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ORIGINAL RESEARCH

RETRACTED ARTICLE: Nanovaccine Confers Dual Protection Against Influenza A Virus And Porcine Circovirus Type 2

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Background: The influenza A virus (IAV) is known for its high variability and poses a huge threat to the health of humans and animals. Pigs play a central role in the cross-species reassortment of IAV. Ectodomain of matrix protein 2 (M2e) is the most conserved protective antigen in IAV and can be used to develop nanovaccines through nanoparticles displaying to increase its immunogenicity. However, the high immunogenicity of nanoparticles can cause the risk of off-target immune response, and excess unwanted antibodies may interfere with the protective efficacy of M2e-specific antibodies. Therefore, it is necessary to select reasonable nanoparticles to make full use of antibody against nanoparticles while increasing the level of M2e-specific antibodies. Porcine circovirus type 2 (PCV2) is the most susceptible virus in pigs and can promote IAV infection. It is meaningful to develop a vaccine that can simultaneously control swine influenza virus (SIV) and PCV2.

Methods: In the present study, M2e of different copy numbers were inserted into the capsid (Cap) protein of PCV2 and expressed in *Escherichia coli* to form self-assembled chimeric virus-like particles (VLPs) as vaccine. BALB/c mice and pigs were immunized with these nanovaccines to explore optimal anti-IAV and anti-PCV2 immunity.

Results: Cap is capable of carrying at least 81 amino acid residues (three copies of M2e) at its C-terminal without impairing VLPs formation. Cap-3M2e VLPs induced the highest level of M2e-specific immune responses, conferring protection against lethal challenge of IAVs from different species and induced specific immune responses consistent with PCV2 commercial vaccines in mice. In addition, Cap-3M2e VLPs induced high levels of M2e-specific antibodies and PCV2-specific neutralizing antibodies in pigs.

Conclusion: Cap-3M2e VLP is an economical and promising bivalent nanovaccine, which provides dual protection against IAV and PCV2.

Keywords: influenza A virus, porcine circovirus type 2, M2e, nanovaccine, virus-like particles, bivalent vaccine

Introduction

It is been 100 years since the 1918 Spanish flu (1918–1919) pandemic ended.[1,2] Vaccines and treatments of influenza A virus (IAV) have continuously improved during the past 100 years.[2] But the IAV remains a deadly threat over humans and animals. The IAV is known for its extreme variability because its genome consists of 8 segments of negative-strand RNA and is highly susceptible to mutation and recombination.[3,4] There are usually no antibodies in human blood against IAV from other species; therefore, once the IAV crosses the species barrier into humans, it can be extremely lethal.[5] Like the 1918 pandemic H1N1 IAV, the 2009 pandemic swine-origin H1N1 IAV was circulated in swine before it emerged in humans.[1,6] The pandemic H3N2 IAV in

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1968 was closely related to avian influenza virus (AIV) and later spread into pigs.[7–9] It is now clear that swine, as "mixing vessel" hosts, contain receptors for IAV of human, avian and swine origin, support replication and reassortment of this IAV.[10–12] The highly pathogenic H5N1 and H7N9 AIV (HP-AIV) have failed to spread widely among humans because these HP-AIVs have not yet stably propagated and spread in human respiratory epithelial cells. However, once these HP-AIVs acquire the ability to fully adapt to human respiratory epithelial cell receptors in the respiratory epithelial cells of pigs, it will be extremely terrible.[13–17] Therefore, it is urgent to establish an IAV immune defense line for pigs to effectively prevent and control the outbreak of pandemic influenza.[17,18]

Vaccines are essential weapons in establishing the immune defense line. Regrettably, current IAV vaccines only provide effective protection against matching strains.[19] More importantly, the global swine influenza virus (SIV) surveillance program is lacking, and it is difficult to effectively detect the changes of IAV in pigs in real time. Therefore, it is imperative to develop a universal influenza vaccine for pigs.[12,20,21] Unlike the high variability of hemagglutinin (HA) and neuraminidase (NA), the matrix 2 protein (M2e) is the most conserved surface protein of all subtypes of IAV.[22] M2e-specific antibodies can be utilized in the treatment of human influenza and protect animals from lethal attacks by various IAV. Therefore, M2e is a promising candidate for the development of a universal IAV vaccine.[23,24] However, M2e is composed of 23 amino acid residues, and it is difficult to induce an effective level of antibodies in a natural state.

Various platforms have been used to improve the immunogenicity of M2e, in which protein nanoparticles are the most dazzling.[23–27] However, protein nanoparticles with strong immunogenicity can induce large amounts of unwanted antibodies leading to off-target effects, which are detrimental to the accurate generation of high levels of M2e-specific antibodies.[26] A reasonable strategy is to select the appropriate insertion site on appropriate protein nanoparticles so that M2e can be fully exposed, such as N-terminal, C-terminal, or loops, while taking advantage of the unwanted antibodies for synergistic effect. Virus-like particles (VLPs) have editable sequences, large molecular weight, and precisely defined surface repeat structures that provide the opportunity to crosslink with more BCR receptors.[28–31] However, these VLPs-M2e vaccines induced only a small fraction of antibodies against M2e, and a large number of antibodies to VLPs are useless. Therefore, it is necessary to select suitable VLPs to display a reasonable copy number of M2e at a suitable site. More importantly, a suitable VLP vector can become a potential bivalent vaccine, which simultaneously induces M2e-specific antibodies and protective antibodies against the corresponding viruses.

Porcine circovirus type 2 (PCV2), the smallest mammalian virus, causes severe immunosuppression and plays an important role in co-infection and multiple infection in pigs.[32,33] Pigs infected with PCV2 promote SIV infection and increase clinical symptoms associated with SIV.[33–35] Excitingly, vaccination with PCV2 vaccine can reduce the susceptibility of pigs to other viruses.[32] Therefore, co-prevention of PCV2 and SIV is very necessary. The unique capsid (Cap) protein of PCV2 can self-assemble to form VLPs, which is an ideal candidate for PCV2 vaccines.[36] The major neutralizing epitopes are located on the outer surface of PCV2 and away from the C-terminal.[37,38]

In this study, we inserted different copy numbers of M2e into the C-terminal of the Cap protein of PCV2 to and self-assembled to form Cap VLPs-based nanovaccines (Figure 1A). Then, we explored the rational display of the number of M2e in the mouse model to reach the best balance between anti-influenza and anti-PCV2. Results showed that the C-terminal of the Cap protein can display 3-sequential repeats of M2e (81 amino acid residues) without affecting the assembly of Cap VLPs (Figure 1B). The Cap-3M2e VLP nanovaccine induced the highest levels of M2e- and PCV2-specific neutralizing antibodies and protected mice from lethal infections of IAV of human, avian and swine origin.

Materials And Methods

Materials

Porcine Kidney-15 (PK-15) cells and MDCK cells were acquired from ATCC (Manassas, VA, USA). The PCV2 strain (GenBank: ADG07991) and several IAV strains (A/swine/Zhucheng/90/2014 (H1N1), A/swine/Henan/1/2010 (H3N2), A/Puerto Rico/8/1934 (H1N1), A/chicken/Guangzhou/GZ/2005 (H9N2) and A/California/07/2009 (H1N1)) used in this research were stored in the laboratory. Carbopol 971P NF polymer (Carbomer) was purchased from Lubrizol (Cleveland, OH, USA). These M2e peptides used in the experiments are given in Table 1.

Expression And Purification Of Recombinant Cap-nM2e Protein

Different sequential repeats of the M2e peptide (SLLTEV ETPTRNGWESRYSDSSD) were combined with the C-terminal of the Cap protein (without nuclear localization

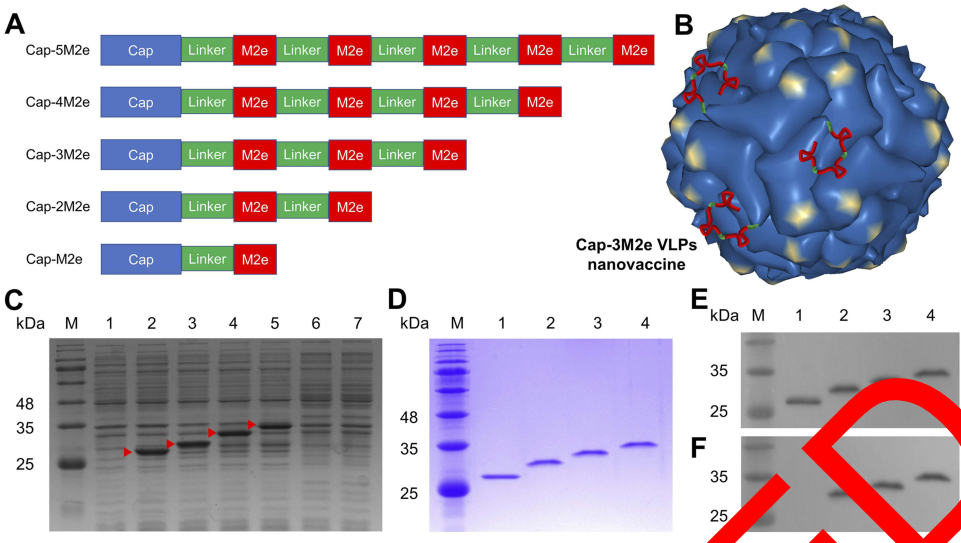

Figure 1 Expression and purification of recombinant Cap-nM2e proteins.

Notes: (**A**) Primary pattern structure of recombinant Cap-nM2e. (**B**) Schematic illustration of Cap-3M2e VLPs nanovaccine. The pattern was drawn from PDB accession number 3R0R. (**C**) SDS-PAGE analysis of Cap-nM2e protein expressed in *E. coli* BL21 (DE3). Lane M: molecular weight markers; Lane 1: SDS-PAGE analysis of total cell lysate of pET28a vector, Cap, Cap-M2e, Cap-2M2e, Cap-3M2e, Cap-4M2e and Cap-5M2e proteins expressed in *E. coli* BL21 (DE3). Red arrow indicates successfully expressed proteins. (**D**) SDS-PAGE analysis of purified Cap-nM2e protein. (**E**) Western blot analysis of purified Cap-nM2e protein using PCV2-specific polyclonal antibody. (**F**) Western blot analysis of purified Cap-nM2e protein using 14C2 mAb (Anti-IAV M2 protein). Lane M: molecular weight markers; Lane 1–4: SDS-PAGE analysis of purified Cap, Cap-M2e, Cap-2M2e, Cap-3M2e.

Abbreviations: Cap, Capsid; M2e, ectodomain of matrix protein 2; VLPs, virus-like particles; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; *E. coli*., *Escherichia coli*; PCV2, porcine circovirus type 2; mAb, monoclonal antibody.

Table 1 M2e Amino Acid Sequences Of Different IAV Strains

Influenza Strains	Abbreviation	M2e Amino Acid Sequence	
Expressed Sequence	M2e	(M)SLLTEVETPTRNGWESRYSDSSD	A
A/swine/Zhucheng/90/2014 (H1N1)	swine-H1N1-challenge	SFLYEVETPTRSGWECRYSDSSD	
A/swine/Henan/1/2010 (H3N2)	swine-H3N2-challenge	SLLTEVETPIRNEWGCRCNDSSD	B
A/Puerto Rico/8/1934 (H1N1)	human-H1N1-challenge	SLLTEVETPIRNEWGCRCNGSSD	
A/chicken/Guangzhou/GZ/2005 (H9N2)	avian-H9N2-challenge	SLLTEVETHTRNGWECRCSDSSD	
A/Brevig Mission/1/1918 (H1N1)	pandemic-1918	SLLTEVETPTRNEWGCRCNDSSD	
A/Shanghai/202/1957 (H2N2)	pandemic-1957	SLLTEVETPIRNEWGCRCNDSSD	B
A/Hong Kong/1/1968 (H3N2)	pandemic-1968	SLLTEVETPIRNEWGCRCNDSSD	B
A/USSR/90/1977 (H1N1)	pandemic-1977	SLLTEVETPIRNEWGCRCNDSSD	B
A/California/07/2009 (H1N1)	pandemic-2009	SLLTEVETPTRSEWECRCSDSSD	
A/swine/Guangdong/1/2013(H1N1)	H1N1	SLLTEVETPTRNGWECRYSDSSD	
A/duck/Hong Kong/273/1978(H2N2)	H2N2	SLLTEVETPTKNGWECRCSDSSD	
A/Florida/8/2013(H3N2)	H3N2	SLLTEVETPTKNEWGCRCNDSGD	
A/duck/Czechoslovakia/1956(H4N6)	H4N6	SLLTEVETPTRNGWECRYSGSSD	
A/chicken/Jilin/hi/(H5N1)	H5N1	SLLTEVETPTRNGWECRCSDSSD	
A/duck/EastChina/54/2002(H6N2)	H6N2	SLLTEVETPTRNGWECKYSDSSD	
A/chicken/Ningbo/S1152/2014(H7N9)	H7N9	SLLTEVETLTRTGWECNCSGSSD	
A/mallard/Interior Alaska/9BM1327/2009(H8N4)	H8N4	SLLTEVETPIRNGWECKCSDSSD	
A/chicken/Jilin/A/2012(H9N2)	H9N2	SLLTEVETPTRNGWGCRCNDSSD	
A/duck/Hubei/137/1985(H10N4)	H10N4	SLLTEVETPTRNGWECKCSDSSD	
A/duck/Zhejiang/727D2/2013(H11N3)	H11N3	SLLTEVETPTRNGWECKCNDSSD	

Notes: (**A**) The underlined amino acid residues are highly conserved among human, swine and avian IAV. (B) The same sequence. The shade indicates the amino acid difference between the M2e of each strain and the expressed M2e sequences.

Abbreviations: M2e, ectodomain of matrix protein 2; IAV, influenza A viruses.

signal) using Gly-Gly-Gly-Gly linker (Figure 1A). Then, these sequences were cloned into the pET28a vector by double digestion with BamHI and HindIII and transformed into *E. coli* BL21 (DE3). Expressed protein amino acid

sequence is available in Note S1 (Supporting Information). For protein expression, these recombinant cells were induced expression at 18°C for 12 hrs by isopropyl-β-d-thiogalactoside (IPTG) at a final concentration of 0.1 mM. These Cap-nM2e proteins were purified by using Ni-NTA His·Bind Resin (Novagen, Madison, WI, USA). These purified Cap-nM2e proteins were determined by SDS-PAGE. Then, in order to determine the reactogenicity of these proteins, Western blotting was performed using M2e-specific monoclonal antibody (14C2) and PCV2 polyclonal antibody. These Cap-nM2e proteins were dialyzed into the assembly buffer (10 mM Tris-HCl, 100 mM NaCl (pH 8.0)). These protein concentrations were determined with a BCA protein assay kit (Thermo) and then were tested for endotoxin concentrations using a ToxinSensor Single Tests Kit (GenScript, USA).

Particle Characteristics Of Cap-nM2e VLPs

The shape, size, and size distribution of the Cap-nM2e VLPs were determined by transmission electron microscopy (TEM) (JEM-1400; JEOL Ltd., Tokyo, Japan). Hydrodynamic diameter and zeta potential of these VLPs were monitored by dynamic light scattering (DLS) (Malvern, Worcestershire, UK) at 25°C.

Antigenic Characterization Of Cap-nM2e VLPs

To further determine whether insertion of the M2e the self-assembly of Cap VLPs and whether M2e the outer surface of these VLPs, we evaluated the binding ability of different monoclonal antibodies to these VLPs by ELISA. Cap VLPs and Cap-nM2e VLPs were coated on 96-well microtiter plates with 100 μL of a 1/2-dilution series of carbonate buffer (pH 9.6) at 4°C overnight. The plates were washed with PBST buffer and blocked with skim milk (5% in PBST) at room temperature for 2 hrs. After washing with PBST five times, M2e-specific rabbit polyclonal antibody (Abcam, USA), PCV2-specific mouse monoclonal antibodies (mAb) 9F4, 6A5, and 8A10 (stored in our lab) were added to the wells and then incubated for 1 hr at 37°C, respectively.[39] Mice were immunized with the commercial Inactivated PCV2 vaccine (Merial) for 4 times. These mAbs were obtained by screening for hybridoma cells capable of secreting mAbs that bind to PCV2 (GenBank: ADG07991). Then, the epitope characteristics corresponding to these monoclonal antibodies were identified. After washing with PBST five times, HRP-labeled goat anti-rabbit or anti-mouse IgG were added and incubated for 1 hr at 37°C. The reaction was developed using 3,3,5,5-tetramethylbenzidine (TMB) as the substrate. The OD value at 450 nm of each well was measured using an ELISA reader.

Immunization And Challenge

Groups of 6–8-week-old female BALB/c mice (24/group) were subcutaneously immunized (100 μL) thrice at an interval of 3 weeks with M2e peptide (2.2 μg), Cap-M2e VLPs (22.2 μg), Cap-2M2e VLPs (24.4 μg), Cap-3M2e VLPs (26.6 μg) or Cap VLPs (20 μg) (Cap molar equivalent). The dose of Cap VLPs is 20 μg to ensure that the candidate vaccine achieves the same level of protection as the commercial PCV2 vaccine. Carbopol 971P, an adjuvant, was mixed with the candidate vaccine at equal volume with a final concentration of 0.1%. Mice in another group were immunized with commercial PCV2 subunit vaccine (Ingelvac CircoFLEX®, Boehringer Ingelheim) (100 μL) as the positive control. Immune component of each group is available in Table 2. Sera samples were harvested at 63 days post-immunization (dpi) and stored at −20°C until use. Nine mice from each group were lightly anesthetized and intranasally challenged with lethal doses of different subtypes of IAV strains at 64 dpi. IAV strains used for challenging were A/swine/Zhucheng/90/2014 (H1N1), A/swine/Henan/1/2010 (H3N2), A/Puerto Rico/8/1934 (H1N1) and A/chicken/Guangzhou/GZ/2005 (H9N2).

Table 2 Immune Component Of Each Group

Immunogen	Adjuvant	Dose And Volume	Number Of Mice
M2e	Carbopol 971P	2.2μg;100μL	24
Cap-M2e VLPs	Carbopol 971P	20μg;100μL	24
Cap-2M2e VLPs	Carbopol 971P	22.2μg;100μL	24
Cap-3M2e VLPs	Carbopol 971P	24.4μg;100μL	24
Cap VLPs	Carbopol 971P	26.6μg;100μL	24
PCV2 subunit vaccine (Ingelvac CircoFLEX®)	Aqueous polymer	Unknown;100μL	24

Notes: Immune component of each group.
Abbreviations: Cap, Capsid; M2e, ectodomain of matrix protein 2; VLPs, virus-like particles; PCV2, porcine circovirus type 2.

Body weight loss and survival rates were monitored daily for 14 days post-challenge (dpc). Weight loss of ≥ 25% was considered as the endpoint at which moribund mice were killed, according to the Institutional Animal Care and Use Committee (IACUC) guidelines.

Fifteen 3-week-old pigs were randomized into three groups. These pigs were obtained from conventional herds and were serologically free of SIV, PCV2. PCV2-specific maternal antibodies could be detected in these pigs (maternal antibodies), but no M2e- and SIV-specific antibodies were detected. Group one were immunized with 266 μg Cap–3M2e VLPs (1 mL). Group two were immunized with commercial PCV2 subunit vaccine (product manual recommended to use 1 mL). Group three were immunized with 1mL PBS (PBS was mixed with the same amount of carbomer adjuvant) (Figure 9A).

Detection Of M2e- And PCV2-Specific Antibodies

M2e- and PCV2-specific antibodies and antibody subtypes in sera were determined by indirect ELISA. M2e peptide (1 μg/mL) was coated on 96-well ELISA plates for detecting antibodies to M2e. Commercial PCV2 antibody test kit (purified and inactivated PCV2 as coated antigens) (BioChek, Reeuwijk, Holland) was utilized to detect anti-PCV2 antibodies. After addition of sera, HRP-labeled goat anti-mouse or pig IgG, IgG1 or IgG2 were added. The color reaction was performed using TMB then stopped with 2 M H_2SO_4. The level of sera M2e-specific IgG or IgG subtype titers was measured by antibody endpoint titer. The highest dilution which gives an OD450 value twice that of the naive group at the same dilution was designated as the antibody endpoint titer.

The native M2 protein has a low abundance on IAV, but is abundantly expressed on IAV-infected MDCK cells, so immunoperoxidase monolayer assay (IPMA) can be used to detect antibody levels against native M2e in sera.[40] Briefly, MDCK cells were seeded in 96-well plates at 2 × 10^4 cells per well. MDCK cells grew to 80% confluency after approximately 12 hrs of incubation. Then, the cells were infected with different IAV strains (A/swine/Zhucheng/90/2014 (H1N1), A/swine/Henan/1/2010 (H3N2), A/Puerto Rico/8/1934 (H1N1), A/chicken/Guangzhou/GZ/2005 (H9N2)) and A/California/07/2009 (H1N1)) (100 μL) of 100 $TCID_{50}$, respectively. After incubation for 24 hrs, these cells were fixed with pre-cooled ethanol at −20 °C for 30 mins. After blocking with 5% skimmed milk at 37 °C for 1 hr, the cells were incubated with 2-fold serially diluted sera. Then, HRP-labeled goat anti-mouse or pig IgG was added. The color was developed with 3-Amino-9-ethylcarbazole (AEC). The highest dilution of wells with red particles was recorded as the titer of native M2e-specific antibodies in serum.

Virus Neutralization Test

IPMA was used to evaluate the PCV2-specific neutralizing antibodies in sera, according to the previous method.[39] Briefly, the sera were inactivated for 30 mins at 56 °C and then serially diluted at 2-fold. The dilute sera were mixed with 200 $TCID_{50}$ of PCV2 at ratio 1:1 and incubated for 1 hr at 37°C. Subsequently, the mixtures were added to 96-well plates containing 100% confluent PK-15 cells. After incubation for 1 hr at 37 °C, cell supernatants were discarded and fresh DMEM (2% FBS) was added. These cells were cultured for 72 hrs at 37 °C, and supernatants were discarded and then cells were fixed with pre-cooled ethanol. After blocking with 5% skimmed milk, the cells were incubated with 9F4 mAb, followed by HRP-labeled goat anti-mouse IgG. The color cells were developed with AEC. Neutralizing antibody titers were evaluated as the reciprocal of the highest dilution that completely protected PK-15 cells.

Lymphocyte Proliferation Assay

Three weeks after the final immunization, spleens were collected from immunized mice (n=3) from each group, and then lymphocytes were isolated. The lymphocytes were seeded into 96-well plates at 100 μL per well (5 × 10^5 cells/mL in RPMI-1640 medium (10% FBS)). Then, lymphocytes were stimulated with M2e peptide (20 μg/mL) or Cap VLPs (20 μg/mL), respectively. The negative control group was stimulated with the medium. There are three parallel repeats per sample. Lymphocyte proliferative responses were determined using CCK-8 (Beyotime, Shanghai, China), 60 hrs after incubation. The stimulation index (SI) was calculated using the following formula:

$$SI = \frac{\text{the mean of OD 450 nm values of M2e or Cap VLPs stimulated wells}}{\text{the mean of OD 450 nm values of medium treated wells}}.$$

Cytokine Detection

Cytokines (IL-2, IL-4, IL-10, IL-12, and IFN-γ) in the supernatant of stimulated lymphocytes were determined

by ELISA kits (R&D Systems, R&D Minneapolis, MN, USA), after lymphocytes were incubated for 72 hrs. There are three parallel repeats per sample.

Determination Of IAV Titers

Mice (n=4) from each challenged group were sacrificed at the time when the virus reached its highest levels in the lungs of mice. Briefly, the lung tissues were homogenized and diluted with DMEM to achieve 10% (w/v) suspension and then centrifuged to remove tissue debris (10,000 rpm, 10 mins). The MDCK cells were seeded at 5×10^4 cells/well in 96-well cell culture plates. After cell adherence, the cells were infected with 100 μL of 10-fold serially diluted lung homogenate supernatant. After incubation for 1hr, supernatants were removed and fresh DMEM was added. After 24 hrs of incubation, cells were fixed, followed by IPMA to determine virus titration via $TCID_{50}$ assay. IAV-specific mAbs were used as primary antibodies. The virus titers were measured by the Reed and Muench method.

Passive Immunization Of Mice

To explore the protective effects of M2e-specific antibody subtypes, 200 μL pooled sera from Cap-M2e VLPs-immunized mice or 34-time diluted from Cap-3M2e VLPs-immunized mice were intraperitoneally transferred to naïve mice. Before the injection, immunized sera were heat-inactivated at 56 °C for 30 mins. Twenty-four hours post transfer, mice were challenged with 2 × LD_{50} of A/Puerto Rico/8/1934 (H1N1). Body weight loss and survival rates were monitored daily for 14 days (Figure 7A).

Cross-Binding Tests To M2e Of Different IAV Subtypes

Cross-binding ability of sera from Cap-3M2e VLPs-immunized mice or pigs to M2e peptides of various IAV strains was measured by ELISA. M2e peptides (1 μg/mL) were coated on 96-well microtiter plates as antigen (M2e peptides were coated directly to the plates). Plates were blocked by skim milk (5% in PBST), followed by an incubation with serially diluted sera samples. Then, the HRP-conjugated secondary antibodies were added. The reaction was developed using TMB. The level of sera M2e-specific IgG or IgG subtype titers was measured by antibody endpoint titer. The highest dilution which gives an OD450 value twice that of the naïve group at the same dilution was designated as the antibody endpoint titer.

Ethics Statement

All BALB/c mice received humane care in compliance with the animal welfare guidelines of the Institutional Animal Care and Use Committee (IACUC) under the approval of the Henan Academy of Agricultural Sciences (Approval number SYXK 2014–0007). All efforts were made to alleviate and minimize animal suffering.

Statistical Analysis

Statistical data analyses were performed via GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA). All data were expressed as means ± SEM. Comparisons among vaccinated groups were performed using one-way ANOVA analysis. Statistical significance was determined at $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).

Results

Characteristics Of Cap-nM2e VLPs

The Cap-nM2e was expressed in *E. coli* and purified using Ni_2^+-NTA column. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot showed that the Cap, Cap-M2e, Cap-2M2e and Cap-3M2e proteins were successfully expressed and purified (Figure 1C–F). The Cap-M2e, Cap-2M2e and Cap-3M2e proteins reacted with the 14C2 mAb and anti-PCV2 polyclonal antibody, indicating that the recombinant protein retained the reactogenicity of M2e and the Cap protein (Figure 1E and F). The Cap-4M2e and Cap-5M2e proteins were not expressed, probably due to the low isoelectric point of M2e, which inhibited expression. The endotoxin contents of these expressed proteins were less than 0.18 EU/mg.

As shown in Figure 2A, transmission electron microscopy (TEM) results indicated that Cap-M2e, Cap-2M2e and Cap-3M2e proteins could self-assemble into VLPs. Diameter distribution of these VLPs was measured by dynamic light scattering (DLS). The results showed that with the increase in the number of M2e, the diameter of these nanoparticles increased gradually, but there was no statistical difference (Figure 2B). However, the zeta potential of these VLPs continues to decrease due to the low isoelectric point of M2e (the isoelectric point of M2e is 4.18) (Figure 2C).

As shown in Figure 2D, M2e-specific antibody could not recognize Cap VLPs. Cap-3M2e VLPs had the highest binding capacity to M2e-specific antibodies, indicating that the M2e was on the outer surfaces and Cap-3M2e VLPs have the potential to induce high levels of M2e-specific antibodies (Figure 2D). The 9F4 and 6A5 are PCV2-specific mAbs,

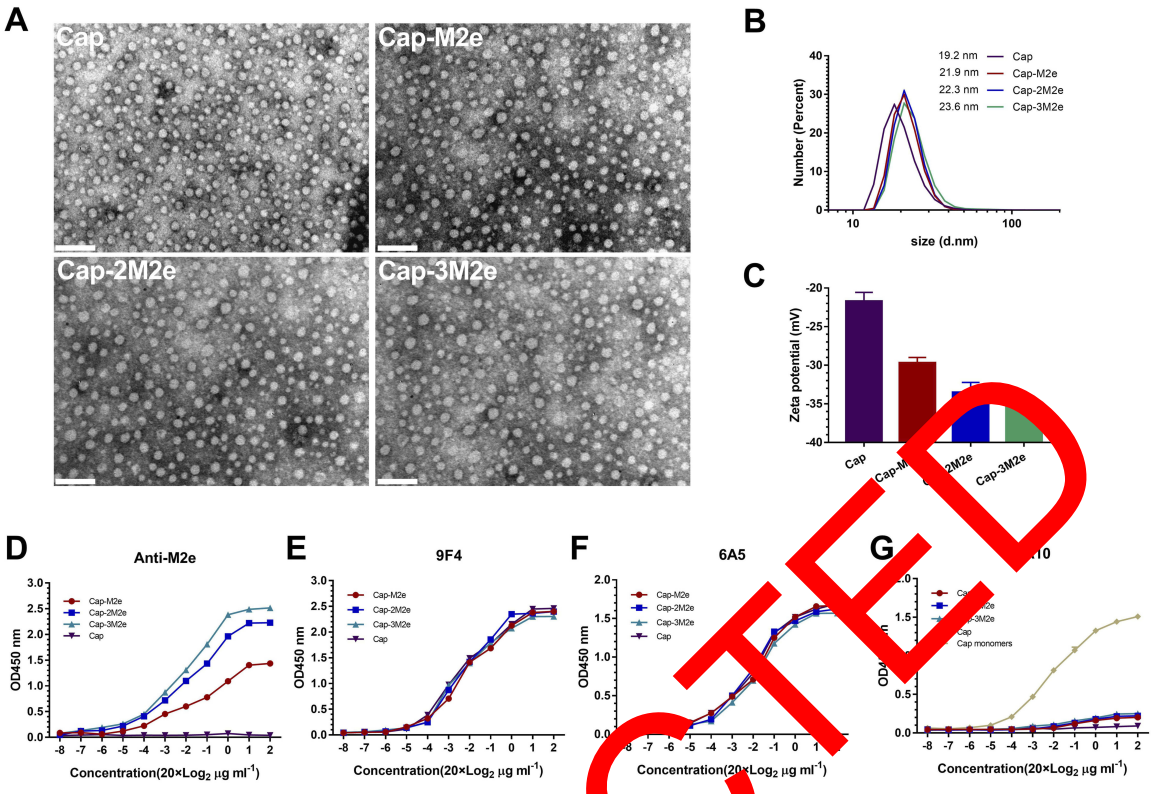

Figure 2 Characterization of Cap-nM2e VLPs.

Notes: (**A**) Transmission electron micrograph of Cap-nM2e VLPs. Scale bars = 100 nm. (**B**) ...namic light ...cattering (DLS) data showing size distribution of Cap-nM2e VLPs (n=5). (**C**) Zeta potential of Cap-nM2e VLPs (n=5). (**D**) Binding capacity ...pecific antibo... ...M2e on Cap-nM2e VLPs was measured by ELISA (n=3). (**E**) Binding capacity of 9F4 mAb to neutralizing epitope on the outer surface of Cap-n... VLPs ...easured by ELISA (n=3). (**F**) Binding capacity of 6A5 mAb to neutralizing epitope on the outer surface of Cap-nM2e VLPs was measured by ELISA (n=3). (**G**) ...inding ...apac... ...8A10 mAb to the decoy epitope Cap (169–180) of Cap-nM2e VLPs was measured by ELISA (n=3).

Abbreviations: Cap, Capsid; M2e, ectodomain of matrix ...tein ...VLPs, virus-like particles; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay.

which recognize neutralizing epitop... on t... ...ter surface of the Cap VLPs. These results in...ate that the ...-terminus of the Cap protein can i...ert ...ree copies ... of M2e without masking the n...tralizing ep...pe of Cap VLPs (Figure 2E and F). H...wever, 8A10 can recognize the decoy epitope Cap (169–...) of ...CV2, which was exposed in Cap protein mon...ers, b... masked ...when forming VLPs.[39] ELISA r...ults sh...ved tha... ...nsertion of three M2e peptides ... not ... the Cap protein to form VLPs (Figure 2C...n general, PCV2 neutralizing epitopes and three copies o...M2e were exposed on the surface of VLPs. The Cap-nM2e VLP has the potential to simultaneously induce antibodies against PCV2 and M2e.

Humoral Immune Effects Of Cap-nM2e VLPs

As shown in Figure 3A, mice were immunized 3 times and sera were collected at 63 dpi. All Cap-nM2e VLP-immunized mice were vaccinated with equal amounts of Cap VLPs. As shown in Figure 3B, Cap-3M2e VLPs induced significantly the highest titers of M2e-specific IgG in all groups than that of the M2e peptides. The highest dilution of M2e-specific IgG in the Cap-3M2e VLP group can reach to 100×2^{15}. The M2e-specific IgG level of the Cap-3Me VLPs group was 34 times that of the Cap-Me VLPs group. The M2e-specific IgG1 level of the Cap-3Me VLPs group was 29 times that of the Cap-Me VLPs group, and the IgG2a level was 83 times (Figure 3C and D). The ratio of IgG1/IgG2a of Cap-3Me VLPs group was lower than Cap-Me VLPs and Cap-2Me VLPs groups. These results showed that M2e-specific antibodies induced by Cap-3M2e VLPs were more biased toward Th1-type immune response than other groups (Figure S1A, Supporting Information). Four IAV strains which were used to challenge mice in this study were utilized to infect MDCK cells for the detection of the binding capacity of M2e-specific antibodies in sera to native M2e from different strains. The immune sera reacted similarly to the native M2e and M2e peptide, suggesting that Cap-nM2e VLPs-induced

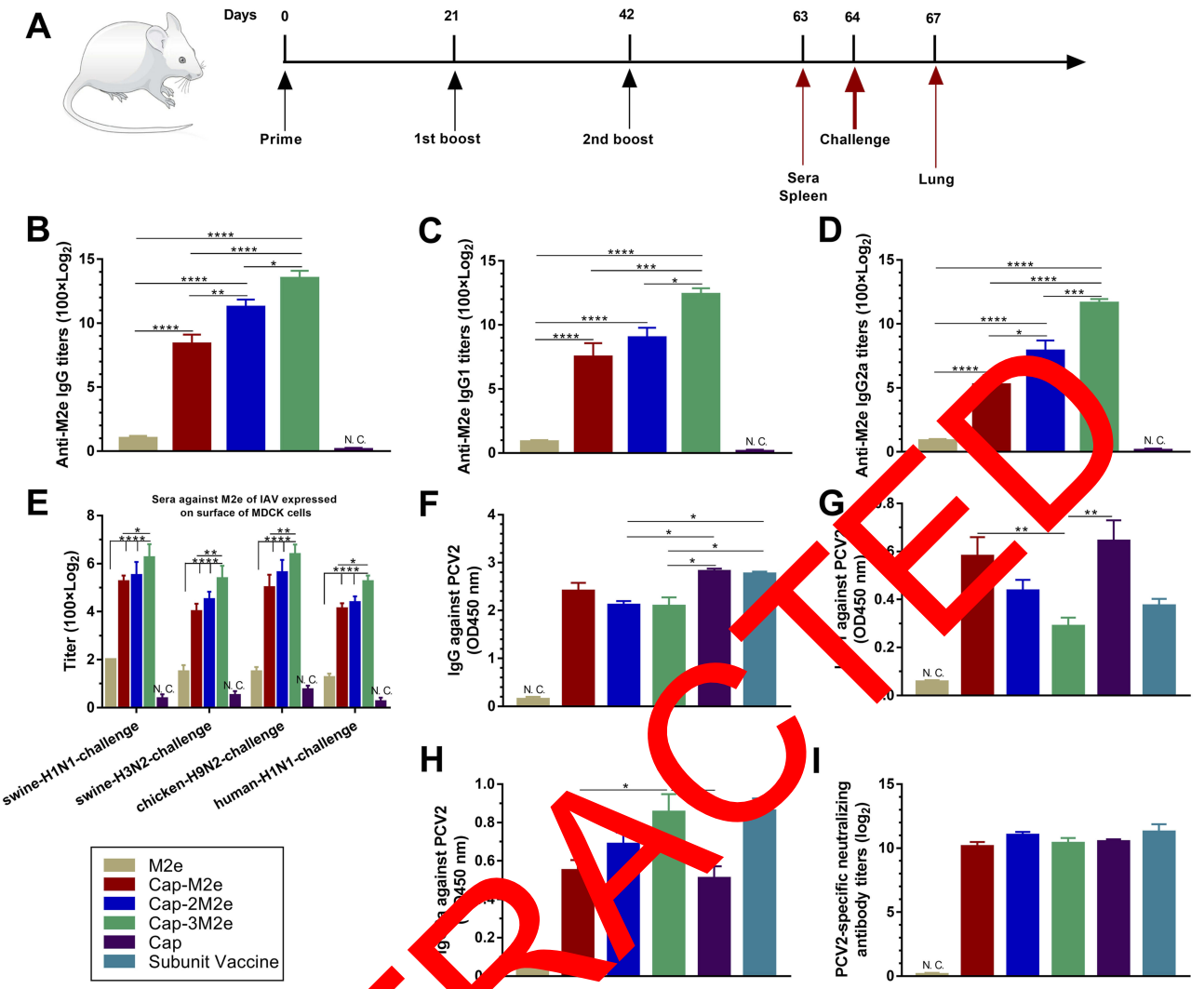

Figure 3 Humoral immune effects of Cap-nM2
Notes: (**A**) Scheme of immunization, virus in tion a mpling. (**B**) M2 pecific IgG level (n=6). (**C**) M2e-specific IgG1 level. (**D**) M2e-specific IgG2a level (n=6). (**E**) Sera IgG to native M2e which expressed on IAV-infected MDC lls (n=6). (**F**) PCV2-specific IgG level (n=6). (**G**) PCV2-specific IgG1 level (n=6). (**H**) PCV2-specific IgG2a level (n=6). (**I**) PCV2-specific neutralizing a odies' level (n=6). 0.05 (*), p < 0.01 (**), p < 0.001 (***), p < 0.0001 (****).
Abbreviations: Cap, Capsid; M2 ectodomain of matrix p ein 2; VLPs, virus-like particles; IAV, influenza A viruses; PCV2, porcine circovirus type 2; MDCK, Madin–Darby canine kidney; N.C ot compa

antibodies can ecogn e conf onal epitopes on native M2e from fferent ins (Figure 3E). These results indicate that the d y of M2e on the surface of Cap VLPs increases the im nogenicity of M2e, while multiple copies of the tandem display of M2e can further increase the level of induced antibodies.

As shown in Figure 3F, Cap-2M2e VLPs- and Cap-3M2e VLPs-induced PCV2-specific antibodies were lower than Cap VLPs and commercial subunit vaccine groups. But there was no significant difference between these groups in PCV2-specific neutralizing antibodies (Figure 3I). This may be because Cap-2M2e VLPs and Cap-3M2e VLPs induced a higher level of PCV2-specific IgG2a, which is more potent in antiviral immunity than IgG1 (Figure 3G and H and Figure S1B).[41] In summary, these Cap-nM2e VLPs induced high levels of M2e-specific antibodies and PCV2-specific neutralizing antibodies simultaneously. The Cap-3M2e VLPs have the most potential to become bivalent vaccines for IAV and PCV2 than Cap-M2e VLPs and Cap-2M2e VLPs.

Lymphocyte Proliferation Assay And Cytokines

As shown in Figure 4A, under the stimulation of M2e, the stimulation index (SI) in the Cap-3M2e VLPs group was

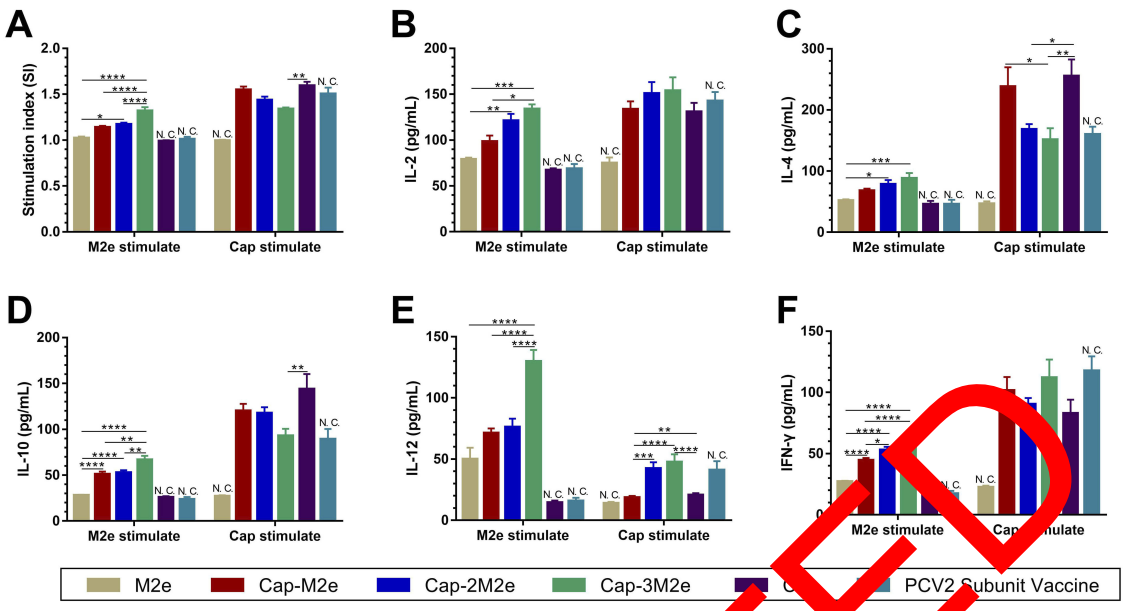

Figure 4 Lymphocyte proliferation assay and cytokines.
Notes: (**A**) Lymphocyte proliferation assay (n=9). (**B**) IL-2, (**C**) IL-4, (**D**) IL-10, (**E**) IL-12 and (**F**) IFN-γ levels in the supernatants of stimulated lymphocytes by M2e or Cap VLPs (n=9). $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).
Abbreviations: Cap, Capsid; M2e, ectodomain of matrix protein 2; VLPs, virus-like particles; IL-2/4/10/12, interleukin-2/4/10/12; IFN-γ, interferon gamma; N.C. not compared.

significantly higher than SI in the other groups. However, the SI in the Cap-3M2e VLPs group was significantly lower than SI in Cap VLPs group when Cap VLPs stimulated. This is caused by Cap-3M2e VLPs that displayed more M2e, while some epitopes on Cap VLPs were masked by 3M2e. IL-4 and IL-10 are Th2-biased cytokines that promote the production of IgG1.[42,43] Cap-3M2e VLPs induced the highest levels of IL-2 and IL-10 when M2e stimulated, but the lowest levels of IL-4 and IL-10 when Cap VLPs stimulated (Figure 4C and D). Correspondingly, the Cap-3M2e VLPs induced the highest M2e-specific IgG1 and the lowest PCV2-specific IgG1 (Figure 3C and G). IL-2, IL-12 and interferon-gamma (IFN-γ) are Th1-biased cytokines that stimulate the production of IgG2a.[42,43] The Cap-3M2e VLPs group induced the highest levels of IL-2, IL-12 and IFN-γ (Figure 4B, E and 4F). Correspondingly, M2e- and PCV2-specific IgG2a in the Cap-3M2e VLPs group was greater than other groups (Figure 3D and H). These results suggest that Cap-3M2e VLPs elicit a balanced Th1/Th2 immune response than Cap-M2e VLPs and Cap-2M2e VLPs groups.

Protective Efficacy Of Cap-nM2e VLPs Against SIV

Mice were challenged with 5 × LD_{50} of A/swine/Zhucheng/90/2014 (H1N1) or A/swine/Henan/1/2010 (H3N2) virus to evaluate the protective efficacy of Cap-nM2e VLPs to different subtype SIV, at 64 dpi. Four mice from each group were sacrificed at 3 dpc, at which time the virus titers were highest in the lungs. As shown in Figure 5, all mice immunized with M2e alone or Cap VLPs died by 5 to 6 dpc (Figure 5B and E) with the highest virus titers in the lungs (Figure 5C and F) and over 25% body weight loss (Figure 5A and D). The protection efficacy of Cap-3M2e VLPs was the highest among all groups and conferred complete protection against H1N1 and H3N2 SIV. The virus titers in the lungs of the Cap-3M2e VLPs group were much lower than that of the Cap VLPs group (18 times lower for H1N1 SIV and 42 times lower for H3N2 SIV). In general, Cap-3M2e VLPs have the best immune protection.

Protective Efficacy Of Cap-3M2e VLPs Against Human And Avian IAV

Mice were challenged with 5 × LD_{50} of A/Puerto Rico/8/1934 (H1N1) or 20 μL 10^9 TCID50/mL of A/chicken/Guangzhou/GZ/2005 (H9N2) to evaluate the protective efficacy of Cap-3M2e VLPs to human and avian IAV, at 64 dpi. Four mice from each group were sacrificed at 4 dpc, at which time the virus titers were highest in the lungs. As shown in Figure 6, all mice immunized with M2e alone died by 4 to 6 dpc (Figure 6B and E) with higher virus titers in the lungs (Figure 6C and F) and over 25% body weight loss (Figure 6A and D).

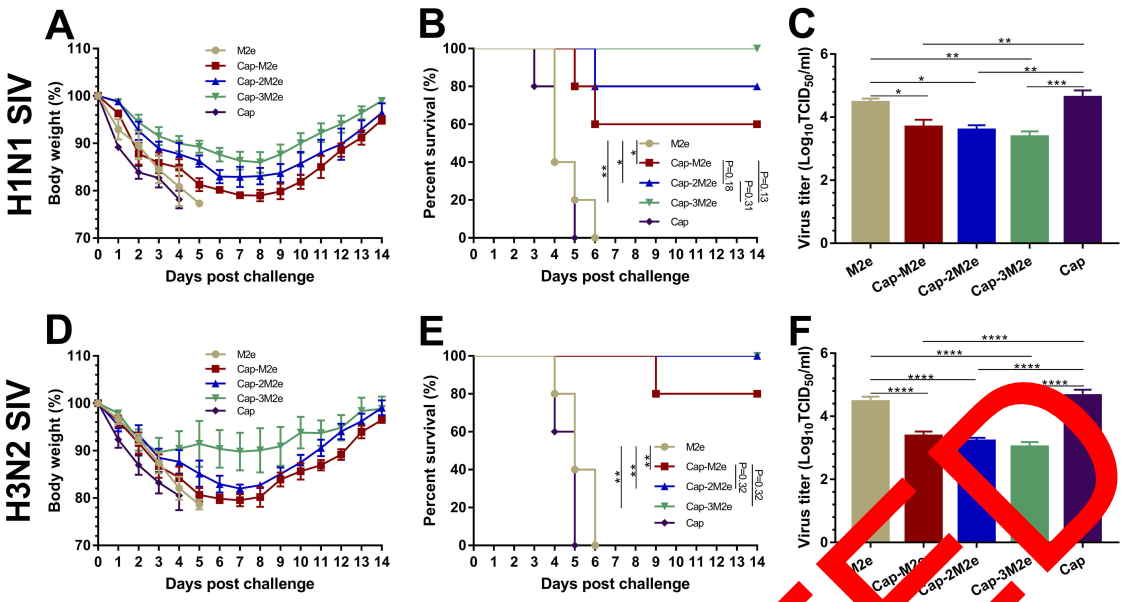

Figure 5 Protective efficacy of Cap-nM2e VLPs against SIV.

Notes: Immunized mice were challenged with 5 × LD_{50} of SIV. Body weight and survival rate changes were monitored daily for 14 days. Virus titer in lungs were determined via $TCID_{50}$ assay at day 3 post-challenge. (**A**) Body weight changes (n=5), (**B**) survival rate (n=5) and (**C**) lung virus titers (n=4) post A/swine/Zhucheng/90/2014 (H1N1) challenge. (**D**) Body weight changes (n=5), (**E**) survival rate (n=5) and (**F**) lung virus titers (n=4) post A/swine/Henan/1/20 (H3N2) challenge. $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).

Abbreviations: Cap, Capsid; M2e, ectodomain of matrix protein 2; VLPs, virus-like particles; SIV, swine influenza viruses; LD_{50}, lethal dose 50%; $TCID_{50}$, tissue culture infective dose 50%.

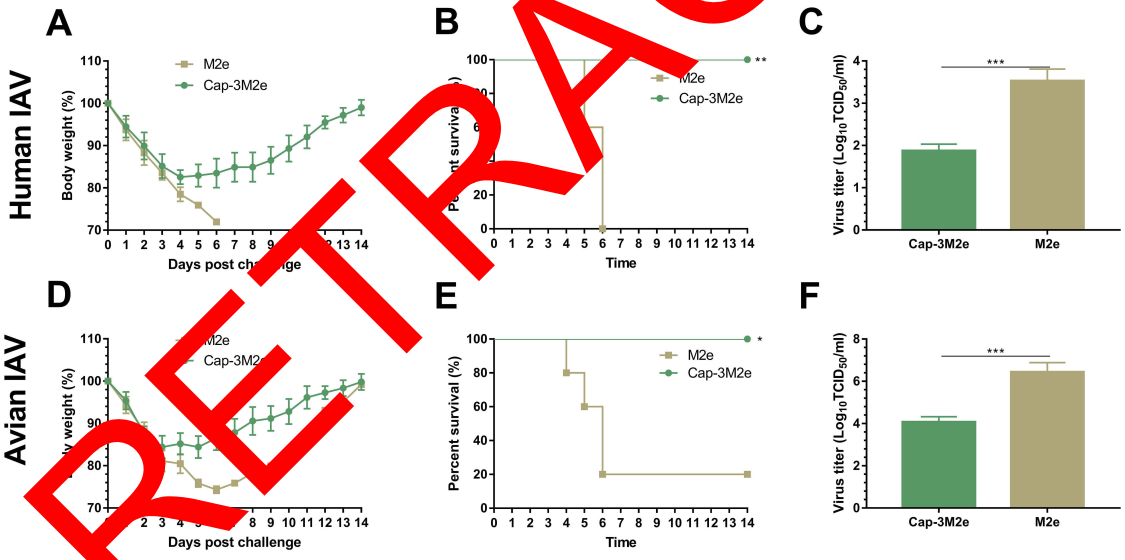

Figure 6 Protective efficacy of Cap-3M2e VLPs against human and avian IAV.

Notes: Immunized mice were challenged with human or avian IAV. Body weight and survival rate changes were monitored daily for 14 days. Virus titer in lungs was determined via TCID50 assay at day 4 post-challenge. (**A**) Body weight changes (n=5), (**B**) survival rate (n=5) and (**C**) lung virus titers (n=4) post 5 × LD_{50} of A/Puerto Rico/8/1934 (H1N1) challenge. (**D**) Body weight changes (n=5), (**E**) survival rate (n=5) and (**F**) lung virus titers (n=4) post 20 μL 10^9 TCID50 mL^{-1} of A/chicken/Guangzhou/GZ/2005 (H9N2). $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

Abbreviations: Cap, Capsid; M2e, ectodomain of matrix protein 2; VLPs, virus-like particles; IAV, influenza A viruses; LD_{50}, lethal dose 50%; TCID50, tissue culture infective dose 50%.

Passive immunization experiment results displayed that Cap-3M2e VLPs-immunized mice sera can provide protection against human and avian IAV (Figure S2). These results further suggest that Cap-3M2e VLPs can induce antibody responses and provide protective immunity to IAV from different origins.

Role Of M2e-Specific Antibody Subtype In Immune Protection

As shown in Figure 3B and D, the IgG2a/IgG ratio of Cap-3Me VLPs group was 2.44 times that of the Cap-Me VLPs group. In order to verify whether M2e-specific IgG2a exerts a better antiviral effect than IgG1, we diluted the Cap-3M2e VLPs-immunized sera 34 fold, which is the same level as the antibody induced by Cap-M2e VLPs. IgG2a in diluted Cap-3M2e VLPs-immunized sera is 2.4 times that of Cap-M2e VLPs-immunized sera. Then, we transferred these sera to naïve mice intraperitoneally. After challenging, the survival rate in diluted Cap-3M2e VLPs-immunized sera group was higher (Figure 7C). In addition, weight loss in diluted Cap-3M2e VLPs-immunized sera group was lower (Figure 7B). These results indicate that M2e-specific IgG2a plays a central role in the anti-IAV process.

Sera Cross-Binding Capability Of M2e Of Various IAV Strains

To investigate whether Cap-3M2e VLPs have the potential ability to confer protection against IAV of different subtypes, we investigated the cross-binding activity of Cap-3M2e VLPs-immunized sera to M2e of different subtypes of IAV (Table 1). ELISA results showed that Cap-3M2e VLPs immune sera can bind to different M2es of different subtypes (from H1 to H11) of IAV including pandemic influenza, indicating that Cap-3M2e VLPs have the potential ability to provide cross-protection against diverse subtypes of IAV strains and can be a promising universal influenza vaccine (Figure 8).

Antibody Response In Pigs

To verify the reactivity of the Cap-3M2e VLPs in pigs, Cap-3M2e VLPs were injected into pigs to detect the antibody response. The commercial PCV2 subunit vaccine as a positive control. The nanovaccine induced high-level M2e-specific antibodies (Figure 9B). The binding capacity of swine sera to M2e of different IAV strains is similar to that of mice sera. The swine sera can react well with native M2e of several IAV strains that included the 2009 pandemic H1N1 IAV (Figure 9C). What is more, the nanovaccine induced high-level PCV2-specific antibodies and neutralizing antibodies which are similar to the commercial subunit vaccine (Figure 9D and E). In addition, swine sera can react well with M2e of various IAV strains, which are similar to mice sera.

Discussion

In this study, we designed and constructed a bivalent VLPs-based nanovaccine which confers dual protection

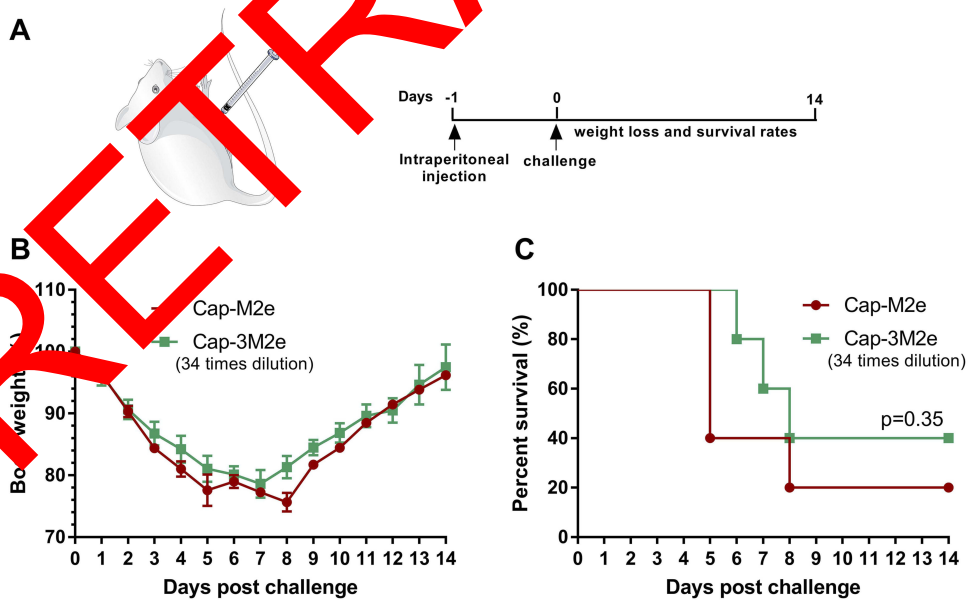

Figure 7 Role of M2e-specific IgG2a antibody in immune protection.

Notes: A total of 200 μL of pooled sera from Cap-M2e VLPs-immunized mice or 34-time diluted of pooled sera from Cap-3M2e VLPs-immunized mice were transferred to naïve mice via intraperitoneal injection (the IgG levels were the same in both groups, but the IgG2a in the Cap-3M2e VLPs group was 2.4 times that of the Cap-M2e VLPs group). Twenty-four hours post-transfer, mice were challenged with 2 × LD_{50} of A/Puerto Rico/8/1934 (H1N1). Mice body weight and survival rate changes were monitored daily for 14 days. (**A**) Scheme of immunization and challenge. (**B**) Body weight changes (n=5) and (**C**) survival rate (n=5).

Abbreviations: Cap, Capsid; M2e, ectodomain of matrix protein 2; VLPs, virus-like particles; IAV, influenza A viruses; LD_{50}, lethal dose 50%.

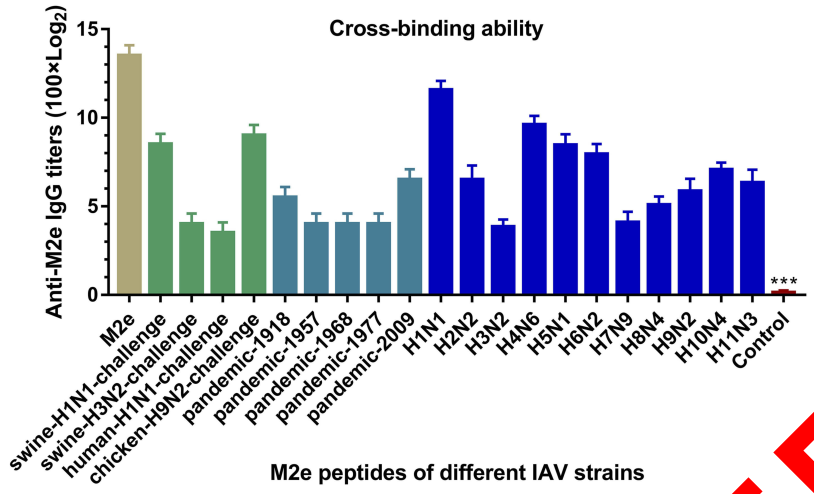

Figure 8 Cross-binding ability of Cap-3M2e VLPs-immunized sera.
Notes: Cross-binding tests of sera from Cap-3M2e VLPs-immunized mice to M2e peptides of IAV of different subtypes (n=6). $p < 0.$ (***).
Abbreviations: Cap, Capsid; M2e, ectodomain of matrix protein 2; VLPs, virus-like particles; IAV, influenza A viruses.

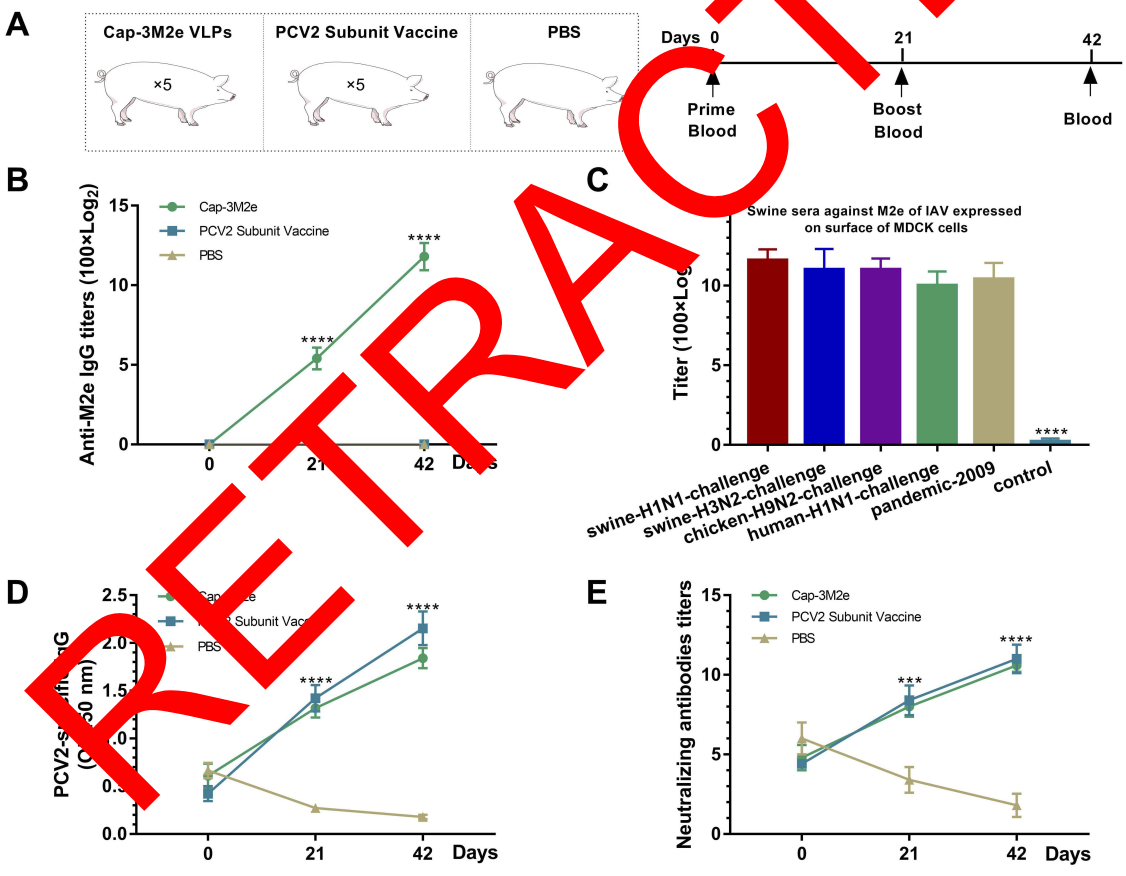

Figure 9 Reactivity of the Cap-3M2e VLPs in pigs.
Notes: Cap-3M2e VLPs nanovaccine was injected into pigs twice to detect the antibody response. (**A**) Scheme of immunization. (**B**) Anti-M2e IgG levels in swine sera (n = 5). (**C**) Anti-native M2e IgG levels in swine sera. MDCK cells were infected with IAV (n = 5). (**D**) Anti-PCV2 IgG levels in swine sera, sera samples were diluted 1:10000 (n = 5). (**E**) PCV2-specific neutralizing antibodies' levels in swine sera (n = 5). These figures show the smallest difference between the PBS group and other two groups. $p < 0.001$ (***), $p < 0.0001$ (****).
Abbreviations: Cap, Capsid; M2e, ectodomain of matrix protein 2; VLPs, virus-like particles; IAV, influenza A viruses; PCV2, porcine circovirus type 2; MDCK, Madin–Darby canine kidney.

against IAV and PCV2. High variability is the most prominent feature of IAV. M2e is the most conserved protective antigen of all subtypes of influenza viruses, and so it is a very promising candidate for universal influenza vaccine. However, there are still some differences in the M2e sequence between different strains, which may affect the protective effect of the nanovaccine.[23] The crystal structure of M2e shows that the first nine amino acid residues, and the 15th tryptophan is essential for the induction of protective antibodies.[44,45] As shown in Figure 5, the protection efficiency of the Cap-nM2e VLPs against A/swine/Henan/1/2010 (H3N2) strain is higher than that of A/swine/Zhucheng/90/2014 (H1N1), because the M2e sequence of H3N2 strain is closer to the expressed M2e sequence (Table 1). The Cap-3M2e VLPs induced the highest levels of M2e-specific immune responses in all Cap-nM2e VLPs and completely prevented the prevention of lethal infection of the H1N1 and H3N2 SIV. The Cap-3M2e VLPs-immunized sera can protect naïve mice against human and avian IAV, suggesting that the nanovaccine has the potential to provide protection against IAV from different species. It is worth noting that the M2e sequence of the A/swine/Henan/1/2010 (H3N2) strain is consistent with the M2e sequence of the 1957, 19[illegible] and 1977 pandemic influenza viruses, and so we speculate that the nanovaccine can withstand the pandemic IAV attack (Table 1).

The precise mechanism by which M2 antibodies provide protection is controversial.[46] Gerlei[illegible] et al demonstrated that natural killer cells play an important role in M2e antibody-mediated protection through FcγRIII-mediated antibody-dependent cellular cytotoxicity (ADCC).[47] However, other studies do not support this view. These studies demonstrate that alveolar macrophages, which express and activate FcγRs, are considered to play the key role in M2-specific antibodies-dependent elimination of influenza A virus-infected cells.[48,49] Moreover, Van den Hoecke Silvie et al revealed that by interacting with multiple activating FcgRs, mouse M2e-specific IgG2a are far more efficient in controlling IAV.[50] In this research, we adjusted the IgG in Cap-3M2e VLPs-immunized sera and Cap-M2e VLPs-immunized sera to the same level, and then intraperitoneally injected into naïve mice, and found that the diluted Cap-3M2e VLPs-immunized sera group had better protection. This result supports the conclusion that M2e-specific IgG2a has a stronger protective effect than IgG1.[50] However, there was only 2.4-fold difference in IgG2a levels between the two groups, and there was no significant difference between the protective effects. In addition, after the immune sera were diluted, the contents of other components in the serum (such as complement and IgG1) changed. Whether these ingredients play a protective role remains unclear, which is a limitation of the experiment. Previous researchers have found that IgG2a plays an important protective role through gene knockout mouse models.[50] However, gene knockout cause mice immunodeficiency and can interfere with experimental results. Overall, the protective mechanisms of M2e-specific antibodies does not solely depend on the level of IgG2a, and more complex protection mechanisms need further study.

VLPs are probably the most precisely defined nanometer-sized protein cage architectures that can be formed by self-assembly and serve as effective stand-alone vaccines.[29,51] Previous studies have demonstrated protection against IAV challenge in animal models using different sites of different VLPs to display M2e, like hepatitis B virus core, human parainfluenzavirus, norovirus and rabbit hemorrhagic disease virus.[24,52–56] In this study, we selected the VLPs of PCV2 to display M2e because PCV2 is often co-infected with SIV and aggravate SIV-related clinical disease.[32] However, peptide insertions may affect the ability of recombinant Cap protein to assemble into VLPs.[57] The N-terminal of the Cap protein is located inside the VLPs and cannot effectively induce antibodies.[58] It is unsuitable for insertion of M2e. The C-terminal of the Cap protein is far from the 2-, 3-, and 5-fold axis of the VLPs and does not participate in the self-assembly of the Cap protein (Figure S3).[36,59,60] More importantly, the C-terminal of the Cap protein is present on the surface of VLPs and can induce PCV2-specific antibodies.[38] Therefore, the C-terminal of the Cap protein was utilized to display M2e. Although several prominent loops in the Cap protein have the potential to allow insertion or substitution of small fragments of foreign peptides, these positions are key neutralizing epitopes of PCV2 and may hinder the formation of VLPs. Insertion of M2e may reduce the level of neutralizing antibodies against PCV2 and interfere with self-assembly.[61] Loop CD may be the most promising insertion site without destroying neutralizing epitopes and the self-assembly capability of the Cap protein (Figure S4). However, current research suggests that it may allow a maximum of 18 amino acid residues to be inserted. Therefore, the loop CD is not suitable for display M2e.[57] Previous studies have shown that high-density display of M2e contributes to the improvement of M2e-specific antibody levels.[62,63] Therefore, M2e-specific antibody levels can

be elevated by tandem expression of multiple copies of M2e at the C-terminal of Cap. However, excessive M2e runs the risk of masking neutralizing epitopes of PCV2. In the research, 3M2e may mask a portion of the epitopes of PCV2, resulting in a decrease in the level of PCV2-specific antibodies induced by Cap-3M2e VLPs (Figure 3F). However, Cap-3M2e VLPs induced a higher level of PCV2-specific IgG2a, and thus the level of neutralizing antibodies induced by Cap-3M2e VLPs did not decrease (Figure 2H and I). The present study demonstrated that Cap is capable of carrying at least 81 amino acid residues at its C-terminal without impairing VLP formation, and without masking neutralizing epitope of PCV2, making it a potential carrier for bivalent nanovaccines.

The bivalent nanovaccine obtained from the *E. coli* expression system can be used as an effective universal influenza vaccine while providing efficient protection against PCV2. In addition, since almost no M2e-specific antibodies can be detected after influenza infection, the nanovaccine can be used as a M2e-labeled PCV2 vaccine to serologically distinguish the natural infection of PCV2, which is very beneficial for the promotion of the nanovaccine. In addition, it is very meaningful to prevent and control these two related respiratory diseases. It will save a lot of manpower and resources. A previous study showed that Cap VLPs could protect pigs against PCV2 and the protect efficiency is consistent with commercial vaccines.[64] Present knowledge of the adaptive immune response against PCV2 infection suggests that neutralizing antibodies play a central role in antiviral responses, while cell-mediated responses play a supporting role.[65] Although PCV2 has multiple subtypes, current vaccines can provide protection against viruses of various subtypes.[65] In this research, Cap-3M2e VLPs can induce high levels of PCV2-specific neutralizing antibodies consistent with widely recognized commercialized PCV2 vaccine in mice and pig models. In the mice model, Cap-3M2e VLPs and commercialized PCV2 vaccine induce similar levels of Th1 (IL-2, IL-12 and IFN-γ) and Th2 (IL-4 and IL-10)-type immune-related cytokines, while PCV2-specific antibody subtypes were at the same level. Therefore, it is reasonable to believe that Cap-3M2e VLPs can provide protection against PCV2 in pigs. Although mice and pigs are suitable for IAV and PCV2 studies, it is hard to construct the co-infection model, and so we did not perform PCV2 challenge experiments in mice and pigs. In addition, previous studies show that M2e-based vaccines contributed to prevent SIV infection in pigs and can effectively reduce the shedding of SIV.[40,66] This Cap-3M2e VLPs nanovaccine induces higher levels of M2e-specific antibodies than previous studies, and so we speculate that the nanovaccine can defend against the challenge of SIV. However, it does not fully indicate that the Cap-3M2e VLPs nanovaccine can provide protection for both PCV2 and SIV in the pig model experiment. Therefore, recommendations for future studies include optimization of assembly efficiency of the nanovaccine and challenge evaluation in pig model.

Conclusion

In summary, we developed an economical bivalent nanovaccine, which can provide protection against IAV from different species and induce high levels of PCV2-specific neutralizing antibodies. Three tandem copies of M2e were inserted into the C-terminal of Cap of PCV2 without burying neutralizing epitopes and expressed in *E. coli* and then self-assembled to form the nanovaccine. Inoculation with Cap-3M2e VLPs nanovaccine induced robust M2e- and PCV2-specific immune responses and provided protection against swine, human and avian IAV. We conclude that Cap-3M2e VLPs nanovaccine has the potential to be used as a PCV2 vaccine and cross-protective influenza A virus vaccine.

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Disclosure

The authors report no conflicts of interest in this work.

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