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Heterosubtypic protection against avian influenza virus by live attenuated and chimeric norovirus P-particle-M2e vaccines in chickens

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ABSTRACT

Avian influenza in poultry continues to be a great concern worldwide, and the currently licensed inactivated influenza vaccines are not effective against the novel strains of influenza virus that continue to emerge in the field. This warrants the development of more broadly protective influenza vaccines or vaccination regimens. Live attenuated influenza vaccines (LAIVs) and subunit vaccines derived from viral peptides, such as the highly conserved ectodomain of influenza virus matrix protein 2 (M2e), can offer a more broadly reactive immune response. In chickens, we previously showed that a chimeric norovirus P particle containing M2e (M2eP) could provide partial but broad immunity, when administered as a standalone vaccine, and also enhanced the protective efficacy of inactivated vaccine when used in a combination regimen. We also demonstrated that a naturally-selected NS1-truncated H7N3 LAIV (pc4-LAIV) was highly efficacious against antigenically distant heterologous H7N2 low pathogenicity avian influenza virus challenge, especially when used as the priming vaccine in a prime-boost vaccination regimen. In this study, we investigated the cross-subtype protective efficacy of pc4-LAIV in conjunction with M2eP using single vaccination, combined treatment, and prime-boost approaches. Chickens vaccinated with pc4-LAIV showed significant reduction of tracheal shedding of a low pathogenicity H5N2 challenge virus. This cross-subtype protective efficacy was further enhanced, during the initial stages of challenge virus replication, in chickens that received a vaccination regimen consisting of priming with pc4-LAIV at 1 day of age and boosting with M2eP. Further, H5N2-specific serum IgG and pc4-LAIV-specific hemagglutination-inhibition antibody titers were enhanced in LAIV-primed and M2eP boost-vaccinated chickens. Taken together, our data point to the need of further investigation into the benefits of combined and prime-boost vaccination schemes utilizing LAIV and epitope-based vaccines, to develop more broadly protective vaccination regimens.

1. Introduction

Avian influenza continues to be a great concern for poultry and humans worldwide. Eradication of avian influenza viruses seems to be the perfect preventive measure to control the disease in poultry [1,2]. However, the experience of the last decade, especially in developing countries, has shown the limitation of traditional biosecurity and stamping-out strategies in the control of avian influenza epidemics [1,2]. Unfortunately, the currently licensed poultry influenza vaccines, consisting of whole inactivated virus particles, are unable to efficiently inhibit or reduce the replication and shedding of newly emerging field strains [1]. This can be due to low cross-reactivity between the vaccine and field strains either as a result of antigenic changes in their surface proteins or appearance of new strains with different hemagglutinin (HA) subtypes [1]. Consequently, novel field strains may still emerge and become established in vaccinated flocks [3,4]. Therefore, efforts should be made to further alleviate the environmental contamination and spread of avian influenza viruses by incorporation of a more broadly protective vaccination regimen into the traditional control strategies [5].

While the conventional inactivated influenza vaccines (IIVs) remain the most accessible means of influenza immunization in

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poultry [5], their protective efficacy is restricted to a narrow spectrum of influenza virus strains that are closely related to the dominant viral surface antigens (mainly HA protein) in each vaccine [5]. In contrast, experimental subunit vaccines based on the highly conserved ectodomain of influenza virus matrix protein 2 (M2e) are broadly effective against a panel of divergent influenza viruses in mice [6,7]. In chickens, we previously showed that a chimeric norovirus P particle containing M2e (M2eP) could elicit high levels of anti-M2e IgG antibodies able to bind to native M2e expressed on the surface of infected cells or on whole viral particles, and inhibit viral replication *in vitro* [8]. However, although M2eP as a standalone vaccine was able to broadly reduce the post-challenge shedding of low pathogenicity avian influenza (LPAI) viruses of different subtypes, the protective efficacy was minimal compared with IIV [8,9]. Interestingly, supplementation of IIV with M2eP in chickens resulted in an enhanced protection against challenge viruses when compared to IIV or M2eP alone, potentially due to the enhanced induction of cross-reactive IgG antibodies to the virus particles [8]. This prompted us to investigate whether the M2eP vaccine can be used to enhance and broaden the protective efficacy of live influenza vaccine in a combination vaccination regimen.

Live attenuated influenza vaccines (LAIVs) are known to elicit robust humoral and mucosal immune responses, as well as broadly-reactive cell-mediated immunity in humans and other species including chickens [10–13]. In chickens, we have demonstrated the ability of a naturally-selected NS1-truncated H7N3 LAIV (pc4-LAIV) [14], with a unique set of biologically active particle subpopulations [15], to protect against antigenically distant heterologous H7N2 challenge virus, especially when it was used as the priming vaccine in a prime-boost vaccination regimen with IIV [14,16,17]. The protective efficacy of pc4-LAIV correlated with its strong ability to promote a rapid induction of high levels of mucosal and systemic innate and adaptive immune responses, even in young, immunologically immature chickens [16,17]. Although the immune response to LAIV is mostly evident against highly variable viral surface antigens and remains mainly subtype-specific [18], the use of LAIV mimics the natural influenza virus infection and induces broadly reactive innate and adaptive immune responses against conserved viral antigens [19–21]. Accordingly, priming with LAIV may provide a pool of broadly protective M2e-specific memory immune cells, which can enhance the boosting effect of M2eP [22–24]. This further encouraged us to investigate the potential priming effects of pc4-LAIV (hereinafter referred to as LAIV) for M2eP vaccine.

In this study, we investigated the cross-subtype protective efficacy of LAIV and M2eP vaccines using single vaccination, combined, and prime-boost approaches against a H5N2 LPAI challenge virus. Our data demonstrated the ability of H7N3 LAIV to significantly reduce the shedding of the challenge virus beyond its vaccine subtype (i.e., heterosubtypic protection). In addition, the protective efficacy of LAIV was enhanced in M2eP-boosted chickens during the early stages of infection. Furthermore, our results demonstrated that the ability of LAIV to induce rapid and robust immune responses [16] can be augmented by the M2eP vaccine [8] to enhance HI antibody response and the presence of cross-reactive whole virus-specific IgG antibodies in the vaccinated birds.

2. Materials and methods

2.1. Chickens and ethics statement

The care, management, and euthanasia of chickens were performed as previously reported in detail [8,16]. In brief, all chickens were maintained, vaccinated, challenged and euthanized according to the protocol #2009AG0002-R2 approved by The Ohio State University Institutional Animal Care and Use Committee (IACUC). This protocol follows the U.S Animal Welfare Act, Guide for Care and Use of Laboratory Animals and Public Health Service Policy on Humane Care and Use of Laboratory Animals. White leghorn chickens were obtained from a specific-pathogen-free (SPF) flock maintained in our on-site facility (Food Animal Health Research Program, The Ohio State University, Wooster, Ohio). No animal died of influenza or unrelated injuries during the entire course of study.

2.2. Virus propagation and purification

All viruses used in this study were obtained from our repository at the Food Animal Health Research Program and passaged once in 10-day-old SPF embryonated chicken eggs (ECEs) to prepare stocks for animal experiments. The LPAI virus, A/chicken/PA/13609/93 (H5N2), was used for challenge infection as described previously [25]. For whole virus ELISA, both the H5N2 challenge virus and the H7N3 vaccine strain were propagated in Madin-Darby canine kidney (MDCK) cells. To purify the whole virus protein for ELISA, infectious cell culture media were purified by equilibrium centrifugation in sucrose gradients as described previously [26].

2.3. Live-attenuated influenza vaccine (LAIV)

The pc4-LAIV used in this study is a mutant of A/TK/OR/71 (H7N3) virus that encodes a C-terminal truncated NS1 protein [14]. This vaccine has been shown to induce high levels of type I IFN in chick embryo cells [15], trigger upregulation of IFN-stimulated genes and rapid adaptive immune response in chickens [14,16,17], and protect chickens from heterologous H7 virus challenge [14,16]. Vaccination was done via intraocular (IO) and intranasal (IN) routes (0.05 ml in each nostril and eye) at a dose of 10^6 median egg infectious doses (EID_{50}) per bird (in 0.2 ml total volume) [8,9,16].

2.4. M2e-P particle (M2eP) chimeric vaccine

The M2eP is a chimeric particle constructed with norovirus P particle and avian influenza consensus M2e (MSLLTEVETPTRNGWECKCSDSSD) that was constructed as described previously [8,9,27]. Each dose of M2eP contained 5 μg of protein/bird and was delivered via the subcutaneous route (SQ) with commercial mineral oil adjuvant (Montanide™ ISA 70 VG, Seppic, Paris, France) (M2eP/Adjuvant, 3:7 V/V ratio; 0.2 ml/dose) [9].

2.5. Experimental design

2.5.1. Trial 1: Combined priming of 2 weeks-old chickens with LAIV and M2eP followed by one M2eP-booster

Two-week-old SPF chickens were divided into 5 groups (n = 7 birds per group) as shown in Table 1: mock control (Mock), single dose of M2eP (M2eP1x), prime-boost with M2eP (M2eP2x), single dose of LAIV (LAIV), and combined LAIV and M2eP2x

Table 1
Vaccination groups and schedule in Trial 1.

Groups	Vaccination and Challenge Schedule		
	2 weeks	4 weeks	6 weeks
Mock	PBS	PBS	Challenge
M2eP1x	M2eP	PBS	Challenge
M2eP2x	M2eP	M2eP	Challenge
LAIV	LAIV	PBS	Challenge
Combined-primed	LAIV&M2eP	M2eP	Challenge

(Combined-primed). Chickens were vaccinated with pc4-LAIV (IO & IN), M2eP (SQ), or both vaccines at 2 weeks of age and boosted once with M2eP at 4 weeks of age in prime-boost groups (Combined-primed and M2eP2x). Mock vaccinated birds were immunized with an emulsified solution of sterile phosphate buffered saline (PBS, Gibco Life Technologies, Grand Island, NY) mixed with ISA70 VG adjuvant. Each bird was intranasally challenged with 10^6 EID_{50} doses (in 0.2 ml volume) of the H5N2 LPAI virus at 6 weeks of age.

2.5.2. Trial 2: Priming of 1-day-old chickens with LAIV followed by three M2eP-boosters

Day-old SPF chickens were divided into 4 groups (n = 8 birds per group) as shown in Table 2: Mock, 3 doses of M2eP (M2eP3x), primed with LAIV and mock-boosted with PBS + ISA70 VG (LAIV), and primed with LAIV and boosted with M2eP for 3 times (LAIV-primed). Chickens were vaccinated with LAIV at 1 day of age, and with either M2eP or PBS + ISA70 VG at 2, 4, and 6 weeks of age. All birds were challenged with the H5N2 LPAI virus at 8 weeks of age as described for the Trial 1.

2.6. Blood and tear sample collection

All birds were bled on a biweekly basis, prior to each vaccination or challenge time-points, during the experiment to collect serum for antibody detection. The serum was separated from other blood components and heat inactivated at 56 °C for 30 min. In addition, tear samples (approximately 50 μl of tears per eye) were collected two weeks after the LAIV vaccination (4 weeks of age) in Trial 1 and one week prior to challenge (7 weeks of age) in Trial 2. Lachrymation was induced as previously described [28]. Briefly, excess lachrymation was induced by sprinkling approximately 3 mg of sodium chloride crystals onto each eye while keeping the eyelids open. Tears were harvested using sterile micropipette tips into sterile tubes and stored at −20 °C until used for antibody detection.

2.7. Determination of hemagglutination inhibition (HI) and M2e-specific IgG in serum, and avian influenza virus-specific secretory IgA in tears

HI antibody titers were determined using two-fold serially diluted serum samples, 8 hemagglutinating units (HAUs) of live virus antigens (H7N3 LAIV and H5N2 challenge viruses) and 1% turkey erythrocyte suspension as previously described [29]. Sera with HI titers $\geq$ 2 HAUs were considered seroconverted.

M2e-specific antibody responses were determined by ELISA using synthetic M2e peptide (Ohio Peptide, Powell, OH) as a coating antigen (200 ng/well) in Nunc MaxiSorp 96-well microtiter plates (Thermo Fisher Scientific, Waltham, MA) as previously described [9,27].

Local IgA antibodies in tears were measured using a commercial avian influenza virus ELISA kit (IDEXX AI Ab Test, Westbrook, ME) according to the manufacturer's recommendation with minor modifications. Briefly, 1:10000 dilution of goat anti-chicken horse reddish peroxidase (HRP) labeled alpha-chain specific goat-anti-chicken IgA conjugates (Gallus Immunotech Inc, ON, Canada) were used as the secondary antibody instead of the anti-IgG conjugate provided in the kit. The coating antigen that detects all subtypes of avian influenza virus is proprietary.

Table 2
Vaccination groups and schedule in Trial 2.

Groups	Vaccination and Challenge Schedule			
	2 weeks	4 weeks	6 weeks	8 weeks
Mock	PBS	PBS	PBS	Challenge
M2eP3x	M2eP	M2eP	M2eP	Challenge
LAIV	PBS	PBS	PBS	Challenge
LAIV-primed	M2eP	M2eP	M2eP	Challenge

2.8. Determination of whole virus specific IgG antibody titers and plaque reduction viral neutralization activity in serum

Pre-challenge sera from both trials were subjected to whole virus IgG ELISA to measure the cross-reactive antibodies using purified vaccine (H7N3 LAIV) and challenge (H5N2 LPAI) viruses as coating antigens. Whole virus specific IgG antibody responses to different virus strains were determined by ELISA using 400 ng of the purified whole virus protein as the coating antigen in each well of the microtiter plate as previously described in detail [8].

Pre-challenge sera from each group (n = 3, selected based on the reduction in the challenge virus shedding) were subjected to plaque reduction viral neutralization assay as previously described [8]. Briefly, MDCK cells were seeded in 6-well plates at a density of 1×10^6 cells/well and allowed to grow to confluence overnight. Pre-challenge sera dilutions (1:4, 1:10, and 1:100) were mixed with pre-diluted 100 plaque-forming units of the H5N2 challenge virus (1:1), and incubated at 37 °C for 60 min. MDCK cells were washed once with PBS before infection with 200 μl of the virus-antibody mixture per well. Virus attachment was done by incubating the cells at 37 °C for 60 min in the CO_2 incubator with rocking them every 15 min. At the end of the attachment step, the plates were washed once with DMEM and overlaid with Minimum Essential Medium plus 0.6% agarose containing 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 dilution, cell control, and virus control were tested in duplicate. As seen in the formula below, plaque reduction (%) is calculated as the complement of the ratio of number of plaques in tested sera to the average number of plaques in mock group times 100.

$$\textit{Plaque reduction}(\%) = 100 \times \left(1 - \frac{\#\ \textit{of plaques in tested sera}}{\textit{average}\ \#\ \textit{of plaques in mock group}}\right)$$

2.9. Tracheal swab collection and quantification of viral RNA

Tracheal swabs (PurFlock Ultra 6″ Sterile Mini-tip Flock Swab, Puritan Diagnostics, Guilford, ME) were collected from all birds at 2 and 4 days post-challenge (DPC) in Trial 1, and at 3 and 5 DPC in Trial 2 to determine the tracheal viral shedding as previously described [8,9]. Briefly, the swabs were eluted in 1 ml of PBS supplemented with gentamicin (10 μg/ml, Gibco Life Technologies, Grand Island, NY) by freeze-thawing (2 times) and vigorous vortexing. Viral RNA was extracted from 100 μl of the supernatant using QIAamp Viral RNA Mini Kit® (Qiagen, Valencia, CA) according to the manufacturer's instructions. Quantification of viral RNA was done by quantitative real-time RT-PCR (qRT-PCR) using primers and probes specifically designed for influenza A virus matrix gene as previously described [30]. Tracheal virus shedding titers were calculated as EID_{50} equivalents based on the threshold cycle (Ct) values in qRT-PCR as described previously [8,9].

2.10. Statistical analysis

All data were analyzed using IBM SPSS Statistics version 24 (IBM, New York, NY) software. Statistical differences among groups were determined by the one-way analysis of variance (ANOVA) followed by least-square difference (LSD) post-hoc test. Analyses

between two groups in experiments with only two groups were performed with the paired-samples t test. Statistical significance was determined at p values (p) ≤ 0.05. All graphs were generated using GraphPad Prism 6.07 (GraphPad Software, San Diego, CA).

3. Results

3.1. Trial 1: Combined priming of 2 weeks-old chickens with LAIV and M2eP followed by one M2eP-booster

In our previous study in SPF chickens, we demonstrated that supplementation of IIV with M2eP, in combined vaccination regimens that started at 2 weeks of age, resulted in enhancement of protective efficacy against heterologous H7N2 LPAI virus challenge [8]. To explore the potential benefit of M2eP supplementation on the heterosubtypic protective efficacy of LAIV, 2 weeks-old birds were primed with a combination of the H7N3 LAIV and M2eP, and boosted with M2eP after two weeks as shown in Table 1. Vaccination schedules for other groups included in this trial are detailed in Table 1 and the Materials and Methods.

3.1.1. Induction of HI antibodies and M2e-specific IgG in pre-challenge serum, and secretory IgA in tears

As expected based on our previous study [17], the median $\log_2$ titers of homologous anti-H7 HI antibodies (homologous to the H7 LAIV) in sera from the LAIV group were 3 at 4 and 6 weeks of age (Fig. 1A). Although the median anti-H7 HI titer in the Combined-primed group was slightly elevated at 4 weeks of age, there was no statistical difference between the LAIV and Combined-primed groups (Fig. 1A). Heterosubtypic anti-H5 HI antibodies against the H5 challenge virus were not detected in the pre-challenge sera. Additionally, anti-H7 and anti-H5 HI antibodies were not detected in non-LAIV vaccinated groups (Mock, M2eP1x, and M2eP2x).

High titers of M2e-specific IgG antibodies were present in M2eP-vaccinated chickens (Fig. 1B), which is in agreement with our previous studies [8,9]. Two weeks after the initial M2eP dose (i.e., at 4 weeks of age), the mean $\log_2$ titers of anti-M2e IgG were around 10 for M2eP1x and M2eP2x groups. At 6 weeks of age, the mean titer in the M2eP2x group was significantly higher compared to the M2eP1x. Low but significant levels of M2e-specific antibodies were present in the LAIV group compared to Mock. No significant differences in the levels of M2e-specific antibodies were observed between the Combined-primed and M2eP2x groups (Fig. 1B).

Vaccine-induced mucosal immunity was evaluated by measuring the levels of influenza virus-specific secretory IgA in tears of 4 weeks-old chickens (i.e., 2 weeks after the first vaccination). The levels of virus-specific tear IgA in the M2eP2x and LAIV groups were not significantly different from the Mock (Fig. 1C). Even though elevated levels of tear IgA were detectable in both LAIV and Combined-primed groups, only the Combined-primed group had a significantly higher mean IgA titer when compared to the Mock and M2eP2x groups (Fig. 1C).

3.1.2. Whole virus-specific serum IgG antibodies and their in vitro cross-neutralization

All vaccinated groups had high levels of serum IgG antibodies that were reactive to the whole virus in ELISA (Fig. 2A). Significantly higher titers of H7N3 LAIV- and H5N2 challenge virus-specific IgG antibodies were present in the LAIV and Combined-primed groups, when compared to the Mock and M2eP (M2eP1x and M2eP2x) groups (Fig. 2A). No significant difference was observed between the LAIV and Combined-primed groups.

The ability of the pre-challenge sera to block the replication of H5N2 challenge virus *in vitro* was assessed by plaque reduction virus neutralization assay. Serum antibodies from LAIV and Combined-primed groups showed a weak (~10–20% plaque reduction in 1:4 dilution of sera), but significant neutralization of the H5N2 challenge virus compared to the Mock and M2eP (M2eP1x and M2eP2x) groups (Fig. 2B). On the other hand, M2eP1x and M2eP2x vaccinations did not induce significant levels of neutralizing antibodies compared with the Mock (Fig. 2B).

3.1.3. Protective efficacy against H5N2 LPAI challenge virus

In accordance with our previous study [9], the H5N2 challenge virus replicated to high titers (mean $\log_2$ EID_{50} titer > 5) in the trachea of mock-vaccinated birds at 4 DPC (Fig. 3). The Combined-primed group showed a significant reduction in tracheal shedding at 2 DPC when compared to the Mock and M2eP1x groups. At 4 DPC, LAIV and Combined-primed groups shed significantly lower virus titers compared to the Mock and M2eP1x groups. The LAIV group also showed significant reduction of virus shedding compared with the M2eP2x group. However, no significant difference was observed between the LAIV and Combined-primed groups at 4 DPC. Additionally, a single or two doses of M2eP vaccine (M2eP1x and M2eP2x) did not significantly reduce the virus shedding in this study, but a slight decrease of virus titer was apparent at 4 DPC (Fig. 3).

3.2. Trial 2: Priming of 1-day-old chickens with LAIV followed by three M2eP-boosters

We previously demonstrated that priming of 1-day-old chickens with LAIV followed by an IIV booster results in the stimulation of robust mucosal and humoral immune responses and almost complete protection against heterologous H7N2 LPAI virus challenge [17]. The benefit of priming with the H7N3 LAIV and boosting with the M2eP vaccine, in protection against the H5N2 challenge virus, was investigated in this trial. After priming 1-day-old chickens with LAIV, three booster doses of M2eP were administered at 2, 4, and 6 weeks of age as shown in Table 2. Vaccination schedules for other groups included in this trial are detailed in Table 2 and the Materials and Methods.

3.2.1. HI antibodies and M2e-specific IgG in pre-challenge serum, and secretory IgA in tears

Most birds in the LAIV and LAIV-primed groups did not have detectable levels of serum anti-H7 HI antibodies (against the H7N3 LAIV) at 2 weeks of age (Fig. 4A). Peak anti-H7 HI titers were observed in 4 weeks-old chickens (i.e., 4 weeks after vaccination with LAIV) for both groups. Interestingly, while the anti-H7 HI antibodies in the LAIV-primed group were maintained at the peak level in 6 and 8 weeks-old birds, there was a significant decrease in the mean titers of the LAIV group at these sampling time-points (Fig. 4A). Anti-H5 HI antibodies (against the H5N2 challenge virus) were not detected in the pre-challenge sera. Additionally, neither anti-H7 nor anti-H5 HI antibodies were detected in Mock and M2eP3x groups.

The titers of M2e-specific serum IgG antibodies in the LAIV and LAIV-primed groups were very low at 2 weeks of age (Fig. 4B). These antibodies remained low in the LAIV group at all pre-challenge sampling time-points. The average titer of anti-M2e IgG in the M2eP3x group was comparable to that reported in our previous study [8]. No significant difference in the mean M2e-specific antibody titer was observed between LAIV-primed and M2eP3x groups (Fig. 4B).

Influenza virus-specific secretory IgA levels were measured using tears collected at 7 weeks of age (1 week prior to challenge) (Fig. 4C). Tear IgA levels in all vaccinated groups were significantly higher compared with the Mock. Among the vaccinated groups, significantly higher tear IgA levels were present in LAIV and

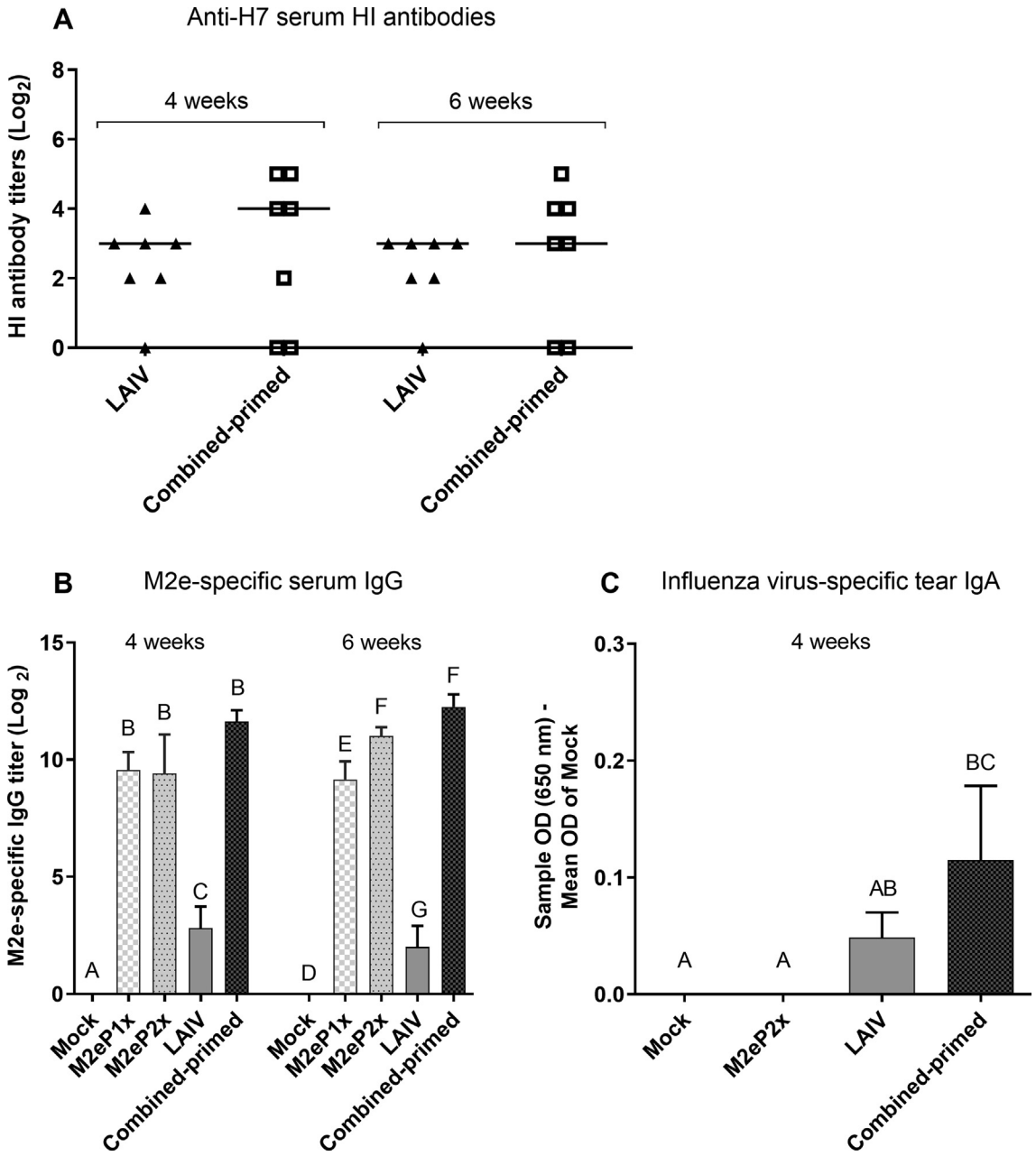

Fig. 1. HI antibody and M2e-specific IgG in serum, and secretory IgA in tears (Trial 1). Chickens were vaccinated as described in Table 1. Sampling time-points are indicated as weeks (of age) on the plots. (A) HI antibody titers against H7N3 LAIV. Horizontal lines represent the median HI titer of the groups. Non-LAIV vaccinated groups were excluded from the graph, as no birds in these groups were seroconverted in the HI assay. The mean HI titer of LAIV vaccinated birds, regardless of M2eP treatment, were statistically significant compared to Mock and M2eP groups. HI titers against the H5N2 challenge virus antigen were below the detection level and are not included in this plot. (B) M2e-specific IgG antibody titers. Log_2 based M2e-specific endpoint IgG titers were determined by ELISA using a synthetic avian M2e peptide as the coating antigen. Each bar represents the mean ± standard error of mean per group. (C) Virus-specific secretory IgA antibody in tears. Avian influenza-specific IgA antibody titers were determined using commercial IDEXX ELISA kit. Each bar represents the mean sample OD – the mean OD of Mock at 650 nm ± standard error of mean per group. Different letters inside the plot denote statistically significant differences among the groups as determined by one-way ANOVA followed by LSD post-hoc test ($p \leq 0.05$).

LAIV-primed groups compared with the M2eP3x group. No significant difference in IgA level was observed between the LAIV and LAIV-primed groups (Fig. 4C).

3.2.2. Whole virus-specific serum IgG antibodies and their in vitro cross-neutralization

Whole virus-specific serum IgG titers of the vaccinated groups were significantly higher than the Mock group regardless of whether the ELISA coating antigen was derived from the H7N3 LAIV or H5N2 challenge virus (Fig. 5A). Among the vaccinated groups, whole virus-specific IgG titers were significantly higher in the LAIV and LAIV-primed groups compared with the M2eP3x group. A slight, but significant, increase of the mean IgG titer against the H5N2 challenge virus was observed in the LAIV-primed group compared with the LAIV group (Fig. 5A).

In addition, low levels of H5N2 virus neutralizing antibodies were detected in all vaccinated birds (Fig. 5B). A significant neutralization of the H5N2 virus was only observed in the LAIV-primed group when compared with the Mock (Fig. 5B).

3.2.3. Protective efficacy against H5N2 LPAI challenge virus

At 3 DPC, the LAIV-primed group was the only group that showed a significant reduction in tracheal shedding of the H5N2 LPAI challenge virus compared with the other groups (Fig. 6). At

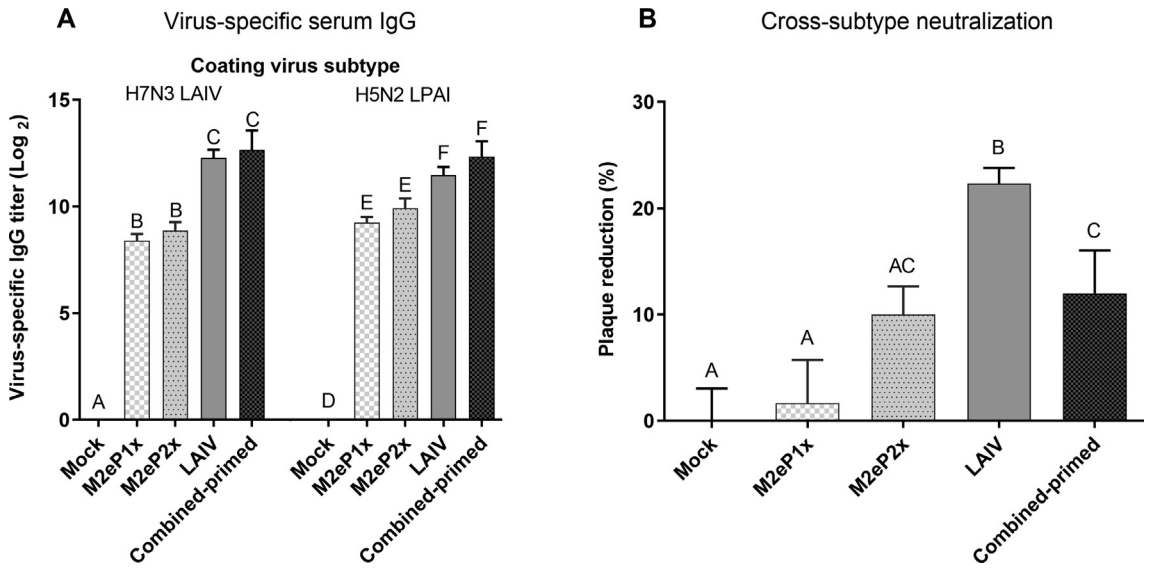

Fig. 2. Whole virus-specific serum IgG antibodies and their *in vitro* cross-neutralization (Trial 1). Chickens were vaccinated as described in Table 1. Pre-challenge sera were taken at 6 weeks of age. (A) Pre-challenge Log_2 based virus-specific endpoint IgG titers were determined by ELISA using purified whole virus preparations from the H5N2 challenge virus and H7N3 LAIV as the coating antigens. Each bar represents the mean ± standard error of mean per group. (B) The neutralization ability of the pre-challenge sera for H5N2 LPAI challenge virus was determined by plaque reduction neutralization assay using 1:4 dilution of serum samples. Each bar represents the mean ± standard error of mean per group. Different letters inside the plot denote statistically significant differences among the groups at each time-points as determined by one-way ANOVA followed by LSD post-hoc test ($p \leq 0.05$).

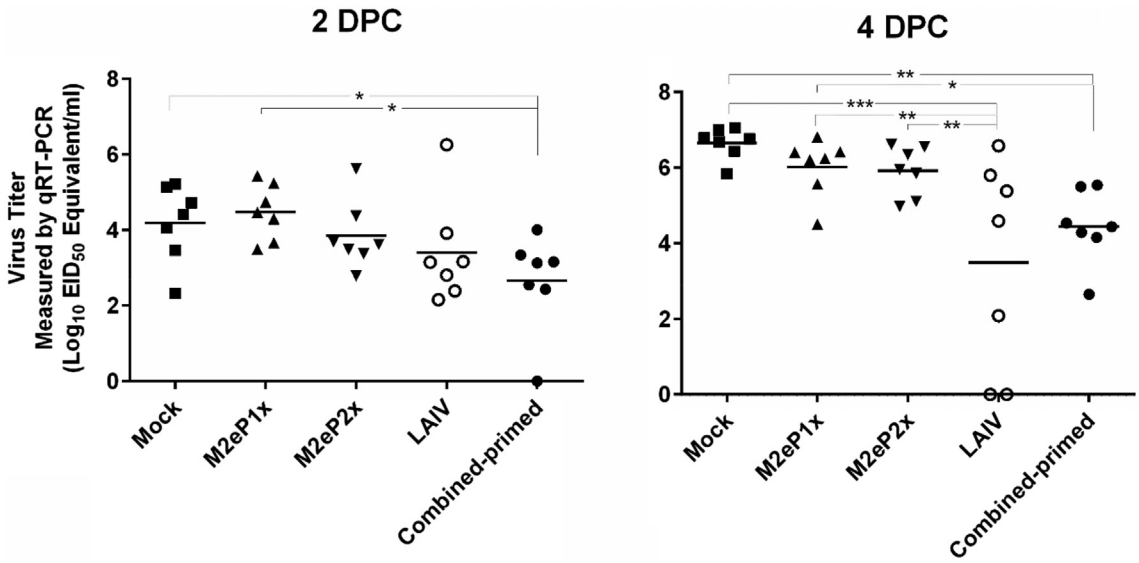

Fig. 3. Protective efficacy of LAIV and M2eP vaccines against heterosubtypic H5N2 LPAI virus challenge (Trial 1). Tracheal virus shedding titers expressed as median egg infectious doses (EID_{50}) equivalent in each ml of the tracheal swab elutes were determined using qRT-PCR. Tracheal swabs were collected at 2 and 4 days post challenge (DPC). Horizontal bars represent the mean viral shedding equivalent per group. Asterisks denote statistically significant differences at $p \leq 0.05$ (*), $p \leq 0.01$ (**), and $p \leq 0.001$ (***) as determined by one-way ANOVA followed by LSD post-hoc test.

5 DPC, the tracheal virus shedding was significantly less in LAIV and LAIV-primed groups compared to the Mock. Further, the tracheal shedding in the LAIV group was also significantly less than M2eP3x group (Fig. 6).

4. Discussion

While M2e-based vaccines have demonstrated robust protection in mice [7,27,31], they are poorly protective as stand-alone vaccines in chickens [8,32]. We recently demonstrated that supplementation regimens consisting of M2eP and IIV were more efficacious compared to each vaccine alone [8]. In this study, M2eP vaccine did not significantly reduce tracheal shedding of the H5N2 challenge virus. However, the addition of M2eP in the LAIV-primed regimen resulted in significant enhancement of anti-H7 HI and H5N2 virus-specific IgG antibody responses and improvement of protection against H5N2 LPAI virus challenge (Figs. 4A, 5A, and 6, compare LAIV with LAIV-primed).

Influenza virus mutants that encode truncated NS1 protein have proved their potential as LAIV candidates in chickens when used against homologous and heterologous LPAI challenge viruses [14,16,17,33]. The current study sought to answer a fundamental question as to whether the broad immunity induced by LAIVs

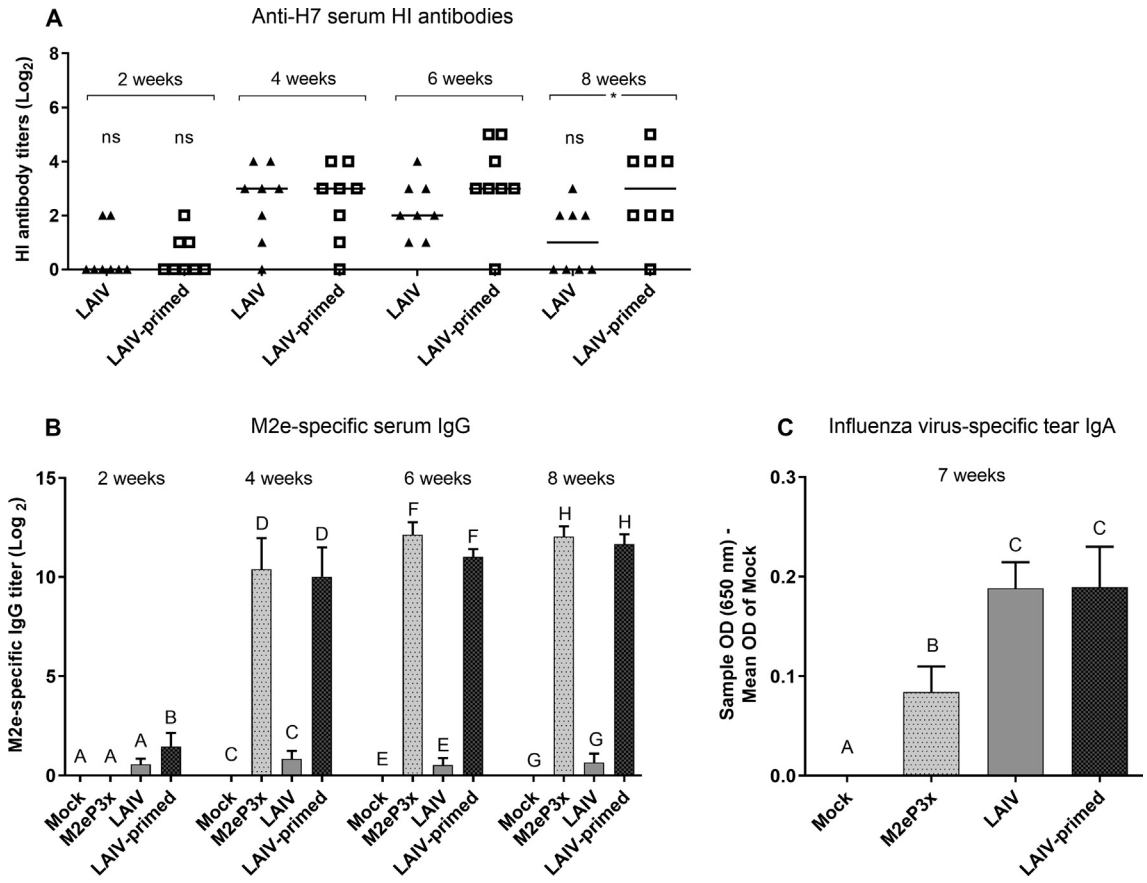

Fig. 4. HI antibody and M2e-specific IgG in serum, and secretory IgA in tears (Trial 2). Chickens were vaccinated as described in Table 2. Sampling time-points are indicated as weeks (of age) on the plots. (A) HI antibody titers against H7N3 LAIV. Horizontal lines represent the median HI titer of the groups. Non-LAIV vaccinated groups were excluded from the graph, as no birds in these groups were seroconverted in HI assay. The mean HI titer of LAIV vaccinated birds, regardless of M2eP treatment, were statistically significant compared to Mock and M2eP groups, unless marked as non-significant (ns) on the graph. The asterisk denotes statistically significant difference between the two groups at the indicated time-point as determined by unpaired *t* test ($p \leq 0.05$). HI titers against the H5N2 challenge virus antigen were below the detection level and are not included in this plot. (B) M2e-specific IgG antibody titers. Log_2 based M2e-specific endpoint IgG titers were determined by ELISA using a synthetic avian M2e peptide as the coating antigen. Each bar represents the mean ± standard error of mean per group. (C) Virus-specific secretory IgA antibody in tears. Avian influenza-specific IgA antibody titers were determined using commercial IDEXX ELISA kit. Each bar represents the mean sample OD – the mean OD of Mock at 650 nm ± standard error of mean per group. Different letters inside the plot denote statistically significant differences among the groups as determined by one-way ANOVA followed by LSD post-hoc test ($p \leq 0.05$).

can protect chickens from heterosubtypic virus infections. Our H7N3 LAIV candidate was able to reduce the tracheal shedding of H5N2 LPAI challenge virus (Figs. 3 and 6) to levels similar to those observed in previous heterologous challenge studies [14,16,17].

Serum HI antibodies may not be required for the LAIV-mediated cross-subtype protection since all pre-challenge sera did not have detectable levels of anti-H5 HI titers. Such a discrepancy between HI titers and the protection elicited from LAIV has been reported in mice and humans [34,35]. Data from the current study shows that high levels of cross-subtype secretory IgA, systemic IgG, as well as low levels of neutralizing serum antibodies are induced by LAIV (Figs. 1C, 2, 4C, and 5). We speculate that these antibodies are partially responsible for the cross-subtype protection provided by LAIV.

Even though the mechanisms involved in the heterosubtypic protection provided by LAIV have yet to be determined, the presence of IgA antibodies in chicken tears might further highlight the importance of mucosal immune responses in the heterosubtypic protection against influenza viruses. Mucosal-associated CD8 + cytotoxic T and B lymphocytes have been proposed to be involved in heterosubtypic protection of mice following mucosal (but not parenteral) vaccination [36,37]. This emphasizes that the complex nature of cross-subtype protection cannot be fully understood without first considering the effects of systemic and mucosal antibodies [38,39], as well as other components of the innate and cell-mediated immunity [13,36,40].

The HI antibodies induced by LAIV in 1-day-old chickens reached peak titers at 4 weeks post-vaccination, and thereafter gradually declined as the bird aged (Fig. 4A). A similar phenomenon was reported in chickens vaccinated with an NS1-trucated LAIV derived from a H9N2 virus [33]. Interestingly, the median HI antibody titer did not decrease after 4 weeks post vaccination in the LAIV-primed group compared with the LAIV group (Fig. 4A). This can be partially explained by the fact that M2e-specific memory CD4 + T helper cells may accelerate the production of IgG antibodies against not only M2e but also hemagglutinin protein as shown in a mice study [23].

The delayed and reduced rise in HI and M2e-specific antibody responses following LAIV vaccination in Trial 2 (relative to Trial 1) was likely due to the lack of proper antibody affinity maturation in 1-day-old chickens. This process is considered necessary for the induction of high avidity IgG antibodies [17,41], and may be a result of functional immaturity and low stimulation of Toll-like receptors of T cells in early age [42]. Indeed, early age vaccination with LAIV induces HI antibodies of lower avidity compared to chickens vaccinated later in life [17,41].

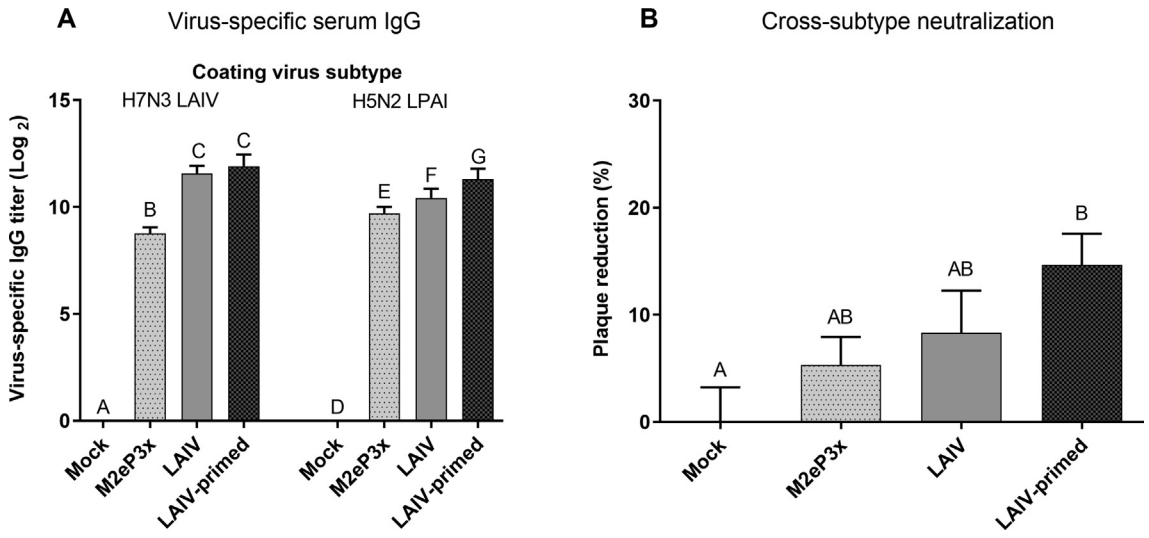

Fig. 5. Whole virus-specific serum IgG antibodies and their *in vitro* cross-neutralization (Trial 2). Chickens were vaccinated as described in Table 2. Pre-challenge sera were taken at 8 weeks of age. (A) Pre-challenge Log_2 based virus-specific endpoint IgG titers were determined by ELISA using purified whole virus preparations from the H5N2 challenge virus and H7N3 LAIV as the coating antigens. Each bar represents the mean ± standard error of mean per group. (B) The neutralization ability of the pre-challenge sera for H5N2 LPAI challenge virus was determined by plaque reduction neutralization assay using 1:4 dilution of serum samples. Each bar represents the mean ± standard error of mean per group. Different letters inside the plot denote statistically significant differences among the groups at each time-points as determined by one-way ANOVA followed by LSD post-hoc test ($p \leq 0.05$).

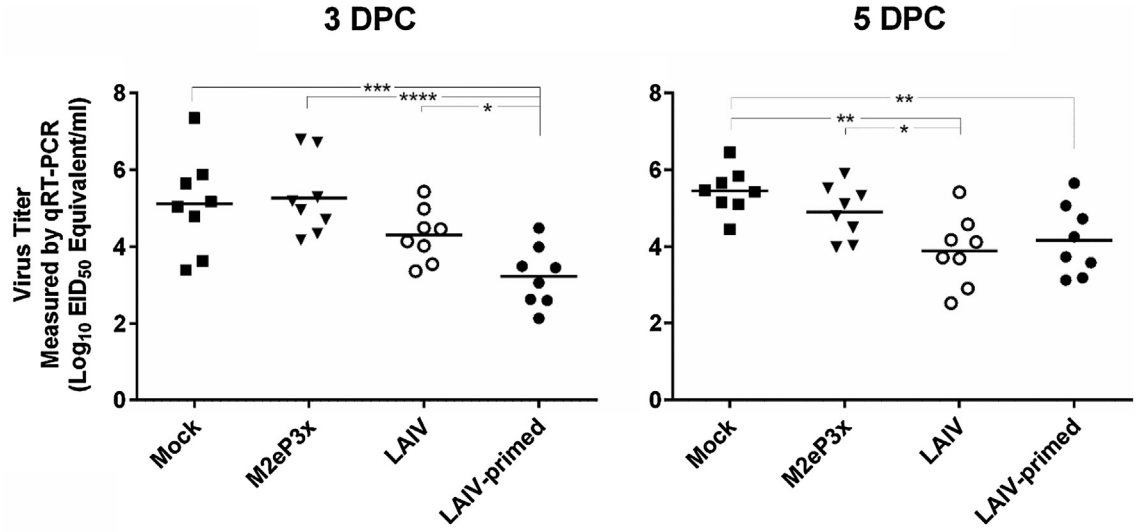

Fig. 6. Protective efficacy of LAIV and M2eP vaccines against heterosubtypic H5N2 LPAI virus challenge (Trial 2). Tracheal virus shedding titers expressed as median egg infectious doses (EID_{50}) equivalent in each ml of the tracheal swab elutes were determined using qRT-PCR. Tracheal swabs were collected at 3 and 5 days post challenge (DPC). Horizontal bars represent the mean viral shedding equivalent per group. Asterisks denote statistically significant differences at $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), and $p \leq 0.0001$ (****) as determined by one-way ANOVA followed by LSD post-hoc test.

The results of the whole-virus ELISA with H7N3 and H5N2 viruses have proven the limitation of HI assay in determining humoral immune response to LAIV [43]. The broadly reactive antibodies detected in ELISA may not efficiently neutralize the heterosubtypic challenge virus as demonstrated in Figs. 2 and 5, but they may facilitate viral clearance through antibody-dependent cell-mediated cytotoxicity (ADCC) [21,44]. The failure of M2eP booster vaccination to increase virus-specific antibody titers in the Combined- or LAIV-primed chickens might also be due to the robust antibody-stimulating attribute of LAIV, which seems to induce highly elevated levels of antibodies after the vaccination (Figs. 2A and 5A).

Unlike IIV in our previous study [8], LAIV vaccination was able to stimulate the production of M2e-specific antibodies, especially following vaccination at 2 weeks of age (Fig. 1B). This observation is in line with the presence of anti-M2e antibodies in convalescent human and mice antisera [44], which may be explained by the abundant expression of M2e on the surface of infected cells [8]. The induction of anti-M2e antibodies by LAIV seems to be highly dependent on the vaccination age (1-day-old vs. 2-week-old) and may be delayed or hampered in young (immunologically immature) birds compared to older birds.

In conclusion, the results of this study demonstrated that pc4-LAIV significantly reduced tracheal shedding of a heterosubtypic H5N2 challenge virus in SPF chickens. The partial protective efficacy of LAIV against heterosubtypic LPAI challenge virus was transiently enhanced by boosting with M2eP. With that said, the boost vaccination with 3 doses of M2eP may not be practical for

poultry under field conditions. Hence, the advancement of the combined vaccination schemes comprised of LAIV and epitope-based vaccines requires further investigation to develop broadly reactive vaccination regimens.

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Conflict of interest

The authors declare no conflict of interest.

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