Vaccine 37 (2019) 5051–5058

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Effective mosaic-based nanovaccines against avian influenza in poultry

Brock A. Kingstad-Bakke [a,b], Shaswath S. Chandrasekar [a], Yashdeep Phanse [b], Kathleen A. Ross [c], Masato Hatta [a], M. Suresh [a], Yoshihiro Kawaoka [a], Jorge E. Osorio [a], Balaji Narasimhan [c], Adel M. Talaat [a,b,*]

[a] Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin, Madison, WI, USA
[b] Pan Genome Systems, Madison, WI, USA
[c] Department of Chemical and Biological Engineering, Iowa State University, Ames, IA, USA

ARTICLE INFO

Article history:
Received 14 February 2019
Received in revised form 15 May 2019
Accepted 24 June 2019
Available online 9 July 2019

Keywords:
Nanoparticle vaccine
Modified vaccinia Ankara
Avian influenza
Vaccine vector

ABSTRACT

Avian influenza virus (AIV) is an extraordinarily diverse pathogen that causes significant morbidity in domesticated poultry populations and threatens human life with looming pandemic potential. Controlling avian influenza in susceptible populations requires highly effective, economical and broadly reactive vaccines. Several AIV vaccines have proven insufficient despite their wide use, and better technologies are needed to improve their immunogenicity and broaden effectiveness. Previously, we developed a "mosaic" H5 subtype hemagglutinin (HA) AIV vaccine and demonstrated its broad protection against diverse highly pathogenic H5N1 and seasonal H1N1 virus strains in mouse and non-human primate models. There is a significant interest in developing effective and safe vaccines against AIV that cannot contribute to the emergence of new strains of the virus once circulating in poultry. Here, we report on the development of an H5 mosaic (H5M) vaccine antigen formulated with polyanhydride nanoparticles (PAN) that provide sustained release of encapsulated antigens. H5M vaccine constructs were immunogenic whether delivered by the modified virus Ankara (MVA) strain or encapsulated within PAN. Both humoral and cellular immune responses were generated in both specific-pathogen free (SPF) and commercial chicks. Importantly, chicks vaccinated by H5M constructs were protected in terms of viral shedding from divergent challenge with a low pathogenicity avian influenza (LPAI) strain at 8 weeks post-vaccination. In addition, protective levels of humoral immunity were generated against highly pathogenic avian influenza (HPAI) of the similar H5N1 and genetically dissimilar H5N2 viruses. Overall, the developed platform technologies (MVA vector and PAN encapsulation) were safe and provided high levels of sustained protection against AIV in chickens. Such approaches could be used to design more efficacious vaccines against other important poultry infections.

1. Introduction

Influenza group A viruses infecting chickens belong to the family orthomyxoviridae [1] with 16 hemagglutinin (HA1-16) and 9 neuraminidase (NA1-9) glycoproteins that decorate the virion spherical surface. Infection with influenza group A viruses results in either mild respiratory distress (coughing, sneezing, ocular/nasal discharge) or a highly contagious respiratory and/or neurological disease accompanied with sudden death [2]. The former is caused by low pathogenic avian influenza (LPAI) while the latter is caused by highly pathogenic avian influenza (HPAI) which includes hemagglutinin serotype 5 (HA5) or HA7 [2,3]. The disease is prevalent worldwide and associated with severe economic losses among broilers (meat producing) and layers (egg producing). To date, sixteen subtypes of influenza virus are known to infect birds with all outbreaks of the highly pathogenic isolates belonging to influenza A viruses of subtypes H5 and H7. Recently, viruses of low pathogenicity were shown to mutate into highly pathogenic viruses after circulation in poultry population [4–6]. During a 1983–1984 epidemic in the US, the H5N2 virus initially caused low mortality, but within six months became highly pathogenic

Abbreviations: CEF, chicken embryo fibroblasts; MDCK, Madin-Darby canine kidney cells; DIVA, differentiation of infected from vaccinated animals; FPV, fowlpox virus; HA, hemagglutinin; H5M, hemagglutinin serotype H5 mosaic gene; PR8, influenza A virus strain A/PR/8/34 (H1N1); n, nanoparticle encapsulated; HPAI, highly pathogenic avian influenza; LPAI, low pathogenic avian influenza; MOI, multiplicity of infection; PFU, plaque-forming units; MVA, modified vaccinia Ankara; SPF, specific pathogen-free; SEL, synthetic early-late promoter.

* Corresponding author at: University of Wisconsin-Madison, Robert P Hanson Biomedical Science Laboratories, School of Veterinary Medicine, 1656 Linden Drive, Room 303, Madison, WI 53706, USA.
E-mail address: adel.talaat@wisc.edu (A.M. Talaat).

https://doi.org/10.1016/j.vaccine.2019.06.077
0264-410X/

resulting in 90% mortality in poultry farms [7]. Although there have been no reported cases of H5N1 virus in domesticated poultry in the US, the same serotype was responsible for several devastating outbreaks in poultry, worldwide [8]. Recently, the Eurasian origin H5N1 virus was recently found in a green-winged teal (bird type) in Washington state. In this report, we evaluated novel approaches to develop effective vaccine constructs against H5N1 serotypes of LPAI that could be also effective against infections with HPAI isolates.

In endemic regions, the main option for reducing the impact of influenza in poultry flocks is vaccination. Several approaches have been tested against AIV including inactivated whole virus, subunit hemagglutinin protein, and recombinant vector vaccines [9,10]. For inactivated vaccines, the production of HPAI, for certain strains, can be lethal to produce in eggs with a long lead time (up to 9 months), which is problematic for emergency vaccination during an outbreak [11–13]. Additionally, growth of wild type HPAI strains presents biosafety and biosecurity challenges. On the other hand, certain viral vectors such as Fowlpox and Newcastle Disease virus, while efficacious, fail to protect chickens pre-immunized with such vectors due to maternally derived antibodies (MDA) [14,15]. Other viral vectors such as turkey herpes virus (HVT), can safely stimulate strong antibody and T cell responses, even in the presence of MDA [16]. Interestingly, synthetic biodegradable polyanhydride nanoparticles (PANs) have been shown to provide controlled release of encapsulated antigens in several animal models (rodents, pigs, cattle, poultry) from a single vaccine dose [17–27], resulting in long-lived immunity, similar to modified live vaccines (MLV). Unfortunately, the potential rise of new virulent strains from genetic re-assortment, in addition to the potential reversion to wild type [28] heighten the concerns of the safety of MLV in poultry. Alternatively, PANs-based vaccines (nanovaccines) were found to be safe with no untoward impact on host tissues and overall health [29,30]. More importantly, these nanovaccines were highly protective against several pathogens [24], including AIV [22]. In addition, PAN-based vaccines induce the robust cellular and humoral immune responses to influenza, and are able to elicit coveted resident memory cell response when delivered by mucosal routes [23]. Similar to other nanovaccine technologies (PLGA, LNPs, PAMs), PAN-based vaccines are also potent but with a sustained antigen-release kinetics that can last several months [31–35].

Previously, mice and monkeys vaccinated with mosaic HA antigen were protected against multiple strains of HPAI [36–38]. In the present study, we evaluated both nanoparticle encapsulation and MVA in chickens to deliver a mosaic H5 antigen (H5M) comprised of >2000 H5N1 HA sequences from Genbank [36]. Our results indicated that H5M antigen was successfully delivered in chickens from both PAN adjuvanted or encoded through MVA vector, compared to unadjuvanted, inactivated vaccine. Vaccinated chickens were found to generate robust cellular and humoral responses against AIV. More importantly, protective levels of immunity were reached against LPAI when challenged 8 weeks post-vaccination, and HI titers from vaccinated chicks were able to neutralize several HPAI isolates.

2. Materials and methods

2.1. Animal immunization and challenge studies

For all animal studies, serum samples collected prior to vaccination were confirmed to be negative for H5 hemagglutinin inhibiting antibodies against inactivated PR8-H5M. The initial study aimed to examine the immunogenicity of vaccine constructs in chickens (immunogenicity study). In this study, 1 day old SPF chicks (Charles Rivers Laboratories) were vaccinated with nH5M (30 μg inactivated PR8-H5M encapsulated in 1 mg nanoparticles), 10^8 PFU MVA-H5M, or 10^8 PFU MVA-GFP (n = 14/group) administered one via the intramuscular (IM) route. Blood samples were collected from all chicks at 21 days post vaccination for HI titers (see below).

For the protective efficacy study, groups of Cornish rock cross chickens (n = 8/group) were obtained from a local farm at one day of age and vaccinated at two days of age with nH5M (20 μg inactivated PR8-H5M encapsulated in 2 mg nanoparticles), inac-H5M (20 μg inactivated PR8-H5M), 10^8 plaque forming units (PFU) MVA-H5M, or as a negative control 10^8 PFU MVA expressing green fluorescent protein, MVA-GFP, in 100 μL PBS by IM thigh muscle injection. Blood samples were collected prior to vaccination at day 1, and at 7, 10, 14 days, and 8 weeks post-vaccination. Chickens were challenged at eight weeks of age by choanal instillation of 10^4 50% tissue culture infectious dose ($TCID_{50}$). The isolate A/northern pintail/Alaska/622/2012 LPAI H5N2 was used for the challenge. Oral/choanal slit/tracheal and cloacal swabs were obtained at days 2, 4 and 8 using flocked nylon swabs collected in brain heart infusion broth (BHI). Also, chicks were euthanized at day 3 post challenge and lungs collected for virus quantification. Lungs were homogenized in MACS (Miltenyi Bio) tubes, and undiluted supernatants clarified and collected from cellular material by centrifugation at 3000 RCF for 15 min for viral titration. To determine viral titer, viral samples were used to infect MDCK cells grown to 90% confluency in 96-well plates. Cells were fixed 3 days later with 10% formalin/0.1% crystal violet (v/v) in PBS and viral titers were determined by observing overt cytopathic effects (CPE) with the aid of a light microscope and calculating the $TCID_{50}$ [39].

2.2. Viruses and cells

Chicken embryo fibroblasts (CEFs) and Madin-Darby canine kidney (MDCK) cells were obtained from Charles River Laboratories, Inc. (Wilmington, WA, USA) and American Type Culture Collection (ATCC; Manassas, VA, USA), respectively. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. The CEFs were used for propagating modified vaccinia Ankara (MVA). Recombinant H5N1 virus encoding the H5 mosaic protein [36,37] was constructed using reverse genetics as described previously [40]. Recombinant H5N1 virus containing the H5 mosaic HA protein [1,2] was generated using reverse genetics as described previously [3]. First, the HA gene encoding H5M nucleic acid sequences [1] was generated by introducing mutations into the HA gene of A/chicken/Vietnam/NCVD5/2003 (H5N1) virus [4] with synthesized nucleotide fragment and mutation primers (IDT, Coralville, IA, USA) by PCR. Additionally, the multi basic cleavage site (MBS, RERRRKKR) of the H5 mosaic HA protein was changed to code for the RETR apathogenic form. This modified DNA fragment was cloned into the reverse genetics plasmid pHH21 and used to generate a virus containing the H5 mosaic HA gene (PR8-H5M), with all other influenza genes from the A/Puerto Rico/8/34 (PR8; H1N1) virus. Generation, growth, and genetic characterization of seed PR8-H5M virus was conducted by the Kawaoka laboratory. All experimental studies with H5N1 viruses were conducted in biosafety level 3+ facilities in compliance with the University of Wisconsin-Madison Office of Biological Safety.

2.3. Inactivation and purification of PR8-H5M virus

Inactivated PR8-H5M virus antigen was prepared by infecting MDCK cells grown to 90% confluency at a multiplicity of infection of 0.01 in serum-free DMEM supplemented with 2 μg/mL TPCK treated with trypsin, 0.5% bovine serum albumin and 20 mM

HEPES (influenza growth media), and harvesting at 72 h post infection when full cytopathic effects (CPE) were observed. Infected cell culture flasks were frozen and thawed once to lyse cells, and virus containing supernatants were clarified and separated from cell debris by centrifugation at 3000 RCF. Virus containing supernatants were chemically inactivated by addition of formaldehyde to a final concentration of 0.2 g/L followed incubation at 4 °C for 7 days, after which chemical inactivation was verified by infection of MDCK cells in influenza growth media. Inactivated supernatants were layered onto a 20% sucrose cushion and ultra-centrifuged in a SW-28 rotor at 112,000 RCF for 3.5 h at 4 °C. Pellet and supernatant fractions at each step were saved for analysis by SDS-PAGE and Western blot. Post sucrose cushion pellets were resuspended in protein free DMEM. Total protein was quantified by Bradford assay.

2.4. Nanoparticle synthesis and characterization

Synthesis of polyanhydride (20:80 CPTEG:CPH) nanoparticles (NP) containing inactivated PR8-H5M antigen (nH5M) was conducted as described previously [22]. Briefly, 20:80 CPTEG:CPH polymer, composed of 1,8-bis(*p*-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) and 1,6-bis(*p*-carboxyphenoxy)hexane (CPH), was synthesized via melt polycondensation. Inactivated PR8-H5M was dialyzed to nanopure water and lyophilized overnight. Next, 20:80 CPTEG:CPH polymer containing 1 wt% PR8-H5M was dissolved 20 mg/mL in methylene chloride. The solution was sonicated to ensure even distribution of the antigen before precipitating into chilled pentane (1:250 methylene chloride:pentane). The resulting nanoparticles were collected via vacuum filtration. The final dose formula was 20 μg/2 mg of inactivated PR8-H5M to nanoparticle ratios. Antigen release kinetics were characterized by incubating the PAN particles in PBS and measuring protein released at regular intervals with a microBCA assay as described previously [41].

2.5. Electron microscopy

Transmission Electron Microscopy (TEM) of inactivated PR8-H5M samples were negatively stained for electron microscopy using the drop method. A drop of sample was placed on a PioloformTM (Ted Pella, Inc.) carbon-coated 300 Mesh Cu grid, allowed to adsorb for 30 s, and the excess removed with filter paper. Next, a drop of methylamine tungstate or uranyl acetate (Nano-W, Nanoprobes Inc.) was placed on the still wet grid, and the excess removed. The negatively stained sample was allowed to dry, and was documented in a Philips CM120 (Eindhoven, The Netherlands) transmission electron microscope at 80 kV. Images were obtained using a SIS MegaView III digital camera (Soft Imaging Systems, Lakewood, CO). For nanovaccine constructs, particle size and morphology were observed with scanning electron microscopy (FEI Quanta 250, FEI, Hillsboro, OR). The average diameter of PAN was quantified using ImageJ software [42].

2.6. Serology and cellular immunity tests

The hemagglutination (HA) titer for inactivated PR8-H5M and other LPAI/HPAI viruses used in this study was defined by serially diluting samples in quadruplicate wells in a 96-well plate, followed by addition of 0.5% washed chicken erythrocytes. The endpoint hemagglutination titer per volume is defined as the final dilution that results in complete agglutination of erythrocytes [43]. For the hemagglutination inhibition (HI) assay, four hemagglutinating units were added to serial dilutions of serum samples in an equal volume of PBS for a total volume of 50 μL. Serum-virus mixtures were incubated at 37 °C for 30 min. After which, 50 μL of a 0.5% suspension of chicken erythrocytes was added to the serum-virus mixture and incubated at 4 °C for 1 h. The endpoint hemagglutination inhibition (HAI) is defined as the final dilution that results in complete inhibition of agglutination of erythrocytes.

For a sub-group of vaccinated chicks (n = 4) from the immunogenicity study, single-cell suspensions of mononuclear cells from spleens were prepared using standard techniques and used for intracellular cytokine staining. Briefly, 10^6 cells per well were plated on flat-bottom tissue-culture-treated 96-well plates. For stimulation, total cells were infected at an MOI of 0.1 with A/northern pintail/Alaska/622/2012 LPAI H5N2, and antigen presentation was allowed to proceed for 8 h before the addition of brefeldin A (1 μL/mL, GolgiPlug, BD Biosciences) followed by 12 h of cytokine accumulation. After stimulation and prior to antibody staining, cells were stained for viability with eFluor® 780. Cells were stained for surface markers (CD4-AF488, CD8-PE, BIO-RAD), and then processed with Cytofix/Cytoperm kit (BD Biosciences, Franklin Lakes, NJ), followed by intracellular staining with rabbit anti-chicken interferon gamma (IFNγ) antibody (Rockland, Limerick, PA) and goat anti-rabbit IgG AF-647 (Thermo-Fisher). Permeabilized cells were transferred to FACS buffer for acquisition. All samples were acquired on an Attune NxT Flow Cytometer (Thermo-Fisher). Data were analyzed with FlowJo software ((BD Biosciences).

2.7. Western blotting

Viral fractions were boiled in Laemmli sample buffer (BioRad, Hercules, CA, USA) and resolved on a 4–20% SDS-PAGE gel (Biorad) by electrophoresis using a Mini-PROTEAN 3 system (BIO-RAD, CA). Polyacrylamide gels were electroblotted onto nitrocellulose membranes using a Turboblot® system. Membranes were blocked in 5% (W/V) skim milk and probed with polyclonal anti-influenza virus H5 HA antibody (BEI resources: NR-2706) and mouse anti-goat HRP-conjugated secondary antibody. Membranes were developed using a solid phase 3,3′,5,5′-tetramethylbenzidine (TMB) substrate system.

2.8. Statistical analysis

Statistical analyses were performed using GraphPad software (La Jolla, CA). Antibody and viral titers, and cellular immune responses were compared using an ordinary one-way ANOVA test with multiple comparisons where $p < 0.05$ = *, $p < 0.005$ = **, $p < 0.0005$ = *** were considered significantly different among groups.

3. Results

3.1. Synthesis and characterization of nanoparticle encapsulated PR8-H5M

The PR8-H5M virus grew productively in MDCK cell culture. After inactivation and purification, a yield of approximately of 1 mg purified, inactivated protein was obtained for each infected T-150 flask of MDCK cells (approx. 20–30 million cells per flask). Purification of virus by ultracentrifugation resulted in high recovery of virus as no HA antigen was detected in supernatants following purification, while the pellet fractions contained HA antigen (Fig. 1A, Western blot). Purified PR8-H5M exhibited multiple virion morphologies with clearly intact surface proteins (Fig. 1B). As these aggregates were too large for encapsulation (>100 nm) in nanoparticles, preparations were briefly (<3 s) sonicated in 1.5 mL microcentrifuge tubes using a bucket style sonicator. This sonication thoroughly disrupted large aggregates and virions, and generated small (<100 nm) particles that maintained envelope proteins (Fig. 1B). The morphology following nanoparticle encapsulation was consistent with prior reports [22]. The average particle diameter estimated by analysis of SEM images was 165 nm.

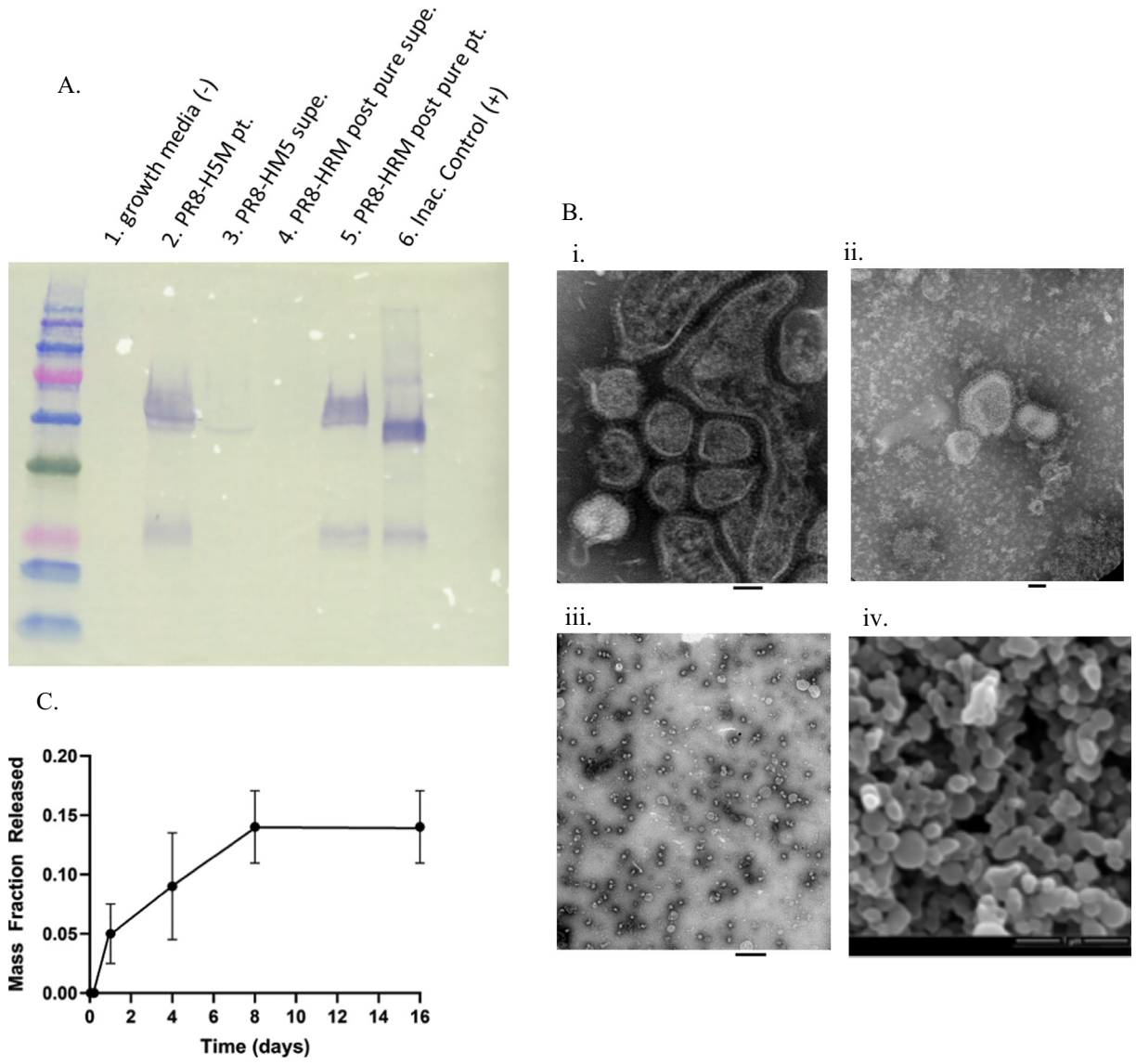

Fig. 1. Characterization of the nH5M (H5M nanoparticle) vaccine constructs. (A) Western blot analysis with anti H5N1 virus polyclonal anti H5 antibody of PR8-H5M samples during purification. Lanes are as follows. 1: MDCK virus growth media (negative control). 2: PR8-H5M post clarification pt. 3: PR8-H5M post clarification supernatant. 4: PR8-H5M post sucrose cushion purification supernatant. 5: PR8-H5M post sucrose cushion purification pellet (final PR8-H5M prep). 6: HA positive control of A/Vietnam/1203/04 stockpiled vaccine virus. (B) Electron micrographs of PR8-H5M virus. i: PR8-H5M virus after purification, TEM scale bar 50 nm. Ii–iii: PR8-H5M virus after sonication, TEM scale bar 50 nm (i, ii) and 800 nm (iii). iv: PR8-H5M after nano-encapsulation (nH5M) SEM scale bar 1 μm. (C) Release kinetics of nH5M particles were characterized by incubating the PAN particles in PBS and measuring protein released at regular intervals with a microBCA.

In vitro, release of antigen from nH5m particles was linear over the first 8 days of incubation, releasing ~15% total protein, then plateaued up to 16 days (Fig. 1C).

3.2. Generation of protective antibodies against multiple strains of LPAI and HPAI viruses

To determine if nanoparticle and MVA vectored vaccines were immunogenic, SPF chicks were vaccinated with nH5M and MVA-H5M vaccines at one day of age. Immunogenicity and protection were compared to unadjuvanted vaccine as to observe any increase in immunity as due to PAN adjuvanticity. All chicks had detectable HAI antibodies against the PR8-H5M virus at 21 days after vaccination (Fig. 2A). In the second study of the commercially sourced chicks, the vaccinated chicks developed detectable HAI titers by D7 post vaccination (Fig. 2B). At all time points, nanoparticle encapsulation increased HAI titers relative to non-encapsulated antigen. By day 14 all chicks in all groups had detectable HAI titers with geometric mean (GMT) endpoint titers as follows: MVA-GFP <= 10, inac-H5M = 15, nano-H5M = 21.5, MVA-H5M = 132.5. These results demonstrate that H5M vaccines can rapidly elicit antibody responses in chickens with a single immunization.

To determine the breadth of the antibody response elicited by the used vaccine constructs, pooled serum samples from vaccinated chickens were tested against multiple clades of H5N1 HPAI virus, as well as two recent LPAI H5N2 viruses. Both nH5M and MVA-H5M elicited HAI antibodies against all strains of H5 viruses tested (Table 1), indicating the breadth of the antibody responses induced by the H5M-based vaccines.

3.3. Protective efficacy of mosaic HA antigen delivered by MVA or polyanhydrides

To evaluate cross protection against a contemporary influenza threat during the lifespan of a broiler, chickens were challenged with LPAI H5N2 (A/northern pintail/Alaska/622/2012 virus) at

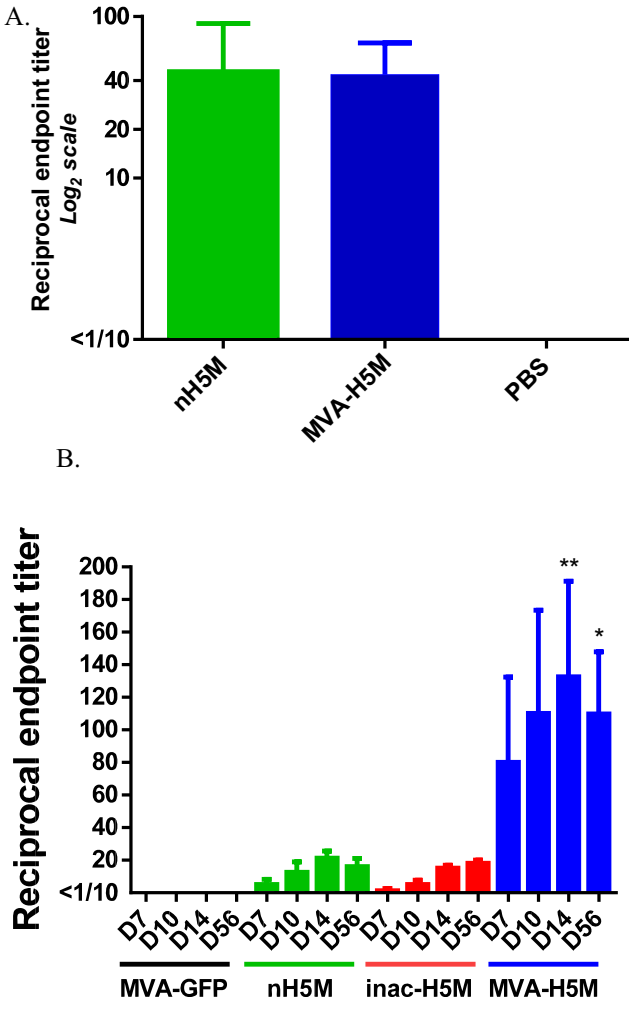

Fig. 2. Humoral immune responses induced by H5M-based vaccine constructs. One day old chicks were immunized with different vaccine constructs. Blood samples were collected at different times post immunization for hemagglutination inhibition assay (HAI). (A) The geometric mean titers (±SD) of HAI antibodies against parent PR8-H5M at 3 weeks after vaccination for the immunogenicity study. (B) The geometric mean titers (±SD) of HAI antibodies against parent PR8-H5M virus after vaccination for the efficacy study.

Table 1
HAI titers against distant AIV isolates in pooled serum samples isolated from chikens vaccinated with MVA-H5M or nH5M.

AIV strain	MVA-H5M	nH5M
A/Vietnam/1203/04 H5N1 HPAI	1:80	1:80
A/Indonesia/5/2005 H5N1 HPAI	1:40	1:80
A/Hong Kong/482/97 H5N1 HPAI	1:40	1:40
A/Chicken/Qalubia-Egypt/1/08 H5N1 HPAI	1:10	1:20
A/northern pintail/Alaska/622/2012 H5N2 LPAI	1:20	1:20
A/American green-winged teal/Alaska/472/2014 H5N2 LPAI	1:10	1:20

8 weeks of age. At this age, all immunized chickens had variable levels of detectable HAI antibodies (Fig. 2). After challenge no major morbidity in terms of body weight changes were observed (Fig. 3A), however significant levels of viral shedding in negative control and inactivated vaccines (Fig. 3B) were detected in swab choanal and cloacal swab samples. Nanoparticles encapsulating the same dose of inactivated antigen reduced shedding levels and duration (Fig. 3B). No shedding was observed in MVA-H5M vaccinated chicks. While viral loads in lungs were highest in PBS control and inactivated chicks, reduced loads were observed in nH5M and MVA-H5M vaccinated chicks (Fig. 3C). These results demonstrate that both nH5M and MVA-H5m vaccines were highly immunogenic and provided protection to a divergent influenza strain.

3.4. Mosaic vaccine constructs elicit significant cellular immune responses

Because chicks vaccinated with nH5M had relatively low HAI titers against homologous virus, we hypothesized that the protection against viral shedding was augmented by cellular immunity since PAN-based vaccines have been shown to promote $CD8^+$ T cell responses in contrast to inactivated vaccines [23]. The numbers of IFNγ-producing influenza-specific $CD4^+/CD8^+$ T cells in spleen after vaccination were quantified by intracellular cytokine staining (ICCS). The percentage of influenza-specific IFNγ producing $CD8^+$ T cells was significantly higher in both MVA-H5M ($p < 0.05$) and nH5M ($p < 0.05$) than cells in spleens of chickens from the PBS group (Fig. 4A). Interestingly, cellular responses from PAN vaccination seemed to be skewed towards $CD8^+$ T cell responses, and in contrast, the MVA-H5M elicited IFNγ secreting $CD4^+$ T cells with a frequency significantly higher ($p = 0.05$) than PBS control chicks (Fig. 4B). Representative dot plots for ICCS are shown (Supplemental Fig. 1).

4. Discussion

LPAI viruses continuously circulate within and between wild bird and poultry populations and have a high degree of recombination and genetic diversity [44–47]. While these viruses typically cause low morbidity and mortality in domesticated poultry, there is significant evidence that LPAI viruses contribute to HPAI genetic diversity through gene swapping, and can even gain virulence and spontaneously give rise to HPAI strains directly [4,48–50]. It is encouraging that these H5M vaccines provided immunogenicity and protection to LPAI strains that are nearly 10% divergent in terms of the H5 amino acid similarity. Limiting the spread of a broad number of AI strains could be important to prevent evolution of viruses in populations where vaccines that provide poor cross protection may drive undesirable evolution of AI viruses [51,52]. Previous studies demonstrated that mosaic H5 hemagglutinin delivered by an MVA vector was highly immunogenic in mice and monkeys [36,37,38]. In the present study, we demonstrate effectiveness of adjuvanted H5M antigen-based vaccines in an avian model. Further, these results demonstrate the effectiveness of both MVA and polyanhydride nanoparticles as viable vaccine vectors that could be used to combat other avian diseases. The utility of these vaccine vectors and nano-formulations to poultry vaccination efforts is highlighted by the rapid immunity elicited using only a single dose regimen.

The PR8-HM5 virus first characterized in these studies appears to be an excellent source of H5 AI virus antigen. Since HPAI virus is lethal to egg layers, the global demand for HPAI vaccine may not be met by egg-based vaccine manufacturing strategies in the event of a mass outbreak in chickens [53]. WT LPAI and HPAI viruses often do not grow to high antigenic densities in widely used mammalian cell lines, complicating the production of AI antigen for use in poultry and humans in such pandemic situations. The recombinant PR8-H5M virus, encoding a completely *in silico* derived H5 gene, grew to remarkably high antigenic densities in MDCK culture. As this H5M gene lacks the multi-basic cleavage site, growing this virus at decreased biosecurity levels should be considered.

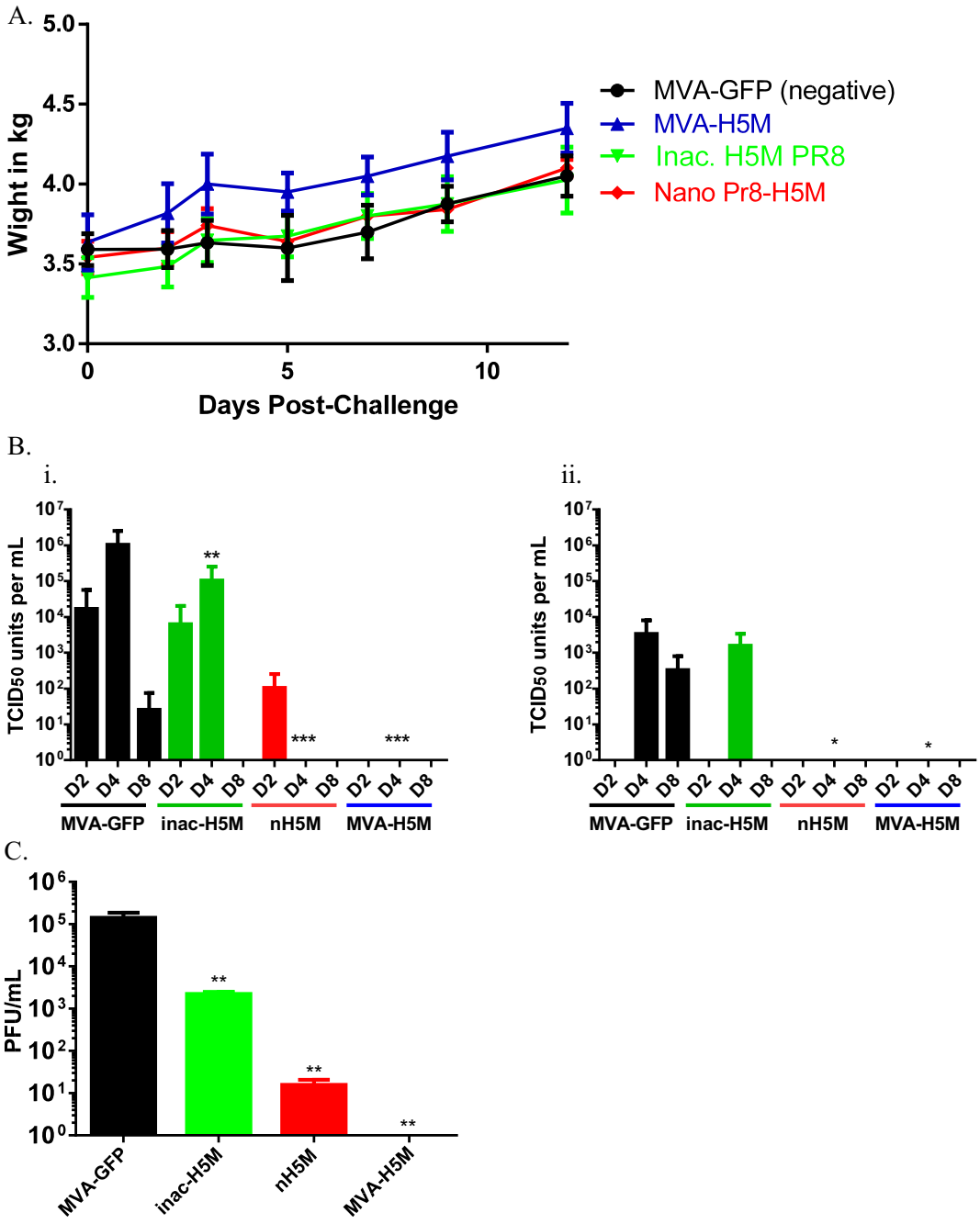

Fig. 3. Long-term protective efficacy of H5M-based vaccine constructs. One day old chicks were immunized with different vaccine constructs and challenged at 8 weeks post immunization with A/northern pintail/Alaska/622/2012 LPAI H5N2 LPAI (efficacy study). (A) The average body weights of chickens after challenge for up to 15 days. (B) Oropharyngeal (i) and cloacal (ii) shedding of H5N2 virus using MDCK cell culturing. (C) Viral isolation from lung samples taken day 3 post challenge using MDCK cell culturing.

In this study, PAN-delivered H5M (nH5M) and MVA-H5M based vaccines elicited HAI antibodies against multiple strains of influenza virus. Neutralizing and HAI antibodies against the influenza globular head of HA are known to provide complete protection against viral replication [54]. Typically, vaccines elicit neutralizing antibodies that are very specific to the vaccine or closely related viruses, leading to low efficacy and breadth in practice [55,56]. Previous studies demonstrated that the MVA-H5M vaccine was superior to traditional vaccines based on a wild type H5 HA sequence [37], with the ability to broaden neutralizing antibodies against the influenza A/Egypt/01/08 strain while maintaining responses to prototypical H5N1 clades. The A/Egypt/01/08 strain is considered to have the potential to transmit between humans with few mutations [57]. Our current studies demonstrate equally broad protection against both Clade 0 to recent Clade 2 strains. Further, H5M vaccines elicited antibodies and protection to highly divergent LPAI field strains.

Another important aspect of AI control is differentiating vaccinated from infected animals (DIVA). While NS specific ELISAs may allow for DIVA analysis using nH5M vaccines containing whole antigen, further refinement of the nH5M vaccine, perhaps using virion splitting techniques, may be required for DIVA

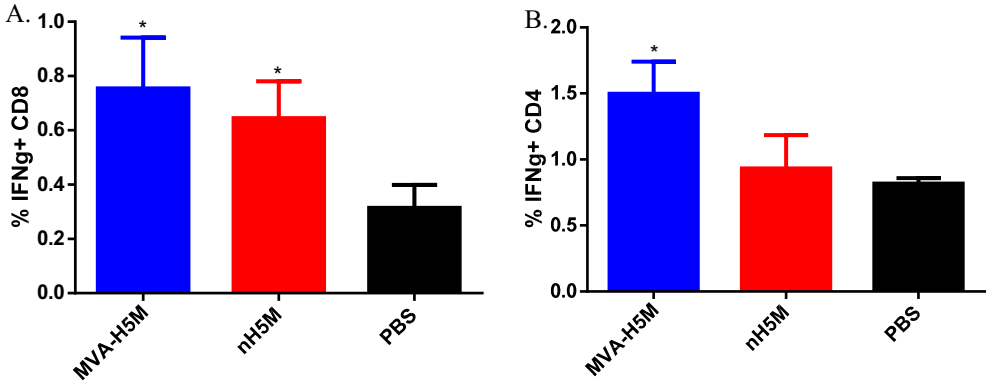

Fig. 4. Evaluation of cellular immune responses elicited by MVA-H5M and nH5M against influenza virus. Chicks were vaccinated by IM injection of MVA-H5M, nH5M or PBS (immunogenicity study) on day 28 after injection, the percentage of IFN-γ-producing $CD8^+$ (A) or $CD4^+$ (B) in splenocytes were quantified by intracellular cytokine staining.

viability. MVA based vaccines that express individual viral proteins are fully compatible with DIVA analysis.

Interestingly, both MVA and PAN based vectors elicited cellular immune responses to live virus detected *ex vivo*. Of note, this study was able to measure IFNγ responses by ICCS and flow cytometry in chicken immune cells, which while possible, has been shown to be challenging in prior studies, as only low levels of IFNγ secreting cells were detected with peptide or mitogens [58]. Indeed, the frequency of IFNγ cells demonstrated by this assay may only represent a fraction of the total IFNγ secreting population, and an even smaller fraction of the total antigen specific T cells. In this study, we used live virus for stimulation, which may have more efficiently loaded antigen on MHC I of antigen presenting cells, in contrast to other studies that stimulated with non-infectious antigen.

Overall, this report described the delivery of mosaic HA antigen by 2 independent approaches for chicken immunization. PANs provided sustained release kinetics of encapsulated antigen, resulting in long-lived, high avidity antibody titers induced with otherwise suboptimal doses of antigen, similar to earlier reports [17,18,24]. Previously, PANs have been shown to be a versatile vaccine adjuvant/delivery platform capable of enhancing the immune response to recombinant proteins, and can be delivered by multiple routes [17,18,24]. Also, presented studies demonstrated the efficacy of MVA as a vaccine vector in chickens, worthy of further consideration for poultry immunization programs. As discussed before, MVA vaccines are highly safe as they only known undergo abortive replication *in vivo* [59], and to date no reports have demonstrated productive replication of virus in a viable host. They are highly stable and are easy to propagate and purify, and like PAN based vaccines, can be delivered by multiple routes. It is noteworthy that prior studies in chickens have compared recombinant NDV and MVA vectors expressing HA antigen to induce immunity to H5N1 challenge, with similar efficacy [60]. While it is well known that viral vectors are highly efficient at stimulating T cell responses, these results demonstrate that PAN based vaccines can elicit $CD8^+$ T cell responses in chickens independent of *in vivo* expression of antigen. While likely due to more efficient cross presentation by PAN particles, these mechanisms are currently unknown in chickens for PAN particles. Further studies in chickens to define the exact types of memory/resident/effector T cell types elicited by these and other vaccines, as well as further understanding of how replicating and non-replicating vaccines induce CD4 and CD8 responses, are critical to design better T cell inducing vaccines. In conclusion, these studies demonstrate proof of concept that adjuvanted H5M-based vaccines elicit robust immunity in chickens, and that both PAN and MVA constructs are promising vaccine vectors in poultry.

Acknowledgements

This work was partially supported by grants USDA NIFA (2015-07821) and University of Wisconsin-WARF Seed Grant awarded to AMT. BN and AMT acknowledge support from the Iowa State University Nanovaccine Institute and BN is grateful to the Vlasta Klima Balloun Faculty Chair.

Declaration of Competing Interest

Adel M. Talaat has an ownership interest in Pan Genome Systems, INC, which is working in the area of animal vaccine development. Brock A. Kingstad-Bakke and Yashdeep Phanse are employed by the same company.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.06.077.

References

[1] Medina RA, García-Sastre A. Influenza A viruses: new research developments. Nat Rev Micro 2011;9:590–603.
[2] Avian Influenza. The Center for Food Security and Public Health; 2014.
[3] Kapczynski DR, Esaki M, Dorsey KM, Jiang H, Jackwood M, Moraes M, et al. Vaccine protection of chickens against antigenically diverse H5 highly pathogenic avian influenza isolates with a live HVT vector vaccine expressing the influenza hemagglutinin gene derived from a clade 2.2 avian influenza virus. Vaccine 2015;33:1197–205.
[4] Monne I, Fusaro A, Nelson MI, Bonfanti L, Mulatti P, Hughes J, et al. Emergence of a highly pathogenic avian influenza virus from a low-pathogenic progenitor. J Virol 2014;88:4375–88.
[5] Banks J, Speidel ES, Moore E, Plowright L, Piccirillo A, Capua I, et al. Changes in the haemagglutinin and the neuraminidase genes prior to the emergence of highly pathogenic H7N1 avian influenza viruses in Italy. Arch Virol 2001;146:963–73.
[6] Horimoto T, Rivera E, Pearson J, Senne D, Krauss S, Kawaoka Y, et al. Origin and molecular changes associated with emergence of a highly pathogenic H5N2 influenza virus in Mexico. Virology 1995;213:223–30.
[7] Bean WJ, Kawaoka Y, Wood JM, Pearson JE, Webster RG. Characterization of virulent and avirulent A/chicken/Pennsylvania/83 influenza A viruses: potential role of defective interfering RNAs in nature. J Virol 1985;54:151–60.
[8] Jhung MA, Nelson DI. Outbreaks of avian influenza A (H5N2), (H5N8), and (H5N1) among birds–United States, December 2014-January 2015. MMWR Morb Mortal Wkly Rep 2015;64:111.
[9] Crawford J, Wilkinson B, Vosnesensky A, Smith G, Garcia M, Stone H, et al. Baculovirus-derived hemagglutinin vaccines protect against lethal influenza infections by avian H5 and H7 subtypes. Vaccine 1999;17:2265–74.
[10] Stone HD. Efficacy of avian influenza oil-emulsion vaccines in chickens of various ages. Avian Dis 1987;31:483–90.
[11] Cox RJ, Brokstad KA, Ogra P. Influenza virus: immunity and vaccination strategies. Comparison of the immune response to inactivated and live, attenuated influenza vaccines. Scand J Immunol 2004;59:1–15.

[12] Ehrlich HJ, Muller M, Oh HM, Tambyah PA, Joukhadar C, Montomoli E, et al. A clinical trial of a whole-virus H5N1 vaccine derived from cell culture. New Engl J Med 2008;358:2573–84.
[13] Wright PF. Vaccine preparedness–are we ready for the next influenza pandemic? N Engl J Med 2008;358:2540–3.
[14] Bertran K, Lee DH, Criado MF, Balzli CL, Killmaster LF, Kapczynski DR, et al. Maternal antibody inhibition of recombinant Newcastle disease virus vectored vaccine in a primary or booster avian influenza vaccination program of broiler chickens. Vaccine 2018;36:6361–72.
[15] Richard-Mazet A, Goutebroze S, Le Gros FX, Swayne DE, Bublot M. Immunogenicity and efficacy of fowlpox-vectored and inactivated avian influenza vaccines alone or in a prime-boost schedule in chickens with maternal antibodies. Vet Res 2014;45:107.
[16] Gelb Jr J, Jackwood DJ, Brannick EM, Ladman BS. Efficacy of recombinant HVT-IBD vaccines administered to broiler chicks from a single breeder flock at 30 and 60 weeks of age. Avian Dis 2016;60:603–12.
[17] Huntimer L, Wilson-Welder JH, Ross K, Carrillo-Conde B, Pruisner L, Wang C, et al. Single immunization of a suboptimal dose of antigen encapsulated into polyanhydride microparticles promotes high titer and avid antibody responses. J Biomed Mater Res 2013;101B:91–8.
[18] Kipper MJ, Wilson JH, Wannemuehler MJ, Narasimhan B. Single dose vaccine based on biodegradable polyanhydride microspheres can modulate immune response mechanism. J Biomed Med Res 2006;76:798–810.
[19] Dhakal S, Goodman J, Bondra K, Lakshmanappa YS, Hiremath J, Shyu DL, et al. Polyanhydride nanovaccine against swine influenza virus in pigs. Vaccine 2017;35:1124–31.
[20] McGill JL, Kelly SM, Kumar P, Speckhart S, Haughney SL, Henningson J, et al. Efficacy of mucosal polyanhydride nanovaccine against respiratory syncytial virus infection in the neonatal calf. Sci Rep 2018;8:3021.
[21] Ross K, Adams J, Loyd H, Ahmed S, Sambol A, Broderick S, et al. Combination nanovaccine demonstrates synergistic enhancement in efficacy against influenza. ACS Biomater Sci Eng 2016;2:368–74.
[22] Ross KA, Loyd H, Wu W, Huntimer L, Ahmed S, Sambol A, et al. Hemagglutinin-based polyanhydride nanovaccines against H5N1 influenza elicit protective virus neutralizing titers and cell-mediated immunity. Int J Nanomedicine 2015;10:229–43.
[23] Zacharias ZR, Ross KA, Hornick EE, Goodman JT, Narasimhan B, Waldschmidt TJ, et al. Polyanhydride nanovaccine induces robust pulmonary B and T cell immunity and confers protection against homologous and heterologous influenza A virus infections. Front Immunol 2018;9.
[24] Ulery B, Kumar D, Ramer-Tait A, Wannemuehler MJ, Metzger D, Narasimhan B. Design of a protective single-dose intranasal nanoparticle-based vaccine platform for respiratory infectious diseases. PLoS ONE 2011;6:e17642.
[25] Haughney SL, Petersen LK, Schoofs AD, Ramer-Tait AE, King JD, Briles DE, et al. Retention of structure, antigenicity, and biological function of pneumococcal surface protein A (PspA) released from polyanhydride nanoparticles. Acta Biomater 2013;9:8262–71.
[26] Wagner-Muniz DA, Haughney SL, Kelly SM, Wannemuehler MJ, Narasimhan B. Room temperature stable PspA-based nanovaccine induces protective immunity. Front Immunol 2018;9:325.
[27] Renu S, Markazi AD, Dhakal S, Lakshmanappa YS, Gourapura SR, Shanmugasundaram R, et al. Surface engineered polyanhydride-based oral Salmonella subunit nanovaccine for poultry. Int J Nanomedicine 2018;13:8195–215.
[28] Mostow SR, Hopkins JA, Wright PF. Behavior of vaccine revertants of temperature-sensitive mutants of influenza virus in ferret tracheal organ culture. Infect Immun 1979;26:193–6.
[29] Vela-Ramirez JE, Goodman JT, Boggiatto PM, Roychoudhury R, Pohl NL, Hostetter JM, et al. Safety and biocompatibility of carbohydrate-functionalized polyanhydride nanoparticles. AAPS J 2015;17:256–67.
[30] Huntimer L, Ramer-Tait AE, Petersen LK, Ross KA, Walz KA, Wang C, et al. Evaluation of biocompatibility and administration site reactogenicity of polyanhydride-particle-based platform for vaccine delivery. Adv Healthc Mater 2013;2:369–78.
[31] Wang Y, Deng L, Kang SM, Wang BZ. Universal influenza vaccines: from viruses to nanoparticles. Expert Rev Vaccines 2018;17:967–76.
[32] Al-Halifa S, Gauthier L, Arpin D, Bourgault S, Archambault D. Nanoparticle-based vaccines against respiratory viruses. Front Immunol 2019;10:22.
[33] Pardi N, Parkhouse K, Kirkpatrick E, McMahon M, Zost SJ, Mui BL, et al. Nucleoside-modified mRNA immunization elicits influenza virus hemagglutinin stalk-specific antibodies. Nat Commun 2018;9:3361.
[34] Spicer CD, Jumeaux C, Gupta B, Stevens MM. Peptide and protein nanoparticle conjugates: versatile platforms for biomedical applications. Chem Soc Rev 2018;47:3574–620.
[35] Lopac SK, Torres MP, Wilson-Welder JH, Wannemuehler MJ, Narasimhan B. Effect of polymer chemistry and fabrication method on protein release and stability from polyanhydride microspheres. J Biomed Mater Res B Appl Biomater 2009;91:938–47.
[36] Kamlangdee A, Kingstad-Bakke B, Anderson TK, Goldberg TL, Osorio JE. Broad protection against avian influenza virus by using a modified vaccinia Ankara virus expressing a mosaic hemagglutinin gene. J Virol 2014;88:13300–9.
[37] Kamlangdee A, Kingstad-Bakke B, Osorio JE. Mosaic H5 hemagglutinin provides broad humoral and cellular immune responses against influenza viruses. J Virol 2016;90:6771–83.
[38] Florek NW, Kamlangdee A, Mutschler JP, Kingstad-Bakke B, Schultz-Darken N, Broman KW, et al. A modified vaccinia Ankara vaccine vector expressing a mosaic H5 hemagglutinin reduces viral shedding in rhesus macaques. PLoS ONE 2017;12:e0181738.
[39] Gao W, Soloff AC, Lu X, Montecalvo A, Nguyen DC, Matsuoka Y, et al. Protection of mice and poultry from lethal H5N1 avian influenza virus through adenovirus-based immunization. J Virol 2006;80:1959–64.
[40] Neumann G, Watanabe T, Ito H, Watanabe S, Goto H, Gao P, et al. Generation of influenza A viruses entirely from cloned cDNAs. Proc Natl Acad Sci USA 1999;96:9345–50.
[41] Torres MP, Determan AS, Anderson GL, Mallapragada SK, Narasimhan B. Amphiphilic polyanhydrides for protein stabilization and release. Biomaterials 2007;28:108–16.
[42] Schneider CA, Rasband WS, Eliceiri KW. NIH image to ImageJ: 25 years of image analysis. Nat Methods 2012;9:671–5.
[43] Killian ML. Hemagglutination assay for influenza virus. Methods Mol Biol 2014;1161:3–9.
[44] Barton HD, Rohani P, Stallknecht DE, Brown J, Drake JM. Subtype diversity and reassortment potential for co-circulating avian influenza viruses at a diversity hot spot. J Anim Ecol 2013.
[45] Gerloff NA, Jones J, Simpson N, Balish A, Elbadry MA, Baghat V, et al. A high diversity of Eurasian lineage low pathogenicity avian influenza A viruses circulate among wild birds sampled in Egypt. PLoS ONE 2013;8:e68522.
[46] Gerloff NA, Khan SU, Zanders N, Balish A, Haider N, Islam A, et al. Genetically diverse low pathogenicity avian Influenza A virus subtypes co-circulate among poultry in Bangladesh. PLoS ONE 2016;11:e0152131.
[47] Chen LJ, Lin XD, Guo WP, Tian JH, Wang W, Ying XH, et al. Diversity and evolution of avian influenza viruses in live poultry markets, free-range poultry and wild wetland birds in China. J Gen Virol 2016;97:844–54.
[48] Suarez DL, Senne DA, Banks J, Brown IH, Essen SC, Lee CW, et al. Recombination resulting in virulence shift in avian influenza outbreak, Chile. Emerg Infect Dis 2004;10:693–9.
[49] Sonnberg S, Webby RJ, Webster RG. Natural history of highly pathogenic avian influenza H5N1. Virus Res 2013;178:63–77.
[50] Ping J, Selman M, Tyler S, Forbes N, Keleta L, Brown EG. Low-pathogenic avian influenza virus A/turkey/Ontario/6213/1966 (H5N1) is the progenitor of highly pathogenic A/turkey/Ontario/7732/1966 (H5N9). J Gen Virol 2012;93:1649–57.
[51] Lee DH, Fusaro A, Song CS, Suarez DL, Swayne DE. Poultry vaccination directed evolution of H9N2 low pathogenicity avian influenza viruses in Korea. Virology 2016;488:225–31.
[52] Read AF, Baigent SJ, Powers C, Kgosana LB, Blackwell L, Smith LP, et al. Imperfect vaccination can enhance the transmission of highly virulent pathogens. PLoS Biol 2015;13:e1002198.
[53] Pandey A, Singh N, Sambhara S, Mittal SK. Egg-independent vaccine strategies for highly pathogenic H5N1 influenza viruses. Hum Vaccin 2010;6:178–88.
[54] Kaur K, Sullivan M, Wilson PC. Targeting B cell responses in universal influenza vaccine design. Trends Immunol 2011;32:524–31.
[55] Aleflali M, Khandaker G, Booy R, Rashid H. Mismatching between circulating strains and vaccine strains of influenza: effect on Hajj pilgrims from both hemispheres. Hum Vaccin Immunother 2015;0.
[56] DiazGranados CA, Denis M, Plotkin S. Seasonal influenza vaccine efficacy and its determinants in children and non-elderly adults: a systematic review with meta-analyses of controlled trials. Vaccine 2012;31:49–57.
[57] Herfst S, Schrauwen EJ, Linster M, Chutinimitkul S, de Wit E, Munster VJ, et al. Airborne transmission of influenza A/H5N1 virus between ferrets. Science 2012;336:1534–41.
[58] Ariaans MP, van de Haar PM, Lowenthal JW, van Eden W, Hensen EJ, Vervelde L. ELISPOT and intracellular cytokine staining: novel assays for quantifying T cell responses in the chicken. Dev Comp Immunol 2008;32:1398–404.
[59] Chahroudi A, Garber DA, Reeves P, Liu L, Kalman D, Feinberg MB. Differences and similarities in viral life cycle progression and host cell physiology after infection of human dendritic cells with modified vaccinia virus Ankara and vaccinia virus. J Virol 2006;80:8469–81.
[60] Veits J, Romer-Oberdorfer A, Helferich D, Durban M, Suezer Y, Sutter G, et al. Protective efficacy of several vaccines against highly pathogenic H5N1 avian influenza virus under experimental conditions. Vaccine 2008;26:1688–96.