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Protective immunity against influenza virus challenge by norovirus P particle-M2e and HA2-AtCYN vaccines in chickens

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ABSTRACT

Development of a broadly reactive influenza vaccine that can provide protection against emerging type A influenza viruses is a big challenge. We previously demonstrated that a vaccine displaying the extracellular domain of the matrix protein 2 (M2e) on the surface loops of norovirus P-particle (M2eP) can partially protect chickens against several subtypes of avian influenza viruses. In the current study, a chimeric vaccine containing a conserved peptide from the subunit 2 of hemagglutinin (HA) glycoprotein (HA2) and *Arabidopsis thaliana* cyanase protein (AtCYN) (HA2-AtCYN vaccine) was evaluated in 2-weeks-old chickens. Depending on the route of administration, the HA2-AtCYN vaccine was shown to induce various levels of HA2-specific IgA in tears as well as serum IgG, which were associated with partial protection of chickens against tracheal shedding of a low pathogenicity H5N2 challenge virus. Furthermore, intranasal administration with a combination of HA2-AtCYN and M2eP vaccines resulted in enhanced protection compared to each vaccine alone. Simultaneous intranasal administration of the vaccines did not interfere with secretory IgA induction by each vaccine. Additionally, significantly higher M2eP-specific proliferative responses were observed in peripheral blood mononuclear cells of all M2eP-vaccinated groups when compared with the mock-vaccinated group. Although tripling the number of M2e copies did not enhance the protective efficacy of the chimeric vaccine, it significantly reduced immunodominance of P-particle epitopes without affecting the robustness of M2e-specific immune responses. Taken together, our data suggests that mucosal immunization of chickens with combinations of mechanistically different cross-subtype-conserved vaccines has the potential to enhance the protective efficacy against influenza virus challenge.

1. Introduction

Avian influenza (AI) continues to be a great concern for poultry health and is considered one of the most economically important diseases of poultry [1,2]. The control of AI virus (AIV) infection in poultry is implemented mainly through programs that utilize education, surveillance, enhanced biosecurity, and elimination of infected birds to prevent spread to uninfected flocks [3]. In addition, vaccination is being used in endemic countries to prevent AIV transmission from flock to flock or spillover to humans [1,4,5]. Several studies have pointed out that a high level of antigenic matching between the vaccine and field viruses is required to ensure high vaccine efficacy [1,3]. However, the currently available AI vaccines and vaccination strategies are narrow spectrum in terms of immunogenicity and protective efficacy likely due to antigenic drifts or shifts in the field strains [1,3]. Therefore, universal or broadly reactive vaccines with ability to provide protection against different AIV subtypes are highly desirable [6-8]. Universal influenza vaccine candidates have been extensively studied but none has been licensed for use in poultry or other species [9-12]. Many of the experimental universal AI vaccine candidates are based on conserved influenza virus antigens such as the ectodomain of matrix protein 2 (M2e) and subunit 2 of hemagglutinin (HA) glycoprotein (HA2) [13,14].

M2e is highly conserved across subtypes of type A influenza virus and has been an attractive target for inducing cross-subtype protection [15,16]. Previous studies have reported varying results regarding the level of correlations between vaccine-induced anti-M2e antibody quantities and protection against influenza

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virus infection in different animal models [17,18]. In our previous chicken studies, although a chimeric vaccine based on the norovirus P particle and M2e (M2eP) provided partial cross-protection against challenge with different AIV subtypes [19,20], the level of protective efficacy was not sufficient enough for M2eP to be used as a stand-alone vaccine in chickens. Alternatively, the M2eP vaccine can be included as a component of universal vaccination regimens that combine conserved epitopes with viral surface proteins such as the hemagglutinin (HA) glycoprotein.

The HA glycoprotein of influenza virus has two subunits, HA1 and HA2, linked by a single disulfide bond [21]. The HA2 subunit has several conserved regions with high sequence homology among different subtypes [22]. Anti-HA2 antibodies induced in human and mice are highly cross-reactive within a given subtype and between different subtypes [14,23,24]. Thus, HA2 is a promising target for designing a universal influenza vaccine [25,26]. Experimental HA2-based vaccines have been demonstrated to induce robust immunological responses and provide high levels of protection against various influenza subtypes in mice [14,23,27-30]. In chickens, oral vaccination with *Lactobacillus plantarum (L. plantarum)* expressing a full HA2 protein was able to induce mucosal and systemic humoral immune responses and provide partial protection against challenge with H9N2 virus [31].

To develop universal influenza vaccines, a myriad of vaccine platforms and technologies have been studied for their ability to induce broad cross-protective immunity through stimulation of both humoral and cell-mediated arms of the immune system [32]. A significant limitation of most experimental universal influenza vaccines in poultry is their failure to confer similar level of protection provided by inactivated vaccines against homologous challenge strains despite having broadened cross-reactivity with several virus subtypes [20,31,33,34]. To overcome this limitation, the focus is shifting toward incorporating a combination of multiple conserved immunogenic proteins or epitopes in vaccines or vaccination regimens to enhance the cross-reactivity and protective efficacy of universal influenza vaccines [35–38]. Simultaneous administration of different vaccines that contain single conserved proteins such as M2e and HA2 can increase cross-protective immunity compared to administration of each vaccine alone. Although this approach has produced promising results in mice [36,38], its efficacy in chickens or other poultry species is unknown.

In the current study, we constructed a novel chimeric HA2 vaccine containing *Arabidopsis thaliana* cyanase protein (AtCYN) [39] and an HA2 protein (HA2-AtCYN vaccine). The HA2-AtCYN vaccine was observed to induce various levels of local IgA and systemic IgG responses in chickens depending on the route of administration. Further, enhanced protection was observed in chickens intranasally vaccinated with a combination of HA2-AtCYN and M2eP compared to other treatment groups. Lastly, we show that tripling the number of M2e copies expressed on the surface of norovirus P particle (3M2eP) did not enhance M2e-specific immune responses or protective efficacy of the vaccine. It did, however, reduce the immunodominance of P-particle epitopes in the chimeric vaccine.

2. Material and methods

2.1. Construction of vaccines

The chimeric M2e vaccine containing the consensus M2e protein (MSLLTEVETPTRNGWECKCSDSSD) of AIV and norovirus P particle (M2eP) was constructed as described previously [19,20,40] and tested in two earlier studies [19,20]. A new chimeric M2e vaccine (3M2eP) was constructed and purified in the same manner by inserting three tandem copies of M2e in loop 2 of the P-particle [41–43].

The peptide sequence (RIENLNKKMEDGFLDVWTYNAELLVLME NERTLDLHDSNVKNL-YDKVRHQLRDNA) encoding residues 76-130 (55 amino acids), within the long α-helix (LAH) region, of the HA2 gene from A/chicken/PA/13609/1993 (H5N2) virus was used for construction of the HA2-AtCYN. LAH domains of HA2 subunits are highly conserved within the two major phylogenetic groupings of HAs of type A influenza viruses [8]. The AtCYN gene encodes a plant cyanase in *Arabidopsis thaliana, which* forms homo-decamer [44]. Briefly, cDNA encoding HA2 peptide and a polyhistidine (His) tag was cloned into the N-terminus of AtCYN gene in pET-15b vector. The recombinant His-HA2-AtCYN fusion protein was expressed in E. coli (BL21, DE3) and purified using TALON Magnetic Beads (Clontech Laboratories, Inc.) according to the manufacturer's instructions. The purified protein was loaded on a size exclusion column Superdex 200 (GE Healthcare Life Sciences) powered by an AKTA FPLC system. The molecular weights of the eluted fractions were calibrated by Gel Filtration Calibration Kits (GE Healthcare Life Sciences). The retrieved protein was confirmed further through SDS-PAGE [41–43].

2.2. Animal experiment

The care, management, treatment, and euthanasia of animals were performed according to the protocol #2009AG0002-R2 approved by The Ohio State University Institutional Animal Care and Use Committee (IACUC). The following categories of vaccine compositions or vaccination regimens were investigated: mock control (Mock), single copy M2eP (M2eP), three copy M2eP (3M2eP), HA2-AtCYN (HA2), and combined vaccine (M2eP + HA2). As shown in Table 1, two-week-old specific-pathogen-free birds were divided into 9 groups according to the vaccine or vaccine combinations and the route(s) of vaccine administration. The vaccines were given via subcutaneous (SQ) route with commercial mineral oil adjuvant (Montanide ™ ISA 70 VG, Seppic, Paris, France) (Antigen/Adjuvant, 3:7 V/V ratio) and/or intranasal (IN) route without adjuvant, depending on the group being treated. Chickens were vaccinated (or mock-vaccinated with phosphate buffered saline, PBS) three times, with 2 week intervals between vaccinations. Each dose of M2eP, 3M2eP, or HA2 vaccines contained 5 μg of total protein (Table 1).

Table 1
Experimental groups with vaccination regimens.

Experimental groups	Vaccine	Route	Adjuvant
Mock control (Mock)	PBS	IN[a]/SQ[b]	No/Yes
Single copy of M2e on one of norovirus P particle loops (M2eP)			
M2eP-IN	M2eP	IN	No
M2eP-SQ	M2eP	SQ	Yes
M2eP-IN/SQ	M2eP	IN/SQ	No/Yes
Three tandem copies of M2e one of norovirus P particle loops (3M2eP)			
3M2eP-IN	3M2eP	IN	No
3M2eP-SQ	3M2eP	SQ	Yes
HA2-AtCYN (HA2)			
HA2-IN	HA2-AtCYN	IN	No
HA2-SQ	HA2-AtCYN	SQ	Yes
Combined vaccine (M2eP + HA2)			
M2eP + HA2-IN	M2eP + HA2	IN	No
M2eP + HA2-SQ	M2eP + HA2	SQ	Yes

Note: Chickens were primed and boosted twice with each vaccine at two weeks interval and challenged with A/Chicken/PA/13609/93 (H5N2) two weeks after the last booster vaccination.

[a] IN: Intranasal route without adjuvant (dose: 5 μg of vaccine protein/bird/vaccination).

[b] SQ: Subcutaneous route with Montanide ISA 70 VG® oil adjuvant (dose: 5 μg of vaccine protein /bird/vaccination).

2.3. Sample collection and quantification of hemagglutination inhibition (HI) antibodies and IgG antibodies in serum, and IgA antibodies in tears

All birds were bled two weeks after each vaccination for serum collection. The serum was separated from the other blood components and heat inactivated at 56 °C for 30 min and assayed for antibody titers as described below. In addition, tear samples (~50 μl from each eye) were collected two weeks after the final (3rd) vaccination as previously described [45].

Serum HI antibody titers were determined using two-fold serially diluted serum samples, 8 HAU of H5N2 challenge virus as antigen, and 1% turkey erythrocyte suspension as previously described [46].

Serum M2e- and HA2-specific IgG antibodies were quantified by ELISA using synthetic M2e and HA2 peptides (Ohio Peptide, Powell, OH) as coating antigens (2 μg/ml) as previously described [20]. Tear IgA antibodies were quantified the same way as IgG antibodies except that horseradish peroxidase (HRP) labeled goat anti-chicken IgA antibody (Gallus immunotech Inc., ON) was used as the secondary antibody instead of the HRP labelled anti-chicken IgG antibody (Sera care, Milford, MA).

2.4. Measurement of M2eP specific cell-mediated immunity

Ten days after the final immunization (3rd vaccination), birds from M2eP (M2eP-IN, M2eP-SQ, and M2eP-IN/SQ) and Mock groups, five birds from each group, were selected for assessment of M2e-specific memory recall response using lymphocyte proliferation assay. Blood and spleen samples were collected and processed as previously described [47,48] with minor modifications. Briefly, blood samples (5 ml per chicken) were collected and immediately transferred into sterile capped tubes with 1% Ethylenediaminetetraacetic acid solution (Affymetrix, Cleveland, OH). Samples were diluted with an equal volume of PBS and carefully layered on the surface of Ficoll-Paque™ PLUS density gradient media (Fisher scientific, Pittsburgh, PA). After centrifugation at 450 g for 30 min without brakes, peripheral blood mononuclear cells (PBMCs) were recovered from the interface and washed twice with PBS. Spleen samples were aseptically collected and placed in sterile 4 °C PBS. Single-cell suspensions were prepared by gently pushing the splenic pulp through a sterile nylon mesh with a pore size of 70 μm (Fisher Scientific). The crude cell preparation was washed, re-suspended in PBS, layered over Ficoll-Paque and centrifuged as described above. Splenocytes were recovered from the interface and washed twice with PBS. The final pellets of PBMCs and splenocytes were re-suspended in RPMI 1640 medium (Gibco, Grand Island, NY) containing 10% heat-inactivated fetal calf serum (Gibco) and seeded in triplicate wells (5×10^5 cells per well) in 96-well plates. Cell monolayers were stimulated under various conditions including: a mixture of 1μg/ml phorbol 12-myristate 13-acetate (PMA) and Ionomycin (PMA/Ionomycin) (Sigma- Aldrich, St. Louis, MO) as a positive control, 5 μg/ml of M2eP, synthetic M2e peptide, and P particle as specific antigens, and RPMI 1640 medium without antigen as a negative control. After 72 h of incubation at 39 °C in a humid atmosphere with 5% CO_2, 20 μl of the combined MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt) and phenazine methosulfate solution from Cell Titer 96® Aqueous Non-Radioactive Cell Proliferation Assay kit (Promega, Madison, WI) was added to each well and the incubation was continued for another 4 h. At the end of incubation period, OD values at 490 nm using an ELISA reader was recorded. Stimulation index (S.I.) was calculated as the ratio of the average OD of antigen-stimulated cells to the average OD of unstimulated cells.

2.5. Viral challenge and quantification of viral RNA

All birds were intranasally challenged with 10^6 median egg infectious doses (EID_{50}) per bird (in 0.2 ml total volume) of A/Chicken/PA/13609/93 (H5N2) virus two weeks after the last vaccination (3rd vaccination). Tracheal swabs were collected from the infected birds at 3 and 5 days-post challenge (DPC) and eluted in 1 ml of PBS supplemented with gentamicin (10 μg/ml) for challenge virus detection and titration. At 7 DPC, all birds were euthanized and bled to determine the post-challenge HI titers as previously described [49].

Viral RNA was extracted from 100 μl of the eluted swab supernatant using QIAamp Viral RNA Mini Kit (Qiagen, Germantown, MD) according to the manufacturer's instructions. Viral genome amounts were determined by quantitative real-time RT-PCR (qRT-PCR) using type A influenza virus matrix gene specific primers and probes as previously described [50,51]. For interpolation of EID_{50} titers of swab samples by the qRT-PCR method, a standard curve was created by plotting cycle threshold (Ct) values generated with RNA extracted from serial 10-fold dilutions of the egg-derived virus stock (with known EID_{50} titer) used for challenge. The curve was used to convert Ct values of tracheal swab viral RNA to EID_{50} titers as previously described [20,52]. It was previously shown that EID_{50} titers derived by the qRT-PCR method employed in this study correlate well with the EID_{50} titers measured in eggs [50].

2.6. Plaque reduction viral neutralization activity

The plaque assay was performed as described by Ghorbani et al. [53]. MDCK cells were seeded in 6-well plates at a density of 1×10^6 cells/well and allowed to grow to confluence overnight. The cells were washed once with PBS before attachment with 200 μl of virus-antibody mixture per well. Pre-challenge sera (n = 3 per group, selected based on the reduction in the challenge virus shedding) were diluted (1:4), mixed with pre-diluted 100 plaque-forming units of the H5N2 challenge virus (1:1), and incubated for 60 min in a CO_2 incubator set at 37 °C. Virus or virus-antibody complex attachment was done at 37 °C for 60 min in the CO2 incubator with rocking at 15 min intervals. After the attachment period, the plates were washed once with serum-free DMEM and overlaid with a medium composed of Minimum Essential Medium, 0.6% agarose and 1 μg/ml of TPCK-treated trypsin. The plates were incubated at 37 °C for 60 h, fixed with 10% formalin, and stained with 0.1% crystal violet to visualize and count the plaques. Each serum was tested in duplicate. Plaque Reduction (%) = 100 × (1 − (#plaques for the test serum ÷ average #plaques for the Mock group)) [53].

2.7. Statistical analysis

All data were analyzed using GraphPad Prism 6.07 (GraphPad Software, San Diego, CA). ELISA, hemagglutination inhibition (HI), and virus titer values were $\log_2$-transformed prior to statistical analysis. Statistical differences among groups were determined by the one-way ANOVA followed by Tukey Post-hoc test. Statistical significance was taken as p values <0.05.

3. Results

3.1. M2eP, 3M2eP, and HA2-AtCYN induced high levels of antigen-specific serum IgG titers in vaccinated chickens

As expected based on our previous studies [19,20], the initial dose of the M2eP vaccine in the M2eP-SQ group induced a high titer of M2e-specific serum IgG antibodies which continued to

increase after each booster vaccination (Fig. 1A). The average IgG titers increased by each booster dose from 10 Log_2 titer at 2 weeks after the first vaccination to an approximate of 12 Log_2 titer in pre-challenge antisera (2 weeks after the 3rd vaccination) (Fig. 1A). No differences were observed in IgG titers between birds vaccinated with M2eP via SQ route (M2eP-SQ) and birds vaccinated with M2eP via IN and SQ simultaneously (M2eP-IN/SQ). The levels of M2e-specific IgG antibodies induced by SQ vaccination were comparable between 3M2eP and M2eP vaccines (Fig. 1A). IN vaccination with M2eP and 3M2eP did not induce detectable levels of M2e-specific serum IgG.

To determine how the number of M2e copies on the chimeric vaccine affects the balance of humoral IgG responses between M2e and P-particle epitopes, sera from M2eP-SQ and 3M2eP-SQ groups (n = 4 per group) were compared side by side. Fig. 1B shows that, while anti-M2e IgG titers were not statistically different between the two groups, there was a significant reduction of P-particle-specific IgG titers (~8-fold reduction) in the 3M2eP-SQ group. As expected, neither anti-M2e nor anti-P-particle antibodies were present in the Mock group (Fig. 1B).

Birds vaccinated with HA2-AtCYN via SQ route (HA2-SQ) developed detectable levels of anti-HA2 IgG antibodies (Log_2 titer of ~8) which significantly increased (by ~ 84-fold) after the 2nd vaccination (first booster dose). However, the 3rd vaccination (2nd booster dose) with HA2-AtCYN did not lead to further increase in the HA2-specific IgG titers (Fig. 1C). Only 1 out of 8 vaccinated birds showed a detectable IgG antibody titer following IN vaccination with HA2-AtCYN (HA2-IN) (Fig. 1C).

Birds vaccinated with a combination of M2eP and HA2-AtCYN vaccines via SQ route (M2eP + HA2-SQ) showed similar levels of M2e-specific and HA2-specific IgG titers when compared with end-point titers induced by M2eP-SQ or HA2-SQ, respectively (Fig. 1A and C).

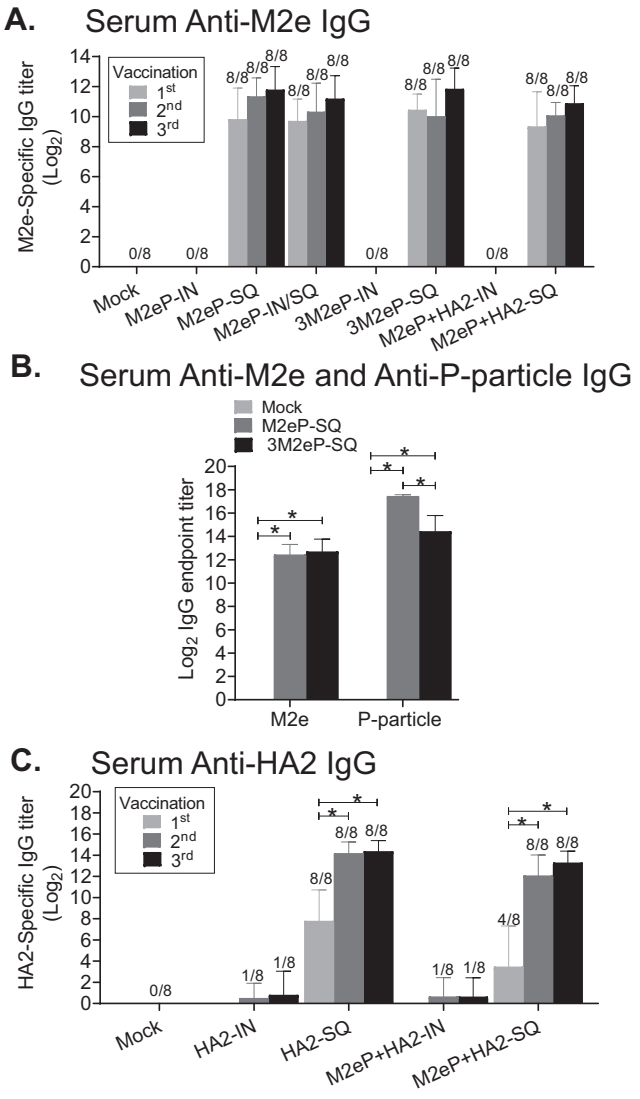

Fig. 1. Serum IgG antibody responses to M2eP and HA2-AtCYN vaccinations. Chickens were vaccinated with M2eP, 3M2eP, HA2-AtCYN, and combination of M2e and HA2 (M2eP + HA2) three times with 2 weeks intervals between vaccinations. IgG antibody responses were measured in the sera collected at 2 weeks after each immunization using free M2e peptide or HA2 peptide as coating antigen by ELISA. (A) Serum anti-M2e IgG titers after each vaccination, (B) Comparison of immunodominance of anti-M2e and ant-P-particle IgG titers after the 3rd vaccination, (C) Serum anti-HA2 IgG titers after each vaccination. Data are presented as the mean ± standard error of mean (n = 8). One-way ANOVA followed by Tukey post-hoc test was used to compare the antibody titers between vaccinations for each group (A and C) and between groups (B). Numbers above the bars indicate #antibody-positive birds/total #birds per group. Asterisks (*) indicate statistically significant difference at $p < 0.05$.

3.2. IN vaccination with M2eP, 3M2eP and HA2-AtCYN vaccines induced antigen-specific local IgA antibodies

M2eP vaccination via IN route without adjuvant induced detectable M2e-specific IgA antibodies in tears of vaccinated chickens (Fig. 2A). Virtually all birds vaccinated with M2eP via the SQ route did not have detectable levels of antigen-specific IgA (only 1 bird converted). The simultaneous administration of M2eP vaccine by IN and SQ routes (M2eP-IN/SQ) induced more uniform IgA titers and a higher conversion rate (86%) than the M2eP-IN group (50%). Chickens vaccinated with 3M2eP intranasally (3M2eP-IN) did not show elevated levels of M2e-specific mucosal IgA response but showed a more uniform mucosal IgA titer when compared to M2eP vaccinated birds (M2eP-IN). As we observed in M2e-specific mucosal IgA antibodies, three times vaccination of chickens with HA2-AtCYN intranasally (HA2-IN) resulted in high levels of mucosal HA2-specific IgA antibodies in tears (Fig. 2B). Further, when HA2-AtCYN was supplemented with M2eP (M2eP + HA2-IN), all birds converted and also displayed more uniform tear IgA titers.

3.3. Vaccination with M2eP vaccine elicited cellular immune response against the vaccine antigen

The antigen recall ability of PBMCs and spleen cells was determined through proliferation assays using M2eP, free M2e peptide, and P-particle as stimulants. The free M2e and P particle did not stimulate proliferation in either of the cell types (Fig. 3). However, M2eP was able to induce a significant proliferative response in PBMCs of vaccinated birds when compared to the mock-vaccinated birds ($p < 0.05$) (Fig. 3A). M2eP-specific proliferative responses were independent of the routes of vaccination (compare IN and SQ route) (Fig. 3A). Although mononuclear cells (PBMCs and spleen cells) from all groups responded similarly to stimulation with the non-specific mitogen (PMA/ionomycin), splenocytes did not respond to any of the vaccine associated antigens (free M2e, P-particle, and M2eP) (Fig. 3B).

3.4. Protective efficacy is enhanced in chickens vaccinated intranasally with a combination of M2eP and HA2-AtCYN vaccines

The protective efficacy of M2eP and HA2 vaccines was evaluated in terms of the level of reduction in virus shedding following challenge with low pathogenicity H5N2 virus (Fig. 4). As we observed in our previous study [20], chickens vaccinated with

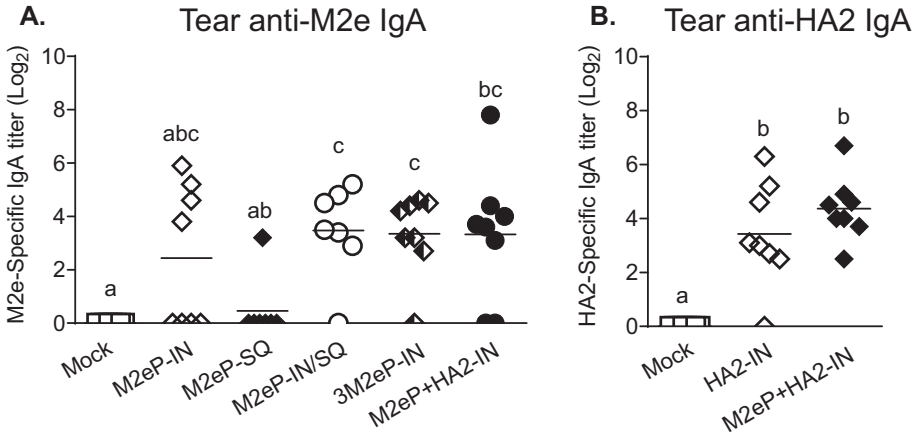

Fig. 2. Tear IgA antibody responses in vaccinated chickens. Tear samples were collected from chickens vaccinated with M2eP, 3M2eP, HA2-AtCYN, and combination of M2e and HA2 (M2eP + HA2) via IN route without adjuvant. IgA antibody responses were measured in the tears collected at 2 weeks after 3rd vaccination using free M2e peptide (A) or HA2 peptide (B) as coating antigen by ELISA. Data are presented as the mean ± standard error of mean (n = 8). One-way ANOVA followed by Tukey post-hoc test was used to compare the antibody titers between different groups. Different letters indicate statistically significant differences between groups ($p < 0.05$), i.e., any two groups are statistically different if they lack at least one common letter between them.

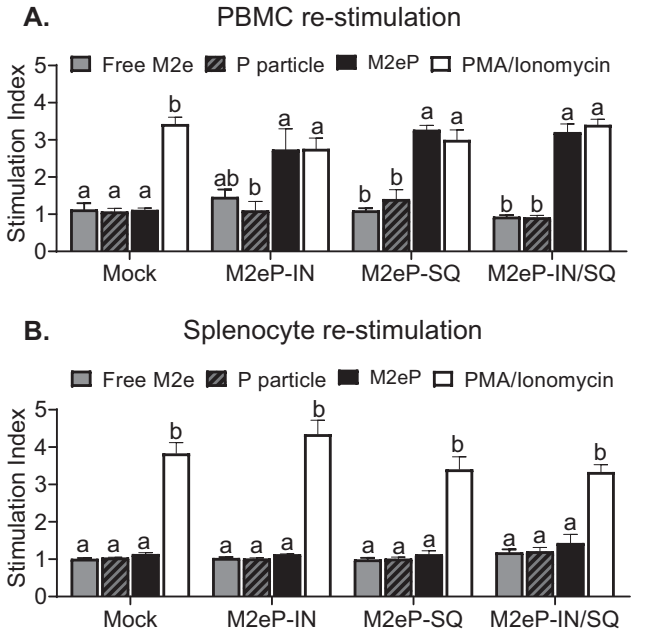

Fig. 3. Cellular immune responses in vaccinated chickens. PBMCs and splenocytes isolated from birds after the third vaccination with M2eP via SQ or IN routes were re-stimulated and specific lymphocyte proliferation was determined against M2eP, P particle, synthetic M2e peptide (5 μg/ml), and combination of phorbol myristate acetate (PMA) with calcium ionophore ionomycin (PMA/ionomycin) (1 μg/ml). Data are presented as the mean ± standard error (n = 5). Different letters indicate statistically significant differences between groups ($p < 0.05$, One-way ANOVA, Tukey post-hoc), i.e., any two groups are statistically different if they lack at least one common letter between them.

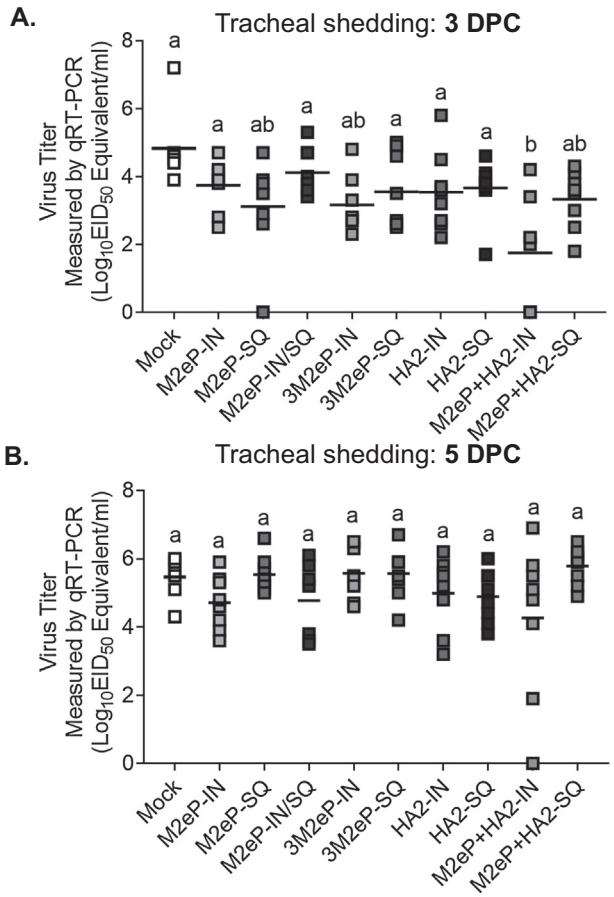

Fig. 4. Protective efficacy of different vaccines in terms of reduction of virus shedding. Tracheal swabs collected at 3 and 5 DPC were used to determine median egg infectious doses (EID_{50}) equivalent titers in each ml of the tracheal swab eluate by interpolation from qRT-PCR Ct values as described in Materials and Methods. Each bar represents the mean ± standard error of mean (n = 8). Different letters indicate statistically significant differences between groups ($p < 0.05$, One-way ANOVA, Tukey post-hoc), i.e., any two groups are statistically different if they lack at least one common letter between them.

M2eP via the SQ or IN routes generally showed a reduction in virus shedding at 3 DPC when compared to the Mock group. We also observed a reduction in virus shedding at 3 DPC in birds vaccinated with 3M2eP or HA2-AtCYN via the IN route as well as with HA2-AtCYN via the SQ route when compared with the Mock group. Notably, only the combined M2eP + HA2-IN vaccination demonstrated a significant reduction in tracheal shedding of the H5N2 virus at 3 DPC when compared to Mock and all other vaccine groups except the M2eP + HA2-SQ group (Fig. 4A). Birds in the M2eP + HA2-IN group still maintained the greatest reduction in shedding at 5 DPC, even though it was not statistically different from other groups (Fig. 4B). All the birds seroconverted after challenge and the post-challenge HI titers detected at 7 DPC were statistically indistinguishable between groups (Fig. 5A).

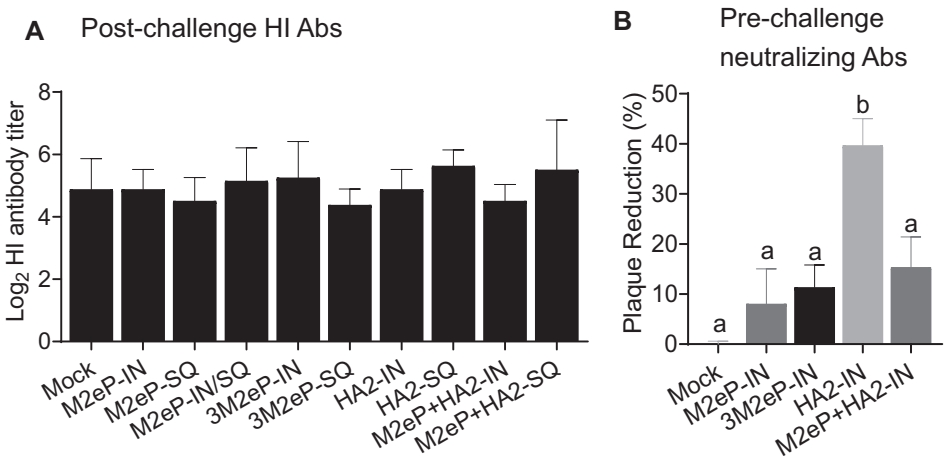

Fig. 5. Post-challenge hemagglutination inhibition (HI) antibody titers induced in vaccinated chickens and pre-challenge neutralizing antibodies. HI antibodies were measured at 7 DPC against the H5N2 challenge virus (A). Activity of neutralization antibodies in pre-challenge sera (after 3rd vaccination) was measured through plaque reduction assay (B). Different letters indicate statistically significant differences between groups ($p < 0.05$, One-way ANOVA, Tukey post-hoc), i.e., any two groups are statistically different if they lack at least one common letter between them.

Based on the enhanced (though transient) protection of chickens in the M2eP + HA2-IN group, and despite the absence of anti-M2e and anti-HA2 IgG antibodies in sera of IN-vaccinated groups (Fig. 1), pre-challenge sera from selected IN-vaccinated groups were tested for virus neutralizing antibodies against the H5N2 challenge virus (Fig. 5B). Sera from the M2eP + HA2-IN, M2eP-IN and 3M2eP-IN groups were weakly neutralizing (~8-15% plaque reduction). In contrast, a stronger and significantly higher plaque reduction was observed with sera from the HA2-IN group (Fig. 5B).

4. Discussion

Because of the frequent antigenic shifts and drifts in type A influenza viruses and the limitations of available vaccines, there is a need for universal vaccines to protect human and poultry against a wide range of virus strains and subtypes. The well-conserved M2e and HA2 proteins are attractive targets for developing broadly cross-protective influenza vaccines [14,16]. In the current study, a peptide from the LAH region of HA2 from H5N2 virus was fused with *A. thaliana* cyanase to construct a chimeric vaccine referred to as HA2-AtCYN. This vaccine was able to induce both systemic and local (mucosal) immune responses and confer partial protection against challenge virus at 3 DPC, especially when combined with the M2eP vaccine and administered via the intranasal route.

Since the LAH peptide in HA2-AtCYN is homologous to the challenge virus used in this study, the extent of cross-subtype protection of chickens by HA2-AtCYN is yet to be tested. However, a synthetic peptide of the LAH region of H3 subtype was able to protect mice from challenge with H1 and H5 virus subtypes [23]. Further, a peptide vaccine made of the fusion peptide and LAH of a H5N1 virus was protective against different influenza virus subtypes in mice [54]. Since the HA2 subunit is highly conserved among different subtypes [22], we expect broad cross-reactivity of HA2-AtCYN with non-H5 subtypes as observed in aforementioned studies. Note that while HA2-based vaccines are broadly reactive, they are significantly weaker in protection compared to inactivated influenza vaccines [19,45]. Interestingly, an enhanced protection was observed in M2eP + HA2-IN combination group compared to all treatment groups at 3 DPC (Fig. 4). The apparent additive nature of protection by M2eP + HA2-IN vaccination, relative to protection conferred by each vaccine alone (Fig. 4), suggests that little or no overlap exists between the protection mechanisms of the two vaccines. Nevertheless, their efficacy of protection was weaker at 5 DPC (Fig. 4). We, and others, have observed similar results with mucosally-administered live-attenuated and subunit vaccines [52,53,55]. We speculate the differential protection is due to low concentrations of mucosal antibodies available to neutralize the virus at 3 DPC in addition to a general increase of virus titers at 5 DPC. Further, the serum antibodies induced by the intranasal vaccines appear to have very minimal, if any, protective roles.

The M2eP + HA2-IN vaccination likely protects chickens through adaptive mucosal immunity since virus neutralizing antibodies are very weak (Fig. 5B) while a stronger association between the level of protection and IgA titers in tears is apparent (Figs. 2 and 4). We recently reported a similar association between tear IgA levels and protection of chickens vaccinated with live-attenuated influenza vaccine [45,53]. Since the levels of HA2- and M2e-specific IgA titers observed in the M2eP + HA2-IN group corresponded to those induced in single vaccine groups (HA2-IN and M2eP-IN) (Fig. 2), there was no interference between the two vaccines during simultaneous administration. IgA is a key component of the chicken respiratory mucosal antibody profile [56]. Because of its ability to translocate across the epithelial barrier in secretions associated with the respiratory system [45,57], IgA can neutralize the virus before it passes the mucosal barrier or clear it in infected epithelial cells [58]. Note that the antibody repertoire of the respiratory mucosal system in chickens and turkeys also include IgG and IgM antibodies [56], which were not tested in the current study. However, we found significantly less IgG in tears from chickens that were IN-vaccinated with live-attenuated influenza vaccine relative to birds vaccinated with inactivated vaccine via the SQ route [45]. IgA may possibly be the dominant local antibody in IN-vaccinated chickens.

Our attempt to detect mucosal IgA in nasal wash samples from IN-vaccinated chickens was unsuccessful [20], while large quantities of IgA were detected in tears (Fig. 2) [45,53]. This might be due to the proximity of the lacrimal glands to the Harderian gland. Mucosal immunization with M2eP, HA2-AtCYN (this study), or live-attenuated influenza vaccine [45,53] may have a direct or indirect stimulatory effect on B-cells in the Harderian gland, resulting in generation of IgA-secreting plasma cells [56]. Additionally, the strong mucosal antibody responses in IN-vaccinated birds may be due to non-specific adjuvant effects of the P-particle and AtCYN platforms used to display the viral antigens [43,44,59]. To the best

of our knowledge, our study is the first to show the ability of M2e and HA2 based vaccines to induce mucosal IgA responses in chicken tears.

Other mechanisms of protection may apply to explain the partial protection conferred by different M2e- and HA2-based vaccines or vaccine combinations and vaccination routes used in our studies. Although anti-M2e and anti-HA2 IgG antibodies are not sufficiently strong to completely neutralize the virus, they may lessen virus replication and spread within the host through different pathways. Previous studies have demonstrated that anti-M2e IgG-mediated cellular cytotoxicity or phagocytosis can induce the removal of infected cells before progeny virus budding and spread [60–63]. Anti-HA2 antibodies can interfere with fusion of the viral and endosomal membranes and thereby block virus entry into host cells as previously reported in mice [64]. Moreover, HA2-specific antibodies were shown to reduce the replication of influenza A viruses of different HA subtypes *in vitro* [65,66].

In addition to inducing mucosal and systemic antibodies, M2e- and HA2-based influenza vaccines were reported to induce robust specific T-cell responses which correlated with protection of BALB/c mice against lethal challenge with influenza virus [36,54,67]. In chickens, we observed high levels of PBMC proliferation in all vaccinated groups in response to M2eP stimulation, but only a minor stimulation was induced by free M2e in one group only (M2eP-IN) (Fig. 3). Free M2e is poorly immunogenic unless it is coupled with an adjuvant or another carrier protein [15,68-70]. Nonetheless, a free M2e peptide was reported to stimulate significant recall responses in PBMCs from chickens vaccinated with recombinant fusion proteins constructed with 4 copies of M2e alone or 4 copies of M2e and HSP70c protein [71]. The discrepancy in PBMC response to free M2e stimulation is possibly due to the 6 amino acid difference between the human influenza A virus-derived consensus M2e peptide used by Dabaghian et al. [71] compared to the avian virus peptide used in our studies [20]. Intriguingly, despite P-particle epitopes being immunodominant over M2e epitopes in terms of humoral antibody responses (Fig. 1B) [20,40], the carrier P-particle did not stimulate proliferation of PBMCs from M2eP-vaccinated birds (Fig. 3). It is possible that the stimulation time and other experimental parameters used in this study were not optimal for induction of PBMC proliferation by the P-particle. Another intriguing observation from this study is the lack of memory recall response in spleen cells, which might be due to the fact that the proportion of chicken T cells is lower in spleen (10–20%) compared to peripheral blood (20–40%) [72].

Although M2eP is not fully protective in chickens, it is broadly reactive with several AIV subtypes [19,20]. Each P-particle in this vaccine has 24 copies of M2e displayed on its surface [43]. Studies utilizing different M2e delivery platforms have reported enhancement of cross-reactivity and protective efficacy of the vaccine by increasing M2e epitope density [69,73-75]. On this basis, we increased the copy number of M2e epitopes displayed on P-particle to 72 to generate the 3M2eP vaccine. Contrary to our expectation, 3M2eP did not show enhanced immunogenicity or protective efficacy compared to M2eP (Figs. 1 and 4). Further, the IgG titers induced by these constructs in mice were not statistically different (data not shown). This indicates that M2eP already contains an optimal M2e copy number and further improvement should focus on other aspects of the vaccine. Data from our previous studies clearly demonstrate that humoral IgG responses to the M2eP vaccine (containing 24 M2e copies per particle) are tilted more toward the carrier-P particle than to the M2e epitope [20,40]. However, in this study, we have lessened the P-particle dominance by increasing the M2e copy number to 72 per particle without reducing M2e-specific IgG responses (Fig. 1B). A similar observation was made with hepatitis B virus core (HBc) where increasing the M2e copy number in M2e-HBc particles dramatically reduced HBc immunodominance in the chimeric vaccine [73]. It might be possible to improve our vaccines further by modifying the immunodominant regions of the carrier particles [73,75].

In conclusion, the AtCYN is a promising antigen delivery platform which can easily accommodate antigens of larger sizes compared to the P particle platform. HA2-AtCYN vaccine was able to induce robust local and systemic antibody responses in chickens depending on the route of administration. Further, the outcome of M2eP + HA2-IN vaccination supports the hypothesis that mechanistically different conserved epitope vaccines can be combined to produce broadly protective regimens against different AIV subtypes. Furthermore, the mucosal system is a promising target for combined vaccination. Mucosal immunization of chickens with combinations of different vaccines carrying cross-subtype-conserved viral epitopes will be a focus of our future studies.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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