 1 h. The plates were washed three times with PBST and HRP-conjugated rabbit anti-mouse IgG, IgG1 or IgG2a (BETHYL) were added at 1:30,000 dilution and incubated 37 °C for 40 min. After washing three times with PBST, the plates were developed using 3,3′,5,5′,-tetramethylbenzidine (Sigma) and the reaction was stopped by adding 2 mol/L H_2SO_4. The OD_{450} was read using a microplate reader (Bio-Rad).

2.8. ELISPOT assay

Antibodies against the mouse cytokines used in our cytokineELISPOT assays were purchased from BD/Pharmingen (San Diego, CA, USA). Briefly, anti-mouse interferon-γ(IFN-γ) and IL-4 antibodies(5 mg/ml in coating buffer) were used to coat multi-screen 96-wellfiltration plates (Millipore, Bedford, MA, USA). Two weeks after the last vaccination, freshly isolated splenocytes (1×10^5 cells) were added to each well in duplicate and stimulated with purified 3M2e-NP-HBc, 3M2e-HBc or HBc-N149 antigen at 10 μg/ml. The plates were incubated for 40–44 h at 37 °C under 5% CO_2. Spots were counted using an automated ELISPOT reader system and ImmunoSpot 3 software (Cellular Technology).

2.9. Viral titers in lung tissues

Viral titers in the lung were determined by the 50% tissue culture infective dose ($TCID_{50}$), calculated by the Reed and Muench method, and expressed as Log_{10} $TCID_{50}$/g of lung tissues.

2.10. Histopathological analysis

Lung tissues of challenged mice were removed and fixed in 4% paraformaldehyde buffer and embedded in paraffin. Sections were made at 4–6 μm thicknesses and mounted on slides. Histopathological changes were detected by hematoxylin and eosin (H&E) staining and observed under a microscope.

2.11. Statistical analysis

The significance between survival curves was analyzed by Kaplan–Meier survival analysis with log-rank testing. Other data were analyzed using a two-tailed Student's *t*-test. All analyses were performed using the GraphPad Prism software.

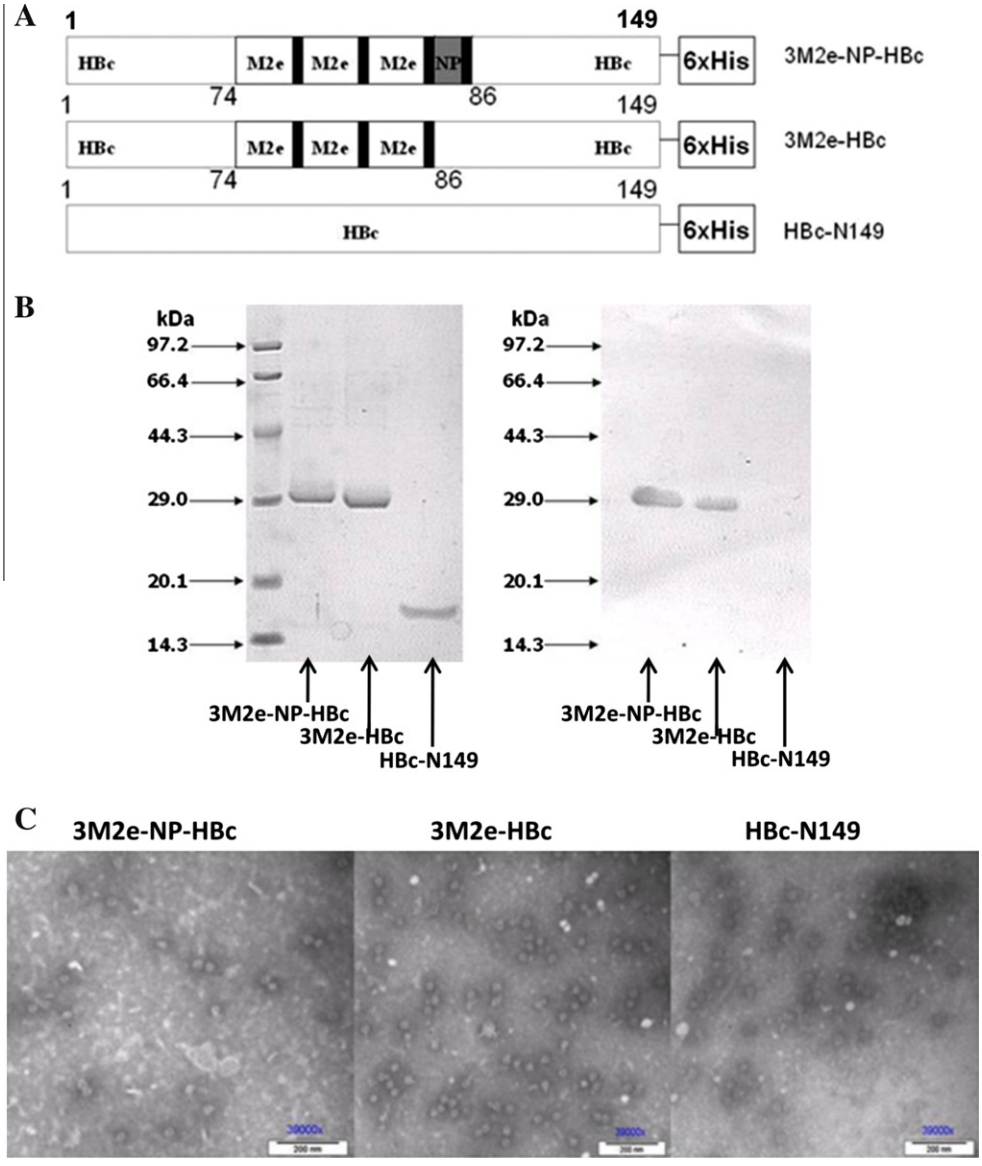

Fig. 1. Construction of plasmids and purification of 3M2e-NP-HBc, 3M2e-HBc and HBc-N149 proteins. (A) Synthetic 3M2e-NP-HBc, 3M2e-HBc or HBc-N149 genes from the PR8 virus were cloned into the pET21a vector. (B) Recombinant proteins expressed in *Escherichia coli* were purified by His-tag affinity chromatography and detected by Western blot using the M2e-specific antibody 14C2. (C) The 3M2e-NP-HBc, 3M2e-HBc and HBc-N14particles observed by electron microscopy.

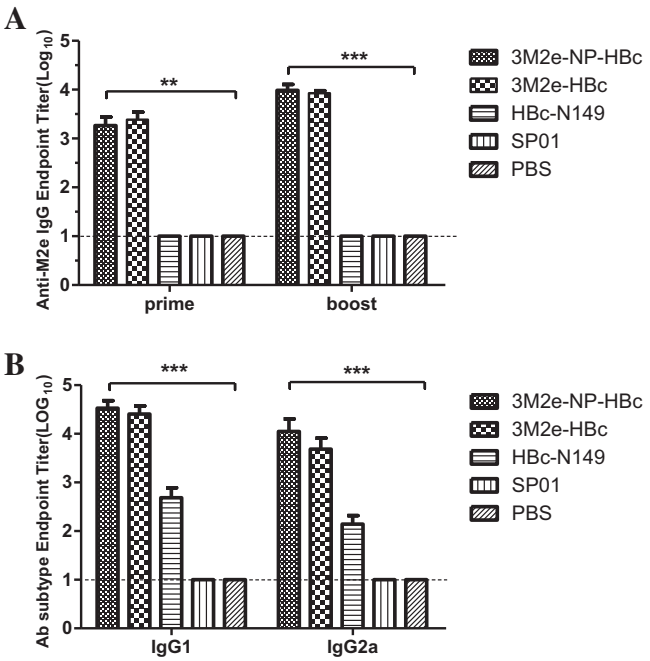

Fig. 2. Isotype specific IgG responses to influenza virus. Groups of mice were immunized twice at a 2-week interval with 3M2e-NP-HBc, 3M2e-HBc and HBc-N149 proteins. Virus specific IgG antibody titers for (A) IgG1 and (B) IgG2a isotypes were determined 2 weeks after a boost vaccination. The data represent mean protein concentrations ± SD from individual mice ($n = 6$; $^{*}p < 0.05$, $^{**}p < 0.01$).

3. Results

3.1. Expression, purification and characterization of 3M2e-NP-HBc, 3M2e-HBc and HBc-N149 particles

The 3M2e-NP-HBc fusion protein was generated by replacing the $MIR_{75–85}$ of HBc-N149 with three tandem repeats of $M2e_{2–24}$ and the $NP_{418–426}$ epitope. The 3M2e-HBc particle was generated by inserting the same $M2e_{2–24}$ sequence without the $NP_{418–426}$ epitope (Fig. 1A). Both the HBc-N149 and fusion proteins (3M2e-NP-HBc and 3M2e-HBc) were propagated in the *E.coli* strain BL21 (DE3). The purified recombinant proteins were analyzed with SDS–PAGE with a molecular mass correspond to 29 kDa for 3M2e-NP-HBc, 27 kDa for 3M2e-HBc and 18 kDa for HBc-N149, which included extra sequence from the plasmid (Fig. 1B). This was consistent with the theoretical expected molecular mass for all proteins and their purity was greater than 90%. Both 3M2e-NP-HBc and 3M2e-HBc fusion proteins were detected by the anti-M2e monoclonal antibody (Fig. 1B). Negative staining electron microscopy (EM) was used to observe that 3M2e-NP-HBc, 3M2e-HBc and HBc-N149 were able to form ~30 nm particles (Fig. 1C).

3.2. Chimeric HBc particles induced high titers of reactive antibody in mice

The humoral immune responses induced by i.p. injection of 3M2e-NP-HBc and 3M2e-HBc fusion proteins were detected by measuring IgG antibodies in the serum of vaccinated mice by ELISA. Both the 3M2e-NP-HBc and 3M2e-HBc vaccine candidates elicited approximately a10^4 rise in M2e-specific antibody endpoint titer 14 days after the last vaccination, while mice immunized with HBc-N149, SP01 alone, or PBS had only background levels of M2e-specific antibodies. There was no statistical difference in the titers of M2e-specific IgG antibodies between the 3M2e-NP-HBc and 3M2e-HBc vaccinated groups (Fig. 2A) and both groups could induce higher IgG1 and IgG2a titers than the PBS groups ($p < 0.001$). In the 3M2e-NP-HBc vaccinated group, the IgG1 and IgG2a titers were 3.9×10^4 and 1.5×10^4, respectively, while in the 3M2e-HBcvaccinated group, the IgG1 and IgG2a titers were $3.2 \times 10^4$and 7.5×10^3, respectively (Fig. 2B). These results indicate that immunization with 3M2e-NP-HBc or 3M2e-HBc fusion proteins stimulates both Th2-(IgG1) and Th1-(IgG2a) associated antibody immune responses.

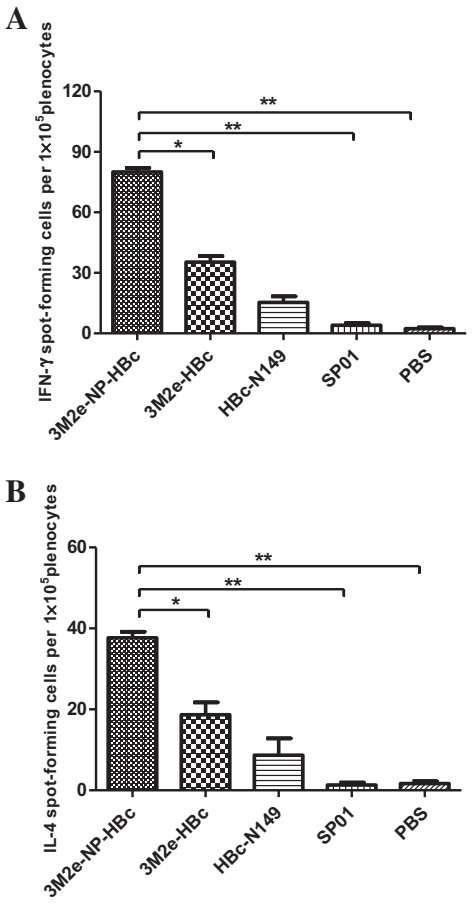

Fig. 3. Analysis of IFN-γand IL-4 by ELISPOT. Mice were immunized twice at a 2-week interval with 3M2e-NP-HBc, 3M2e-HBc and HBc-N149 proteins. On day 14 after the final immunization, three mice in each group were sacrificed and single-cell suspensions prepared from the spleens. The cells were cultured at 1×10^5 cells in 200 ml with 10 μg/ml 3M2e-NP-HBc, 3M2e-HBc and HBc-N149 proteins. (A) IFN-γ and (B) IL-4 secretion by splenic lymphocytes was detected by ELISPOT after culture for 40–44 h. The data represent the means ± SD from three experiments.

3.3. 3M2e-NP-HBcchimericparticles effectively induce cellular immune responses

To estimate the ability of the 3M2e-NP-HBc fusion protein to elicit a cellular immune response, IFN-γ and IL-4 producing cells were detected by ELISPOT. As shown in Fig. 3A, 3M2e-NP-HBc induced a significantly higher level of IFN-γ producing T cells than 3M2e-HBc ($p < 0.05$), SP01 alone ($p < 0.01$) and PBS groups ($p < 0.01$). We also that found 3M2e-NP-HBc induced a significantly higher level of IL-4 producing T cells than 3M2e-HBc (Fig. 3B, $p < 0.05$). Moreover, the number of IFN-γ-secreting cells was greater than IL-4-secreting cells, indicating that there appears to be a greater Th1-type than a Th2-type immune response in the vaccinated group. These results suggest that both types of immune responses are elicited in the vaccinated animals, but it is unclear

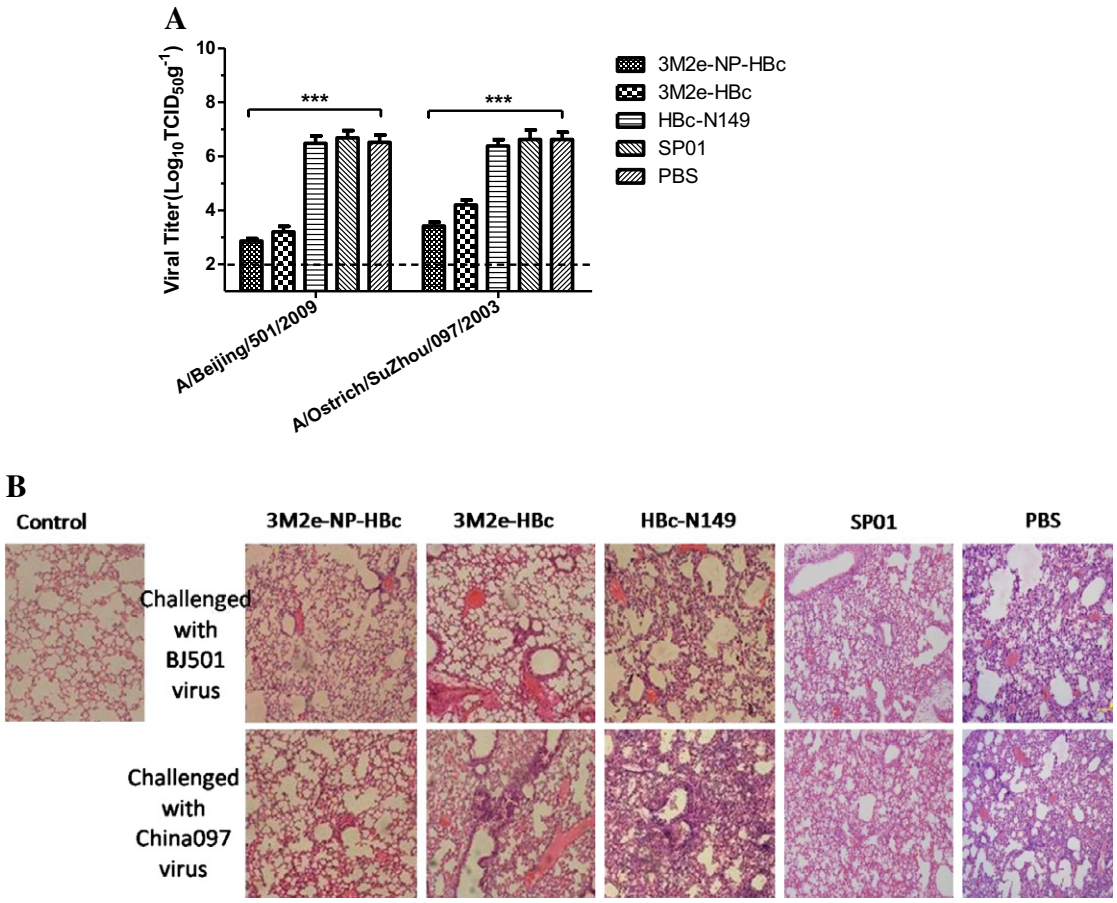

Fig. 4. Viral titers and histopathological changes in lungs of vaccinated mice following challenge withpandemic 2009 H1N1 and HPAI H5N1 virus. Two weeks after the last immunization, vaccinated mice were challenged with a lethal dose ($10LD_{50}$) of influenza virus A/Ostrich/SuZhou/097/2003 (H5N1) or A/Beijing/501/2009 (H1N1) and lung tissues were collected four days later. (A) Viral titers in lungs of infected mice. The data are expressed as $\log_{10}TCID_{50}/g$ tissues; $^{*}p < 0.05$, $^{**}p < 0.01$. (B) Histopathological changes in the lungs following virus challenge. Representative images of histopathological damage from hematoxylin and eosin stained lungs are shown from 5 mice per group at a 200× magnification.

which type of response is required to protect the animals from influenza challenge.

3.4. 3M2e-NP-HBc chimeric particles restrict viral replication and attenuate virus-induced lung pathology

Lungs of vaccinated mice were collected 4 days post-challenge with HPAI A/Ostrich/SuZhou/097/2003 (H5N1)(China097) or A/Beijing/501/2009 (H1N1)(Beijing501) viruses to determine viral titers and histopathological lung damage. When challenged with the Beijing501virus, the mean viral titer in the3M2e-NP-HBc vaccinated group was $10^{3.0}TCID_{50}/g$ and that in the 3M2e-HBc vaccinated group was $10^{3.6}$ $TCID_{50}/g$, which represents a significant decrease in H1N1 viral titer in lung tissues of both vaccinated groups compared to the control groups (Fig. 4A, $p < 0.001$). Slight histopathological damage, such as pulmonary vascular dilatation and congestion, sparing fragmentation of alveolar walls, and infiltration of lymphocytes were observed in the 3M2e-NP-HBc and 3M2e-HBc groups, whereas severe histopathological damage was observed in the HBc-N149 and PBS groups (Fig. 4B). These results reveal that replication of the H1N1 virus in the lungs of mice decreased with 3M2e-NP-HBc VLP vaccinations.

Significantly decreased China097 viral titers were detected in the lungs of 3M2e-NP-HBcvaccinated mice ($10^{3.7}$ $TCID_{50}/g$) compared with the 3M2e-HBc mice ($10^{4.2}$ $TCID_{50}/g$; Fig. 4, $p < 0.05$). Limited pulmonary vascular dilatation and slight thickening and fragmentation of alveolar walls were observed in lung tissues of the 3M2e-NP-HBc vaccinated group. However, dramatic histopathological damage, such as exfiltration of large numbers of lymphocytes followed by exudation and severe edema, severe fragmentation or thickening of alveolar walls was present in the 3M2e-HBc and HBc-N149 vaccinated groups (Fig. 4B). This demonstrated that 3M2e-HBc would provide poor restriction against the H5N1 virus. Combined, these results suggest that the 3M2e-NP-HBc VLP vaccine could efficiently induce protective immunity against H5N1 viral replication in lung tissues.

3.5. 3M2e-NP-HBc fusion proteins provide effective cross-protection from lethal challenge with pandemic 2009 H1N1 virus and HPAI H5N1 virus

To further confirm the potential subtype cross-protective immunity induced by 3M2e-NP-HBc against influenza viruses, mice were respectively challenged with Beijing501 ($50LD_{50}$) and China097 viruses ($10LD_{50}$) two weeks after the last immunization. The 3M2e-NP-HBc vaccinated groups showed powerful resistance when challenged with Beijing501 virus, all the mice survived for more than 14 days after the lethal challenge, and body weight recovered quickly 7 days post-challenge. As a comparison, the survival rate in the 3M2e-HBc group was 90% (Fig. 5A and B) and no mice in the HBc-N149, SP01 and PBS groups lived longer than 9 days after the virus challenge. Remarkably, mice in the 3M2e-NP-HBc vaccinated group also presented additional resistance against a challenge from the HPAI China097 virus and 90% survived

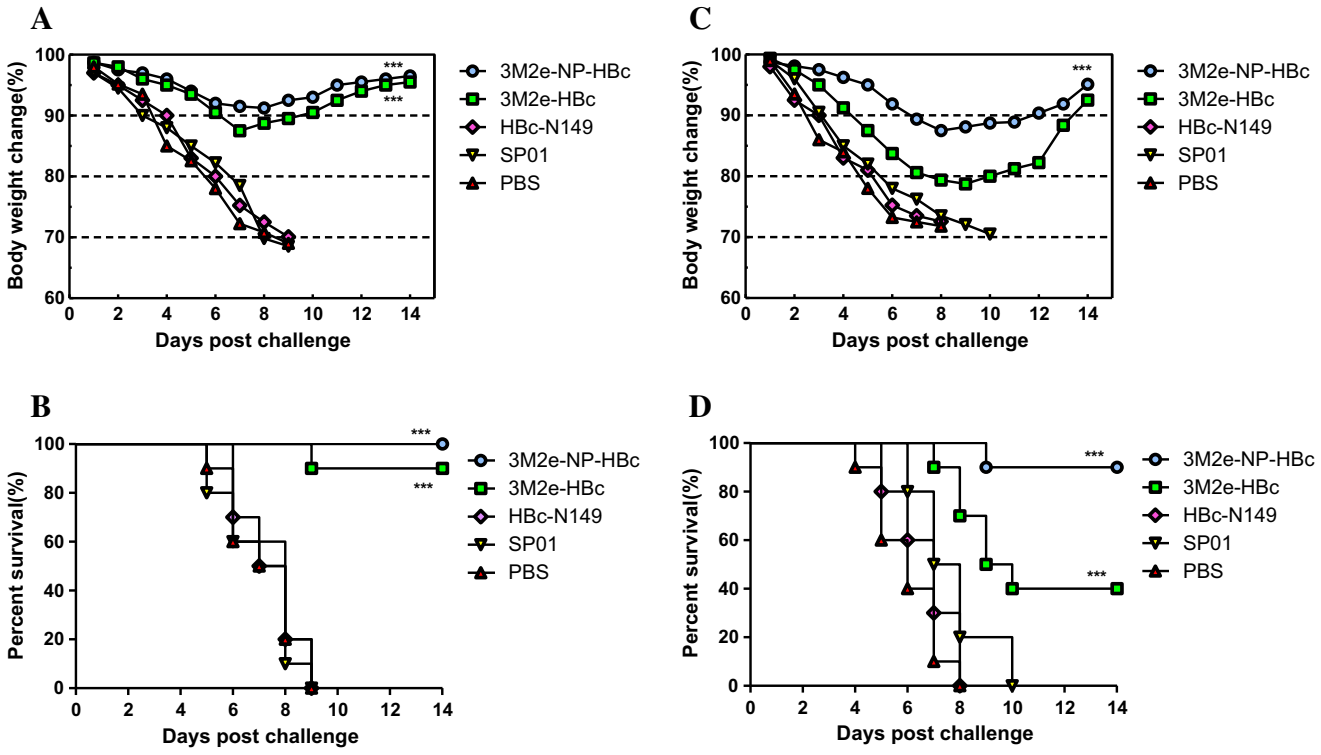

Fig. 5. Cross-protection of 3M2e-NP-HBc vaccinated mice against a lethal challenge of pandemic 2009 H1N1 and HPAI H5N1 virus. 3M2e-NP-HBc vaccinated mice were challenged with a lethal dose of influenza virus A/Beijing/501/2009 (H1N1) ($50LD_{50}$) or A/Ostrich/SuZhou/097/2003 (H5N1) ($10LD_{50}$) and monitored daily for two weeks post challenge. (A, C) Percent change (%) in mouse body weight. Each point represents the mean body weight of 10 mice per group. (B, D) Survival rate (%). $^{***}p < 0.001$ compared to the control group.

for more than 14 days after the lethal challenge. These mice also exhibited attenuated body weight loss, which occurred on the third day after challenge and continued slowly with a lowest mean percentage of body weight change of 12%. In contrast, in the 3M2e-HBc vaccinated group, body weight loss occurred acutely on the second day after challenge with a lowest mean percentage of body weight change of 20%and only a 40% survival rate. The survival curves between 3M2e-NP-HBc and 3M2e-HBc vaccinated groups are shown in Fig. 5C and D and were significantly different ($p < 0.05$). These results demonstrate that the 3M2e-NP-HBc VLP vaccine could provide effective cross-protection against a lethal challenge with pandemic 2009 H1N1 virus and HPAI H5N1 virus.

4. Discussion

Current influenza vaccines contain strain-specific HA and NA glycoproteins that must be re-evaluated annually and fail to provide effective cross-protection in humans. The emergence of the 2009 pandemic influenza A (H1N1) virus and the possibility of a new human influenza pandemic have increased the demand for an influenza vaccine that provides broad-spectrum protection across a wide range of strains and subtypes (Michaelis et al., 2009a, b).

M2e is highly conserved among influenza A viruses and has been considered as a promising universal influenza vaccine antigen. A number of strategies have been developed to induce cross-protection using M2e-based influenza VLP vaccines (De Filette et al., 2006a,b; Jazi et al., 2012). Although studies have confirmed that an M2e-specific monoclonal antibody can reduce viral duplication *in vitro* and antibodies induced by M2e could provide protection *in vivo*(Zou et al., 2005), M2e has reduced capacity to induce cellular immune responses compared to other viral antigens such as HA, NA and NP(Patel et al., 2009; Wu et al., 2009a; Wu et al., 2009b).These shortcomings limit the functional capacity of an M2e-based influenza VLP vaccine.

We investigated the $NP_{418-426}$ epitope as a potential complementary constituent of an M2e-based influenza VLP vaccine that could provide better subtype cross-protection. To accomplish this, a fusion protein was generated by inserting three tandem copies of the M2e sequence to the $NP_{418-426}$ epitope to produce the 3M2e-NP-HBc VLP. We observed that inserting93 amino acids of foreign sequence did not impact the physical characteristics of the HBc protein, and both fusion proteins were able to form chimeric HBc particles (Fig. 1C), which is in accordance with previous reports (Geldmacher et al., 2005). We also utilized a convenient *E. coli* expression system and a Ni-chromatography column purification protocol to produce ample quantities of highly purified proteins. Efficacy of an M2e-based vaccine relies on the production of high-titer M2e-specific antibodies, which induce complement-mediated cytotoxicity and/or antibody-dependent cell-mediated cytotoxicity (Jegerlehner et al., 2004). Both 3M2e-NP-HBc and 3M2e-HBc particles elicited robust M2e-specific IgG antibodies in vaccinated mice (Fig. 2). Previous studies have reported that repeated copies of M2e induce higher antibody titers when fused to the N-terminus of protein carriers (De Filette et al., 2006a; De Filette et al., 2006b; Zhao et al., 2010a; Zhao et al., 2010b). Both 3M2e-NP-HBc and 3M2e-HBc induced significant Th1- and Th2-associated antibody responses (Fig. 2). Notably, fusion with the$NP_{418-426}$ epitope elicited enhanced cellular immune responses in the 3M2e-NP-HBc vaccinated group compared to the 3M2e-HBc vaccinated group, as indicated by increased IFN-γ and IL-4 secreting lymphocytes (Fig. 3). $NP_{418-426}$ is a proven immunodominant epitope that is highly associated with IFN-γ production and activity of the human influenza virus-specific CTL response *in vitro*. Loss of the $NP_{418-426}$ epitope leads to a significant reduction of IFN-γ-

expressing $CD8^+$ T cells and a decrease in the virus-specific CTL response (Berkhoff et al., 2007).

Previous studies indicated that internal influenza proteins such as NP, which is highly conserved among all influenza A subtypes, can induce cross-reactive T-cells across all subtypes of influenza A virus in different animal models (Breathnach et al., 2006; Laddy et al., 2009). Zhou et al. (2010) described a vaccine regimen based on sequential immunization with two serologically distinct chimpanzee-derived replication-defective adenovirus vectors expressingM2e from three divergent strains of influenza A virus fused to NP that induced M2e antibodies and virus-specific CD8 + T cells to NP (Zhou et al., 2010). Xu et al. (2009) reported that a chimeric peptide antigen composed of M2e and two NP CTL epitopes of avian influenza virus provided good chicken immunogenicity (Xu et al., 2009). Our study has demonstrated that M2e-based chimeric particles fused with the CTL epitope of NP (3M2e-NP-HBc) enhanced cellular immune responses in mice. The 3M2e-NP-HBc vaccine was also more effective in protecting against a lethal challenge with the Beijing501 or China097 virus by reducing body weight loss and increasing survival rate. It is important to note, however, that even though immunization with 3M2e-NP-HBcVLPs increased survival, the mechanism of this response is not clear. Viral titers in lung tissues and histopathology also revealed that 3M2e-NP-HBc could decrease viral replication in lung tissues (Figs. 4 and 5).These data suggest that a cellular immune response to NP is responsible for this enhanced response, but an overall enhancement in the immune response, as illustrated in Fig. 3, could also be involved and requires further study. Overall, our results indicate that the chimeric HBc particle vaccine candidate 3M2e-NP-HBc not only provided full protection against the 2009 pandemic H1N1 influenza virus but also enhanced protection against the HPAI H5N1 influenza virus, suggesting that this new formulation could be developed further as a universal influenza vaccine candidate.

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