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Sequential Aerosol and Oral Immunization with a Bivalent H9N2/H5N2 Vaccine Protects Against H5N1 and H9N2 Avian Influenza Challenges

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ABSTRACT

Avian influenza continues to be a major threat to poultry and public health. This study investigated the efficacy of a bivalent H9N2/H5N2 modified live virus (MLV) vaccine delivered through a sequential aerosol priming and drinking water boosting in chickens. We hypothesized that this vaccination strategy would induce robust protective immunity against both low pathogenic H9N2 and high pathogenic H5N1 avian influenza viruses. The results demonstrated that the bivalent MLV vaccine provided sterilizing immunity against homologous and antigenically drifted H9N2 virus challenges. While anti-H5 antibody responses after MLV vaccination were generally weak, a robust anti-N2 antibody response was observed. Notably, the bivalent MLV prime-boost group demonstrated 90% survival against a high-dose H5N1 HPAI challenge. These findings highlight the potential of this mass vaccination approach, utilizing aerosol delivery complemented by drinking water administration, as a convenient and cost-effective method for avian influenza control, particularly H9N2 and H5N1 HPAI.

KEYWORDS

Avian influenza, mass vaccination, vaccine development, H9N2, H5N1, HPAIV, LPAIV.

INTRODUCTION

Avian influenza (AI) continues to pose a significant global threat to both poultry production, agriculture, and public health. Highly pathogenic avian influenza (HPAI) viruses, particularly those of the H5N1 subtype, are responsible for severe disease and high mortality in poultry, leading to substantial economic losses [1]. These viruses also present a zoonotic risk, with documented cases of human infection [2, 3]. Concurrently, low pathogenic avian influenza (LPAI) H9N2 viruses are enzootic in many regions and can cause milder respiratory disease in poultry [4, 5]. H9N2s are also zoonotic viruses themselves with an unusual ability to recognize human-like sialic acid receptors [6, 7]. In addition, H9N2s are also recognized for their potential to reassort with HPAI viruses and contribute to the emergence of novel strains [6]. The continuous evolution and spread of both HPAI H5N1 and LPAI H9N2 underscore the urgent need for effective control strategies, with vaccination being a cornerstone of disease prevention and mitigation.

Traditional vaccination approaches, such as inactivated vaccines, have been widely used in poultry. However, these methods often require multiple doses, leading to increased costs and labor-intensive administration [8]. Moreover, inactivated vaccines may not consistently induce a robust, broad-spectrum immune response against diverse influenza virus strains [6, 8-10]. In contrast, modified live influenza virus vaccines (MLVs) offer an attractive alternative due to their ability to mimic natural infection, thereby eliciting both humoral and cellular immune responses [11]. A significant advantage of MLVs is their potential for rapid and widespread administration, facilitating the vaccination of a large number of animals in a short timeframe [12]. Despite their promise, the progress of MLV development for avian influenza in poultry has faced challenges related to concerns of reassortment and unintended transmission [8]. Our laboratory has previously established the safety and efficacy of genome rearrangement (RAM) as a robust platform for MLV development [13-15]. We have previously demonstrated that the monovalent H9N2 vaccine (MLV-H9N2-IL) did not undergo reassortment with wild-type viruses in chicken

coinfection studies, nor did it transmit from infected to naïve direct-contact animals [13]. These RAM vaccines have demonstrated effective protection in previous challenge studies, and the incorporation of additional molecular markers and immunomodulators, such as chicken interleukin (IL)-18, further enhances their protective capabilities and immune responses [13].

In poultry, mass vaccination is a common practice for millions of animals, with coarse spray vaccination being a widely adopted method for administering live vaccines against diseases such as infectious bronchitis, Newcastle disease, and coccidiosis [16-18]. This method typically employs sprayers that produce coarse droplets (100-150 microns) to ensure vaccine delivery to the upper respiratory tract. Vaccines are generally reconstituted with fresh, clean, non-chlorinated water to maintain viral viability. This vaccination method is frequently performed on day-old chicks at the hatchery and can be used in the field for booster vaccinations [19]. However, effective coarse spray vaccination relies on even distribution of the vaccine over the flock, which can be challenging, particularly with larger droplet sizes that are more susceptible to uneven coverage. Environmental control, such as turning off fans to minimize vaccine drift and evaporation, is also crucial.

Aerosol vaccination in poultry, while theoretically effective due to its ability to target the primary site of viral entry, is not traditionally performed as a widespread method because it has not been fully developed and optimized for mass application. To address this gap, we have designed a calm-aerosol settling chamber system [20]. In this report, we show that this accessible and cost-effective system has the potential for aerosol vaccination of large numbers of birds simultaneously, targeting the respiratory tract, which is a primary site of viral entry and replication for avian influenza viruses. Subsequent oral boosting via drinking water provided a convenient and stress-free method for re-exposing birds to the vaccine, reinforcing systemic and mucosal immunity. Using this system, we developed a bivalent MLV-H9N2-IL/MLV-H5N2-IL vaccine and investigated its efficacy using a sequential aerosol priming followed by drinking

water boosting. We hypothesized that this dual-route vaccination strategy would induce robust protective immunity against both H9N2 LPAI and H5N1 HPAI challenges. Our data revealed a strong anti-H9 and anti-N2 antibody response after vaccination. However, we observed lower anti-H5 antibody responses. Despite the lower anti-H5 antibody titers, our findings demonstrate robust protection against a high-dose H5N1 challenge. Furthermore, the bivalent vaccine provided strong protection against both homologous and antigenically drifted H9N2 strains. These results highlight its potential as a valuable vaccine candidate for avian influenza control.

RESULTS

Limited replication of the MLV vaccine after aerosol administration in one-day-old chickens

Day-old chickens were immunized via a 15-min aerosol exposure to a 5 mL inoculum containing 1×10^7 EID50/mL. Following a 5-min chamber purge, the chickens were transferred to isolators. The vaccinated groups received either a monovalent MLV-H9N2-IL vaccine or a bivalent MLV-H9N2-IL/MLV-H5N2-IL vaccine. For comparison, a whole-virus-inactivated-adjuvanted (WIV-adj-H9N2/H5N2) vaccine was administered subcutaneously at a dose of 512 hemagglutination units (HAU) (Fig. 1a). Following the initial aerosol vaccination, virus shedding was transiently detected from the monovalent and bivalent MLV groups only on day 1 post-prime (dpp) in oropharyngeal (OP) swabs, becoming undetectable by day 3 (Fig. 1b). This finding aligns with the previously established limited replication of these MLVs in chickens**.** No viruses were detected in cloacal (CL) swabs (Fig. 1b). Booster vaccinations were subsequently administered: the monovalent MLV group received a second dose via drinking water, half of the bivalent MLV group received a second bivalent dose also via drinking water, and the WIV-adj group received a subcutaneous boost. Virus shedding was not assessed after these booster doses, as previous studies established that MLV boosting results in undetectable shedding [13].

Aerosol and drinking water vaccination induce antibody responses against H9, N2, and NP, but weak antibody responses against H5

Blood samples were collected from all groups at 14 days post-prime (dpp) and 14 days post-boost (dpb). All vaccinated groups developed hemagglutination inhibition (HI) titers against H9 after the prime vaccination (14 dpp), including the monovalent MLV, bivalent MLV, and the WIV-adj-H9N2/H5N2 (Fig. 2a). HI titers against H9 were similar between the monovalent and bivalent MLVs. Not surprisingly, the HI titers in the WIV-adj-H9N2/H5N2 group were at least two-fold higher compared to the MLV groups after the initial vaccination (Fig. 2a). Following the boost vaccination, animals that received a booster, including the monovalent MLV-H9N2-IL and the bivalent MLV-H9N2-IL/MLV-H5N2-IL prime-boost groups, showed at least a two-fold increase in H9 HI titers compared to prime vaccination. A similar increase in titers was also observed in the WIV-adj-H9N2/H5N2 group (Fig. 2a). Conversely, animals that did not receive a boost (bivalent MLV, prime only) did not show an increase in anti-H9 titers. In contrast, the HI response against H5 was mostly below the limit of detection (Fig. 2b). None of the MLV vaccination strategies, either with a single prime or a prime-boost regimen, elicited a detectable H5 response. Only few animals in the WIV-adj-H9N2/H5N2 group developed detectable H5 titers (Fig. 2b).

We further characterized serum antibody responses using a nanoluciferase-based virus neutralization assay (VNluc) [21] using H9N1-Nluc and H5N1-Nluc surrogate viruses. In addition, enzyme-linked immunosorbent assays (ELISA) were performed to detect responses against the H9 and H5 HAs, the nucleoprotein (NP), and the N1, N8, and N2 NA antigens. VNluc assays, performed using sera collected post-boost, corroborated HI data. Titers against H9 were highest in the bivalent WIV-adj group (Log_2 ISD_{50} = 8.88), followed by the bivalent MLV prime-boost (Log_2 ISD_{50} = 8.1) (Fig. 2c). Overall, significant neutralization titers were observed across all groups against the H9N1-Nluc virus. Conversely, only the bivalent WIV-adj group elicited

detectable levels of neutralization antibodies against the H5N1-Nluc virus (Fig. 2d). Similarly, anti-H9 antibody titers measured by ELISA were readily detectable after prime vaccination and increased after boost across all groups (Fig. 3a). Conversely, anti-H5 antibody levels remained low to negative, with detection limited to few MLV- and WIV-vaccinated animals post-boost (Fig. 3b). After prime vaccination, the bivalent MLV group exhibited the highest levels of anti-NP antibodies compared to the other groups (Fig. 3c). However, following the boost vaccination, anti-NP antibody levels were comparable across all groups (Fig. 3c). As anticipated, given the presence of N2 antigens in the vaccine preparations, anti-N2 antibodies were readily detected by ELISA at 14 dpp and increased by 14 dpb (Fig. 3d), while antibodies against N1 and N8 NA antigens were absent (Figs. 3e and f). Collectively, these findings indicate that aerosol and drinking water vaccination methods elicit robust antibody responses against H9 HA, NP, and N2 NA antigens, but a weak response against H5.

The bivalent MLV-H9N2-IL/MLV-H5N2-IL vaccine provides sterilizing immunity against homologous and antigenically drifted H9N2 LPAI virus challenges

We previously demonstrated that the MLV-H9N2-IL vaccine, when administered via drinking water in a prime-boost regimen, provided complete protection against a homologous H9N2 viral challenge [13]. Building on these findings, our current study investigated whether this robust protection is maintained when the prime vaccination is delivered by aerosol. Furthermore, we expanded our investigation to assess if the MLV vaccine candidates induce broader protection against an antigenically drifted H9N2 challenge. To evaluate cross-reaction between the vaccine strain (ck/EGY) and the antigenically drifted challenge virus (ck/SX), HI assays were performed using homologous MLV sera (ck/EGY) against the drifted strain (ck/SX). No cross-HI reaction was observed when homologous MLV sera were tested against the drifted H9N2 strain (Fig. 4a). In contrast, high HI titers were consistently observed when homologous MLV sera were tested against

the homologous vaccine strain (Fig. 4a). The control WIV-adj-H9N2 sera also showed limited but discernible cross-reactivity against the ck/SX strain.

Following challenge with the homologous ck/EGY H9N2 strain, chickens in the mock-vaccinated/challenge group showed virus shedding in OP swabs for the first 6 days and in CL swabs for the first 4 days post-challenge (Figs. 4b and c). No virus shedding was detected in any of the vaccinated groups, which aligns with prior research [13]. Importantly, the results indicate that aerosol priming alone provides complete protection (note lack of virus detection in the bivalent MLV prime only group) (Figs. 4b and c). Upon challenge with the antigenically drifted H9N2 ck/SX strain, the MLV-vaccinated groups exhibited significantly lower virus shedding in OP samples compared to the non-vaccinated controls (Fig. 4d). Of relevance, the bivalent MLV conferred complete protection against the drifted strain in both prime-only and prime-boost regimens (Figs. 4d and e). Conversely, the WIV-adj group showed similar levels of virus in OP swabs compared to the non-vaccinated controls on days 2, 3, and 4 post-challenge (Fig. 4d). While cloacal shedding was limited across all groups, 2 chickens in the monovalent MLV-H9N2-IL and 1 chicken in the bivalent WIV-adj groups did show shedding levels comparable to controls on days 3 and 4 post-challenge, respectively (Fig. 4d and e). Tissue titration data collected at 3 days post-challenge indicate complete protection against both homologous and antigenically drifted H9N2 viruses in MLV-vaccinated groups (Fig. 4f). In contrast, the bivalent WIV-adjuvanted vaccine failed to protect against the drifted H9N2 virus (Fig. 4f). These findings indicate that the bivalent MLV vaccine, whether administered as a prime-only or prime-boost strategy, induces the best protective immunity against both homologous and antigenically drifted H9N2 strains. This observation further supports the conclusion that MLV vaccination offers superior protection compared to WIV-adjuvanted vaccination.

The bivalent MLV-H9N2-IL/MLV-H5N2-IL vaccine provides strong protection against lethal H5N1 challenge

We conducted a lethal dose 50 (LD50) study in chickens using the prototypical clade 2.3.4.4b H5N1 strain, A/turkey/Indiana/22-003707-003/2022 (H5N1) – ty/IN, to determine the appropriate challenge dose for the subsequent vaccination-challenge study. Chickens infected with 10^5 EID_{50}/chicken rapidly succumbed to the disease within 2 days post-infection (Fig. 5a). All animals infected with 10^4 EID_{50}/chicken died within 5 days post-infection and exhibited severe clinical signs. The animals infected with lower doses (10^3 and 10^2 EID_{50}/chicken) neither died nor exhibited clinical signs (Fig. 5a). Based on the proportion of deaths, the calculated LD50 was established as 3.162×10^3 EID_{50}/ml.

Despite the lack of discernible anti-H5 responses after MLV vaccination, we proceeded to establish the level of protection against HPAI H5N1 in vaccinated chickens. On day 28 after the initial vaccination (prime), or 14 days after the booster vaccination, ten chickens from each group —monovalent MLV, bivalent MLV prime only, bivalent MLV prime-boost, WIV-adj prime-boost, and non-vaccinated controls—were challenged via the oculo-nasal-tracheal-cloacal routes with 50 chicken LD50 (1.58×10^5 EID_{50} per chicken). As anticipated, the unvaccinated control group rapidly succumbed to the disease with a mean death time (MDT) of 43.2 h (Fig. 5b). Chickens vaccinated with the monovalent MLV-H9N2-IL also died within 3 days post-challenge. However, their MDT of 57.6 h was slightly delayed compared to the unvaccinated/challenged control group. The bivalent MLV prime-only group showed 30% survival, and among the non-survivors, the MDT was 60 h, consistent with partial protection. More importantly, we observed 90% survival in chickens that received both the prime and boost with the bivalent MLV vaccine (1 out of 10 died). The WIV-adj-H9N2-H5N2 vaccine group had 100% survival after challenge (Fig. 5b). Notably, none of the animals in the bivalent MLV prime-

boost and WIV-adj-H9N2-H5N2 vaccine groups exhibited clinical signs associated with HPAI H5N1 virus challenge.

Analysis of virus shedding in OP and CL samples after H5N1 challenge aligned with survival data, with one notable exception (Figs. 6a and b). Specifically, the prime-boost bivalent MLV-H9N2-IL/MLV-H5N2-IL vaccine group consistently exhibited lower virus titers and/or fewer virus-positive chickens compared to all other experimental groups, including the bivalent WIV-adj group (Figs. 6a and b). Only two animals in the bivalent MLV group showed low levels of challenge virus in OP and CL swabs (Figs. 6a and b). On day 3 post-challenge, tissue samples were collected for virus titration. Detectable levels of H5N1 were observed in the sinuses of only one animal within the bivalent MLV group and one animal within the bivalent WIV-adj group (Fig. 6c). However, it is important to note that these tissue data represent a single time point post-challenge, thus warranting cautious interpretation. Collectively, these findings indicate that the bivalent MLV-H9N2-IL/MLV-H5N2-IL vaccine provides robust protection against a high-dose lethal H5N1 challenge.

DISCUSSION

This study evaluated the efficacy of a bivalent H9N2/H5N2 MLV vaccine administered via a sequential aerosol priming followed by a drinking water boost. Our central hypothesis, proposing that this dual-route immunization strategy would elicit robust protective immunity against both LPAI H9N2 and HPAI H5N1 strains, was largely supported by the experimental findings. In addition, this strategy mimics those followed by the poultry industry. The vaccine demonstrated complete protection against homologous and antigenically drifted H9N2 challenges and robust protection against a lethal H5N1 challenge. These findings highlight its potential as a valuable tool for controlling avian influenza.

Our previous research demonstrated that the MLV-H9N2-IL vaccine did not reassort with wild-type H9N2 strains *in vivo* or transmit to naïve direct contact chickens [13]. This vaccine candidate induced sterilizing immunity in chickens when administered via drinking water using a prime-boost strategy. Our current study expanded upon that work by evaluating whether this candidate could protect against an antigenically drifted H9N2 virus, thereby assessing its ability to confer broader protection. Consistent with our previous findings, the monovalent MLV-H9N2-IL or bivalent MLV-H9N2-IL/MLV-H5N2-IL vaccines effectively conferred sterilizing immunity against the homologous ck/EGY H9N2 virus when administered via an aerosol prime followed by a drinking water boost. Notably, the aerosol prime alone was also sufficient to induce sterilizing immunity. No viral shedding was detected in any vaccinated groups following challenge with the homologous H9N2 strain, indicating complete protection. Similarly, the bivalent MLV-H9N2-IL/MLV-H5N2-IL vaccine also induced sterilizing immunity against the antigenically drifted ck/SX H9N2 in a prime-only or prime-boost strategy. MLV-vaccinated groups demonstrated significantly reduced viral shedding compared to non-vaccinated controls after ck/SX H9N2 challenge. In many instances, shedding in vaccinated animals was negative. Notably, only two MLV-vaccinated (monovalent) animals exhibited detectable, albeit low, levels of challenge virus shedding post-challenge. In contrast, the prime-boost WIV-adj group showed comparable levels of oropharyngeal virus shedding to non-vaccinated controls on days 2, 3, and 4 post-challenge with antigenically drifted ck/SX H9N2. Notably, on day 3 post-challenge, detectable virus levels were found in the sinuses and trachea of birds in the bivalent WIV-adj group, whereas samples from all MLV-vaccinated groups were negative. Our findings align with numerous other studies, underscoring the limited efficacy of WIV-adj vaccines against antigenically mismatched strains [22-24]. This presents a significant concern for developing effective vaccination strategies. While achieving sterilizing immunity in large poultry populations under field conditions is likely impractical, these findings consistently demonstrate that MLV vaccination offers improved and broader protection compared to WIV-adj vaccination.

The bivalent MLV-H9N2-IL/MLV-H5N2-IL vaccine provided strong protection against a high-dose H5N1 challenge. Crucially, the bivalent MLV prime-boost group achieved 90% survival rate, with only one out of ten birds succumbing to the challenge. This death could be a per acute HPAIV case, which is characterized by rapid, systemic organ failure and death that often occurs before the onset of common HPAIV clinical signs such as respiratory distress, swelling of the head and wattles, or neurological symptoms. The sudden and late timing of this death, combined with the absence of clinical signs in the other birds, precluded a necropsy. While a secondary bacterial infection could have played a role, the specific cause of death was neither confirmed nor accompanied by evaluation of virus titers due to extenuating circumstances beyond our control. However, a notable trend towards reduced H5N1 shedding and lower virus titers was observed in the bivalent MLV prime-boost vaccine group compared to the WIV-adj-H9N2-H5N2 vaccine group, particularly in oropharyngeal samples between days 3 and 5 post-challenge. The robust protection conferred by the bivalent MLV-H9N2-IL/MLV-H5N2-IL prime-boost strategy following H5N1 challenge, despite the absence of detectable anti-H5 antibodies, presents an intriguing observation. Although high levels of anti-N2 antibodies were detected post-vaccination, their lack of cross-reactivity with anti-N1 antibodies suggests that NA antibodies are not the primary mechanism for the observed protection against H5N1 in this study [25-27]. This observed protection warrants further investigation into the underlying mechanism, particularly to determine if shared epitopes within the stalk domain of group 1 HAs, particularly H9 and H5 HAs, contributed to the observed protection. Nevertheless, NA antibodies are known to possess antiviral activity [27, 28]. This inherent activity likely contributes to the cross-protection observed when vaccinated chickens were challenged with an antigenically drifted H9N2 strain, even in the absence of pre-existing HI antibodies specific to that drifted strain. Importantly, the lack of cross-protection by the monovalent MLV-H9N2-IL vaccine against an H5N1 challenge underscores the necessity of including an H5 vaccine antigen. Beyond HA and NA antibodies, MLV vaccines are recognized for their ability to induce robust mucosal IgA responses and cell-mediated immunity,

both of which contribute to enhanced protective immunity against influenza virus [2, 29, 30]. The incorporation of IL-18 downstream of the NA gene in our vaccine candidates offers a promising strategy for further boosting protective immunity. IL-18 is crucial for the development of Th1 immune responses, which are associated with broader cross-protection [31]. Furthermore, IL-18 significantly enhances interferon-gamma (IFN-γ) production from T cells, natural killer (NK) cells, and B cells [32]. Future research will aim to elucidate the precise mechanisms of protection conferred by these vaccines, especially in contexts where neutralizing antibodies may be absent.

An important observation from our study is the limited efficacy of the monovalent H9N2 vaccine against an H5N1 challenge. These chickens exhibited severe clinical signs and succumbed to H5N1 infection within three days post-challenge with significant viral shedding, comparable to non-vaccinated controls. However, these chickens showed slightly delayed mean death time compared to non-vaccinated controls, which highlights a critical concern: vaccination strategies that offer only minimal heterosubtypic protection against HPAI viruses, rather than complete protection, could inadvertently lead to substantial virus transmission and potentially accelerate HPAI virus evolution. This risk is not exclusive to MLV strategies but applies to any vaccine approach that provides only partial cross-protection against highly pathogenic strains. Given these risks, the implementation of avian influenza vaccines must be coupled with robust surveillance systems to monitor virus circulation across all subtypes. Judicious vaccine use is paramount, as there is a risk of infection not only from the primary subtype of concern but also from other seemingly less critical subtypes. Regardless of the specific subtype, avian influenza vaccines should be deployed strictly as part of comprehensive virus eradication strategies. In this context, our new aerosol vaccination approach deserves further development. This method is particularly well-suited for use in controlled laboratory settings, which minimizes the risk of the vaccine virus being released into the environment or mixing with other wild-type strains while

still producing a strong immune response. Complemented by drinking water administration, our MLV approach offers a convenient and cost-effective vaccination method.

Vaccination remains one of the most effective methods for controlling influenza outbreaks in poultry flocks [33]. However, the presence of vaccine-induced antibodies can interfere with serologic surveillance and affect international trade [34]. Therefore, it is crucial to differentiate between naturally infected and vaccinated animals, a concept known as “Differentiating Infected from Vaccinated Animals” (DIVA). While a DIVA strategy is not always considered essential for H9N2, it is vital for H5N1. Our studies using an MLV with the H5N2 subtype demonstrated that vaccinated animals developed a strong antibody response to the N2 component of the vaccine, but not to the N1 neuraminidase found in a natural H5N1 infection. This clear difference in antibody profiles allows for straightforward differentiation. Consequently, a simple ELISA can effectively distinguish between a flock that has been infected with H5N1 and one that has been vaccinated with our H5N2 candidate. However, further research is necessary to develop more robust alternatives to the neuraminidase-based approach, as the reassortment of field viruses could render the NA-based DIVA strategy impractical. It is important to note that our current MLV vaccine constructs incorporate a *Thosea asigna* 2A (Ta2A) protease, which possesses an immunogenic C-terminal end [35]. We are currently exploring whether this Ta2A incorporation can facilitate a DIVA strategy that would not be affected by NA gene segment reassortment. For instance, animals vaccinated with our constructs may elicit an immune response against the Ta2A sequence, thereby enabling a marker-based detection strategy.

Our findings demonstrate that the bivalent MLV-H9N2-IL/MLV-H5N2-IL vaccine, administered via aerosol followed by a drinking water boost, is immunogenic and protective against challenge with both H9N2 and H5N1 avian influenza viruses. This controlled aerosol delivery system operates within a closed environment, thereby meeting the necessary biosafety requirements for both operators and the vaccinated animals. We acknowledge, however, a current limitation

related to the capacity of the aerosol chamber, which presently accommodates only 50 chickens. To achieve the rapid delivery required for large-scale applications, the chamber design will necessitate re-engineering and upscaling. Nonetheless, the efficacy and inherent DIVA strategy of this vaccine and system position them as a highly promising candidate for mass vaccination campaigns and comprehensive avian influenza control.

METHODS

Cells and Eggs. Madin-Darby canine kidney (MDCK) and human embryonic kidney 293T (HEK293T) cells were kindly provided by Dr. Robert Webster, St. Jude Children's Research Hospital, Memphis, TN, USA. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich), 1% antibiotic/antimycotic (10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Amphotericin B; AB, Sigma-Aldrich), and 1% L-glutamine (Sigma-Aldrich). Cells were maintained at 37°C in a 5% CO_2 atmosphere. Specific pathogen-free embryonated chicken eggs used for virus propagation were purchased from Charles River (Wilmington, MA, USA).

Generation of reverse genetics plasmids. The genome rearrangement strategies (RAM) employed to produce MLVs have been previously described [13-15]. Both MLVs (MLV-H9N2-IL and MLV-H5N2-IL) were generated in the backbone of A/guinea fowl/Hong Kong/WF10/1999 (H9N2) [36, 37]. Briefly, genome rearrangements (RAM) included a modification in segment 2 to encode a chimeric PB1-M2 open reading frame (ORF) separated by a glycine-glycine-glycine-glycine-serine (G4S) spacer, and the *Thosea asigna* virus 2A protease (2a Tav). Segment 7 was modified by introducing multiple early stop codons in the M2 ORF via site-directed mutagenesis to prevent its expression. Therefore, the only source of M2 in the RAM virus is from the PB1-M2 chimeric construct. Segment 4 encoding either the H9 HA from A/chicken/Egypt/A15068/2018 (ck/EGY) strain or the H5 HA from A/turkey/Indiana/22-003707-003/2022 (ty/IN) strain was

358 modified to insert a 58-amino-acid-long sequence previously described [13], which included the
359 unique 8-amino-acid peptide aspartic acid-arginine-proline-alanine-valine-isoleucine-alanine-
360 asparagine (DRPAVIAN). This peptide was placed downstream of the HA signal peptide
361 sequence but upstream of the mature HA open reading frame (ORF). Additionally, the H5 HA
362 HPAI cleavage site was replaced with that of the A/Puerto Rico/8/1934 (H1N1) (PR8) strain. The
363 mature protein-coding sequence of chicken IL-18 (ckIL18, Genscript, Piscataway, NJ) was
364 subcloned in frame with the NA ORF of the WF10 virus at the C-terminus [13]. Plasmids were
365 propagated in TOP10 *E. coli* competent cells (ThermoFisher, Waltham, MA, USA) and purified
366 using the QIAGEN Plasmid Maxi Kit (Qiagen, Gaithersburg, MD, USA). Plasmid sequences
367 were confirmed by Sanger sequencing (Psomagen, Rockville, MD, USA).

368 *Generation of MLVs by reverse genetics*. Recombinant viruses were rescued by reverse
369 genetics using the 8-plasmid system and helper plasmids in a coculture of HEK293T and MDCK
370 cells [38]. The MLV-H9N2-IL virus was generated from the combination of reverse genetics
371 plasmids pDPPB2WF10, pDPPB1M2WF10, pDPPAWF10, pDPDRPAVIAN-HA-ck/EGY (H9),
372 pDPNPWF10, pDPNAWF10-ckIL18, pDPM1ΔM2WF10, pDPNSWF10. The generation of MLV-
373 H5N2-IL was performed using all the previously mentioned plasmids, except for the HA plasmid,
374 which was replaced with pDPDRPAVIAN-HA-ty/IN (H5). Both transfections were performed
375 using protein expression helper plasmids encoding the polymerase complex of PR8
376 (pcDNA762-PB2, pcDNA787-PA, pcDNA693-NP, and pcDNA774-PB1) [39]. A coculture of
377 HEK293T and MDCK was prepared the day before transfection. On the day of transfection, 1 µg
378 of each plasmid was mixed with the TransIT-LT1 transfection reagent (Mirus Bio LLC, Madison,
379 WI, USA) at a ratio of 1 µg plasmid DNA to 2 µL of transfection reagent, in a final volume of 1
380 mL of Opti-MEM media (Fisher Scientific, Hampton, NH, USA). This mixture was incubated for
381 45 min and used to overlay the cell coculture overnight. The following day, the transfection
382 mixture was replaced with fresh Opti-MEM media containing 1% AB (Sigma-Aldrich). One

μg/mL of tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK) treated trypsin (Worthington Biochemicals, Lakewood, NJ, USA) was supplemented to the cells at 24- and 72-h post-transfection. Viral stocks were generated in 10-day-old specific pathogen-free (SPF) eggs. Viruses were titrated by tissue culture infectious dose 50 ($TCID_{50}$) and egg infectious dose 50 (EID_{50}) and established by the Reed and Muench method [40]. The MLV-H9N2-IL stock reached titers of $6.81x10^7$ EID_{50}/ml while the MLV-H5N2-IL stock reached titers of $1x10^6$ EID_{50}/ml. Virus sequences were confirmed by Sanger sequencing (Psomagen).

Lethal dose 50 (LD50) study. To determine the LD50 of the ty/IN H5N1 virus, 24 chickens (n=6/group) were inoculated with one of four doses (10^2 to 10^5 EID_{50}/ml) in a 500 μL virus suspension via the oculo-nasal-tracheal-cloacal routes. Chickens were monitored for five days, and the LD50 was calculated based on the proportion of deaths.

Vaccine-challenge studies in chickens. Animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Georgia (Animal Use Protocol A2022 09-016-Y3-A8). Vaccination and H9N2 challenge studies were conducted under ABSL2 conditions, while H5N1 studies were performed under ABSL3 conditions. On day 0, one-day old SPF White Leghorn chickens (Charles River Laboratories International Inc., Wilmington, MA, USA) were vaccinated via aerosol using an aerosol chamber [20]. The aerosol chamber system, extensively described elsewhere for infecting cells with various viruses [20, 41], was used here to deliver the MLV vaccines to chickens. Included within the aerosol chamber setup is an air pump (flow rate 13.3 L/min), Buxco mass dosing controller (Data Sciences International, MN, USA; flow rate 8 L/min), Aeroneb lab control module (Kent Scientific, CT, USA), Aeroneb lab nebulizer unit (Small VMD; Kent Scientific, CT, USA), mass dosing exposure chamber (Data Sciences International, MN, USA), SKC BioSampler (SKC, PA, USA), BioLite+ High-volume sample pump (SKC, PA, USA; 12 L/min), SKC vacuum pump (SKC, PA, USA; 3.5 L/min), liquid traps (Fisher Scientific, NH, USA), HEPA-CAP filters (VWR, PA, USA), and multiple sizes of plastic tubing and tubing

adapters [20]. The first group of 1-day-old chickens (n=50) was placed in the aerosol chamber. A 5 mL volume of MLV-H9N2-IL was loaded into the Aeroneb lab nebulizer, resulting in an average exposure of $1x10^6$ EID_{50}/chicken, or $1x10^6$ EID_{50}/100 μl. The exposure lasted for 15 min, followed by a 5-min purge of the chamber to remove any remaining aerosols before the chicks were moved to isolators. This same procedure was then repeated in two separate batches (n=50/batch) for the bivalent MLV-H9N2-IL/MLV-H5N2-IL. Additionally, a third group (n=50) was vaccinated subcutaneously with 512 HAU per chicken of a bivalent H9N2/H5N2 whole-inactivated-virus adjuvanted vaccine (WIV-adj). A fourth group (n=50) included non-vaccinated controls. On day 14 post prime (14 dpp), chickens were boosted via drinking water at $1x10^6$ EID_{50}/ml. MLVs were diluted in distilled water containing Vac-Pac Plus (Best Veterinary Solutions, Columbus, GA, USA) to neutralize residual chlorine and adjust the pH. Chickens were deprived of water for 6 h prior to vaccination. The MLV vaccines were administered via drinking water using bell drinkers over a 2-h period. All animals in the monovalent MLV-H9N2-IL group received the boost. However, in the bivalent MLV-H9N2-IL/MLV-H5N2-IL vaccine group, only half of the animals were boosted, creating both prime-only and prime-boost subgroups. All chickens in the WIV-adj vaccine group received the boost. On day 28 corresponding to four weeks after prime vaccination or two weeks after the boost (14 dpb), three subsets of chickens from each group were challenged with three different viruses: the homologous H9N2 strain (A/chicken/Egypt/A15068/2018) – ck/EGY, the antigenically drifted wild-type H9N2 strain (A/chicken/Shanxi/BJ-2021-544/2021) – ck/SX, and the homologous H5N1 strain (A/turkey/Indiana/22-003707-003/2022) – ty/IN. The challenge doses for both H9N2 strains were $1x10^8$ EID_{50}/chicken, while the H5N1 dose was 50 chicken lethal dose 50 (approximately $1.58x10^5$ EID_{50}/chicken). Challenge was performed via the oculo-nasal-tracheal-cloacal routes in a total volume of 500 μL of virus dilution.

Hemagglutination Inhibition (HI) assays. Blood was collected from a subset of chickens per group at 14 dpp, and at 14 dpb. Serum samples were analyzed using the HI assay [13, 42], to detect antibodies that neutralize HA's ability to agglutinate red blood cells (RBCs). Briefly, sera were treated twice with 50% RBCs to remove nonspecific inhibitors and then diluted 1:10 in PBS. A 50 µL aliquot of this dilution was added to the first column of a 96-well plate and 2-fold serially diluted. Subsequently, the diluted sera were mixed with 4 HAU/25 µL of the homologous virus. The virus/sera mixture was incubated for 30 min at room temperature, and the HI activity was determined after 30 min of incubation with 0.5% of chicken RBCs. HI titers below 10 were arbitrarily assigned a value of 10.

Virus neutralization assays. Blood samples were collected from a subset of chickens in each group at 14 dpp and 14 dpb. Serum was isolated and pre-treated twice with 50% RBCs. Virus neutralization (VN) assays were performed as previously described [42], with modifications for Nanoluciferase activity (VNLuc) detection [21]. We used reverse genetics to generate homologous H9N1 and H5N1 influenza viruses using 7 gene segments derived from the laboratory adapted A/Puerto Rico/8/1934 (PR8) strain, expressing a Nanoluciferase gene downstream of the PB1 gene [21]. Serum samples were serially diluted in 96-well plates and incubated with 100 $TCID_{50}$ of the homologous virus (H9N1 or H5N1). The serum-virus mixture was incubated for 1 h at 37°C and then inoculated onto MDCK cells, following incubation for 15 min at 4°C and 45 min at 37°C. Then, the mixture was removed, and cells were supplemented with Opti-MEM-AB and TPCK-treated trypsin and incubated for 48 h as previously described. VNLuc titers were determined using the Nano-Glo Luciferase Assay System (Promega, Madison, WI, USA) and quantified with a Victor X3 multilabel plate reader (PerkinElmer, Waltham, MA, USA).

ELISA. Blood was collected from a subset of chickens per group 14 days post-prime and 14 days post-boost. The levels of nucleoprotein (NP) antibodies were determined using the IDEXX AI MultiS-Screen Avian Influenza Virus Antibody Test kit (IDEXX Laboratories Inc., Westbrook,

ME, USA). The presence or absence of antibodies was determined by comparing absorbance values at 650 nm to the negative control mean. Following the manufacturer's recommendations, an S/N ratio of ≥ 0.6 was considered negative. For other viral antigens, direct ELISAs were performed. Briefly, Immulon 4HBX plates were coated with recombinant proteins at a concentration of 2 µg/ml at 4°C overnight. The influenza virus H9 and N2 (A/chicken/Hong Kong/G9/1997), H5 and N1 (A/bald eagle/FL/W22-134OP/2022), and N8 (A/mallard/Sweden/50/2002) proteins were produced using the baculovirus expression system. Blocking was done using 3% non-fat dry milk in PBS-T (phosphate-buffered saline with 0.05% Tween 20) for 1 h. Serum samples were added at an initial dilution of 1:30 in 1% non-fat dry milk PBS-T and further diluted in 1:3 serial dilution steps. After 2 h of incubation at 20°C, plates were washed three times with PBS-T. Goat anti-chicken IgY (H+L) secondary antibody, horseradish peroxidase (HRP) (Invitrogen) was used at a dilution of 1:3000 in 1% non-fat dry milk in PBS-T. Fifty µl of the secondary antibody mixture was added and incubated for 1 h. After another three times wash with PBS-T, 100 µl of O-phenylenediamine dihydrochloride (Sigma Aldrich) was added and incubated for 10 min. The reaction was stopped with 50 µl of 3 M hydrochloric acid. The developed plates were read at 490 nm with a Synergy H1 microplate reader. Area under the curve (AUC) values were calculated using GraphPad Prism software version 10 (GraphPad Software Inc., San Diego, CA, USA).

Virus Titration. Oropharyngeal (OP) and cloacal (CL) swabs were collected after prime vaccination on 1 and 3 dpp to assess MLV replication in the intestinal and respiratory tracts. Additionally, OP and CL swabs were collected at 1-, 2-, 3-, 4-, 5-, and 6-days post-challenge (dpc). Swab samples were serially diluted and inoculated into 96-well plates containing 1.5×10^4 MDCK cells per well. Virus titers were then determined using the Reed and Muench method [40]. Similarly, tissues collected at 3 dpc were also titrated to determine protection after challenge. Briefly, tissue homogenates were prepared from sinuses, trachea, lungs, brain, pancreas, and

intestine using the TissueLyser II (Qiagen, Hilden, Germany). 1 mL of PBS-AB was added to each sample with tungsten carbide 3 mm beads (Qiagen, Hilden, Germany) in the tubes. Samples were homogenized for 10 min and then centrifuged at 15,000 g for 10 min. Supernatants were collected, aliquoted, and directly inoculated into a 96-well plate containing 1.5 x 10^4 MDCK cells/well. Virus titers were also established by the Reed and Muench method [40].

Graphs/Statistical Analyses. All data analyses and graphs were performed using GraphPad Prism software version 10. We used ordinary one-way or two-way ANOVA as appropriate, followed by Tukey's multiple comparison tests to calculate P values. A mixed-effects model was employed for analyses with missing data. A P value of less than 0.05 was considered statistically significant.

DATA AVAILABILITY

All data generated or analyzed during this study are included within this published paper. Genbank accession numbers for the wild-type A/turkey/Indiana/22-003707-003/2022 (H5N1) virus are OQ965225.1 to OQ965232.1. The Genbank accession number for the HA segment of A/chicken/Egypt/A15068/2018 (H9N2) is MN038183. Genbank accession numbers for the MLV backbone, A/guinea fowl/Hong Kong/WF10/1999 (H9N2), are MN267495.1 (PB2), MN267496.1 (PB1), MN267497.1 (PA), MN267499.1 (NP), MN267500.1 (NA), MN267501.1 (M), and MN267498.1 (NS). The A/chicken/Shanxi/BJ-2021-544/2021 (H9N2) strain used in this study is a reassortant virus generated by reverse genetics containing the PB2 and PB1 gene segments derived from A/Cygnus columbianus/Hubei/50/2020 (H5N8) (MW505374.1 and MW505381.1, respectively), the PA, HA, NP, NA, and M segments with ≥99% nucleotide identity to A/chicken/Shanxi/3-27JZRL22-C/2021(H9N2) - PV372693.1 (PA), PV373458.1 (HA), PV374410.1 (NP), PV375280.1 (NA), PV376141.1 (M) -, and the NS segment with ≥99% nucleotide identity to A/chicken/Shanxi/9.3JZRL8-O/2021(H9N2) (PV381492.1). The chicken IL-

18 GenBank accession number is AJ277865.1. Raw data are available and will be provided upon request to dperez1@uga.edu.

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AUTHOR CONTRIBUTIONS

F.C.F. and D.R.P. designed the animal study. F.C.F. performed the cloning and virus rescue experiments. D. R. K. provided the H5N1 challenge virus. F.C.F., L.C.G., D.R., and T.S.M. conducted the in vivo experiment and analyzed the data. R. H. performed and interpreted the HA and NA ELISA data. F.C.F. and D.R.P. interpreted the results, analyzed the data, and wrote the manuscript. F. K., D. R. K., and D.R.P. edited the manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

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FIGURES AND FIGURE LEGENDS

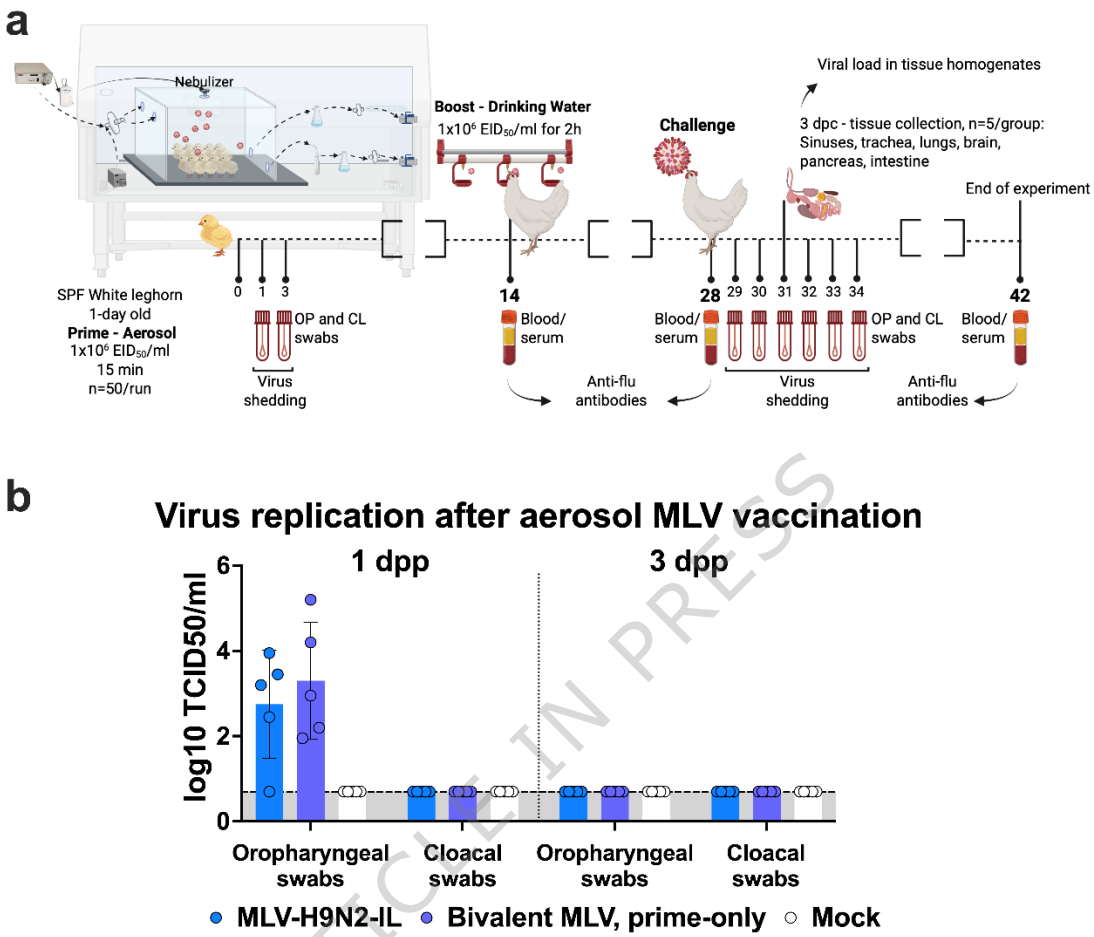


Figure 1. Limited replication of the MLV vaccine after aerosol administration in one-day-old chickens. (a) One-day-old chickens were vaccinated on day 0 via aerosol and boosted on day 14 via drinking water with a monovalent MLV-H9N2-IL or a bivalent MLV-H9N2-IL/MLV-H5N2-IL vaccine. A subset of animals was vaccinated with a bivalent H9N2/H5N2 whole-inactivated-virus adjuvanted vaccine (WIV-adj). Swab samples were collected after prime to analyze virus replication, while blood samples were collected after both the prime and boost for serological analysis. On day 28, three distinct subsets of chickens were challenged with either a homologous H9N2 strain, an antigenically drifted H9N2 strain, or a homologous H5N1 strain. Swabs and tissues were subsequently collected post-challenge to evaluate vaccine protection. **(b)** Oropharyngeal and cloacal swabs (n=5/timepoint) were collected at 1- and 3-days post-prime (dpp) to analyze virus replication after aerosol MLV vaccination. Bars represent the mean titers ± standard deviation (SD) Log_{10} $TCID_{50}$/mL. The limit of detection (LOD) is shown at 0.6999 $TCID_{50}$/mL.

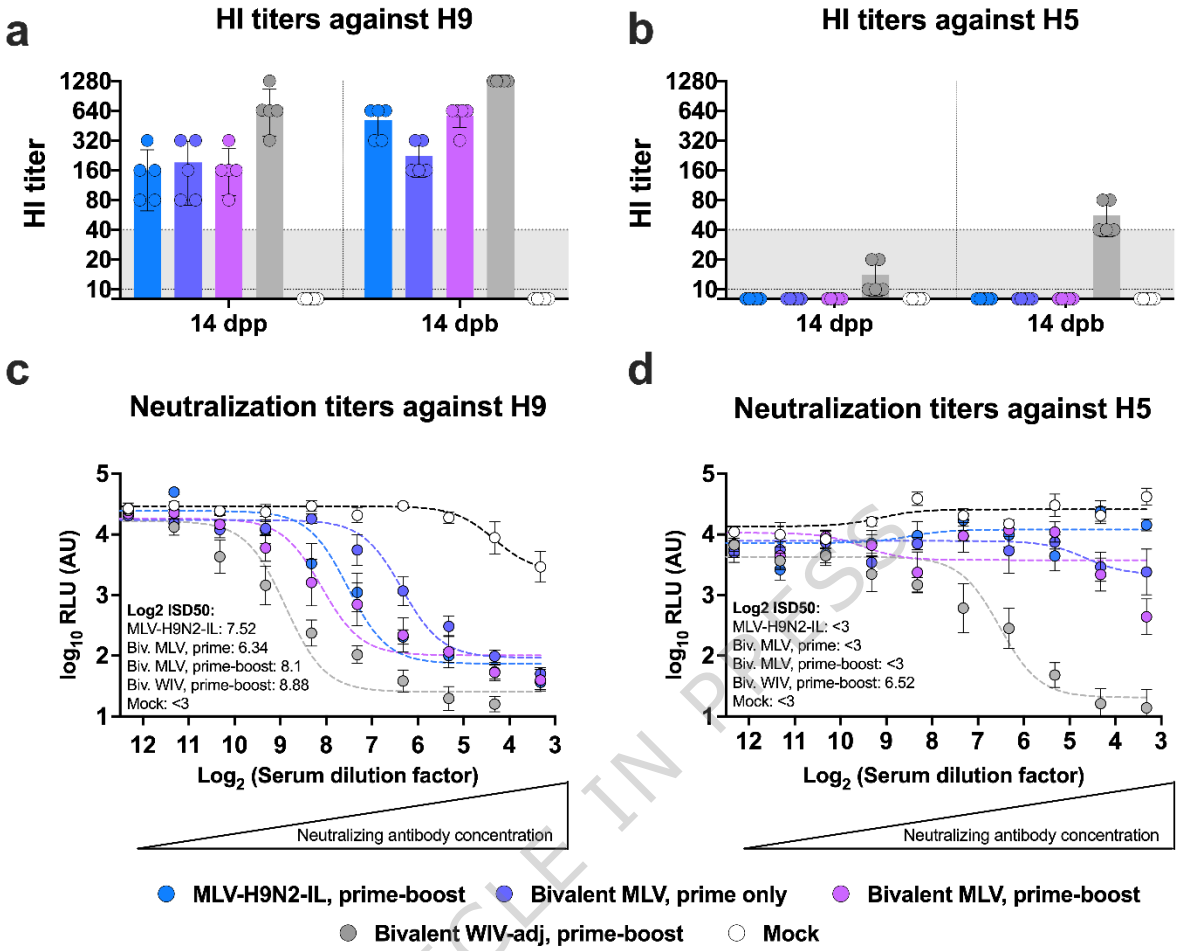


Figure 2. Aerosol and drinking water vaccination induced strong HI responses against H9, but weak responses against H5. Blood samples (n = 5/group/timepoint) were collected at 14 dpp and 14 dpb, and sera were prepared to establish levels of seroconversion against H9 **(a)** and H5 **(b).** Bars represent the mean HI titers, and error bars represent the standard deviation. Additionally, sera were also used to perform virus neutralization assays against H9 **(c)** and H5 **(d).** VNluc titers were plotted as arbitrary Log10 relative light units (Log10 RLU (AU)) versus the Log2 sera dilution. Nluc activity was measured at 48 hpi. The Log2 inhibitory sera dilution 50 (Log2 ISD50) for each serum group is shown.

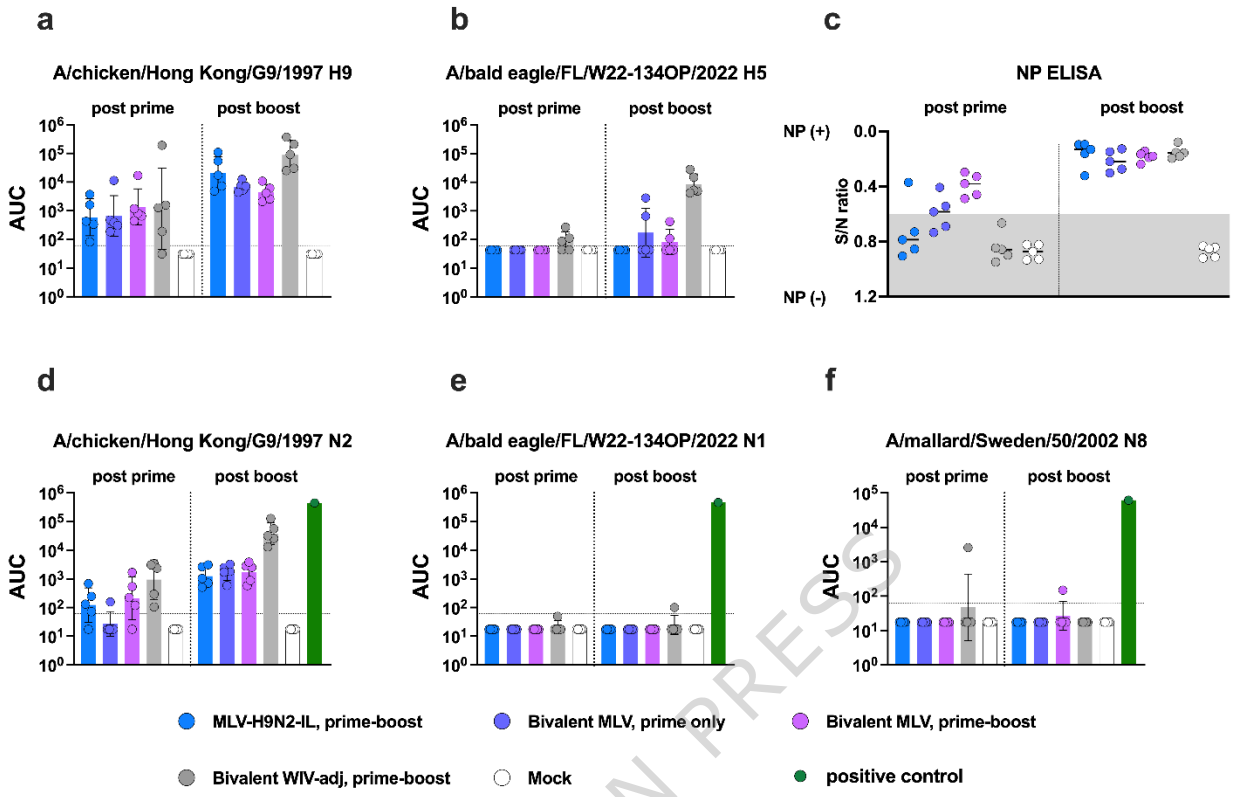


Figure 3. Robust anti-H9, anti-N2, and anti-NP antibody responses were observed by ELISA. Blood samples (n = 5/group/timepoint) were collected at 14 dpp and 14 dpb. Sera were then prepared and subjected to ELISAs to detect antibodies against the H9 HA (a), H5 HA (b), or NP (c). Similarly, ELISAs were performed to detect antibodies against the N2 NA (d), N1 NA (e), and N8 NA (f). For HA and NA ELISAs, the specific viral strains used for each assay are indicated in their respective graphs, with results expressed as areas under the curve (AUC). Bars represent the geometric mean titer with error bars displaying the geometric standard deviation. For the NP ELISA, antibody levels are presented as the signal-to-noise (S/N) ratio against the serum collection time point. An S/N ratio of ≥ 0.6 was considered negative.

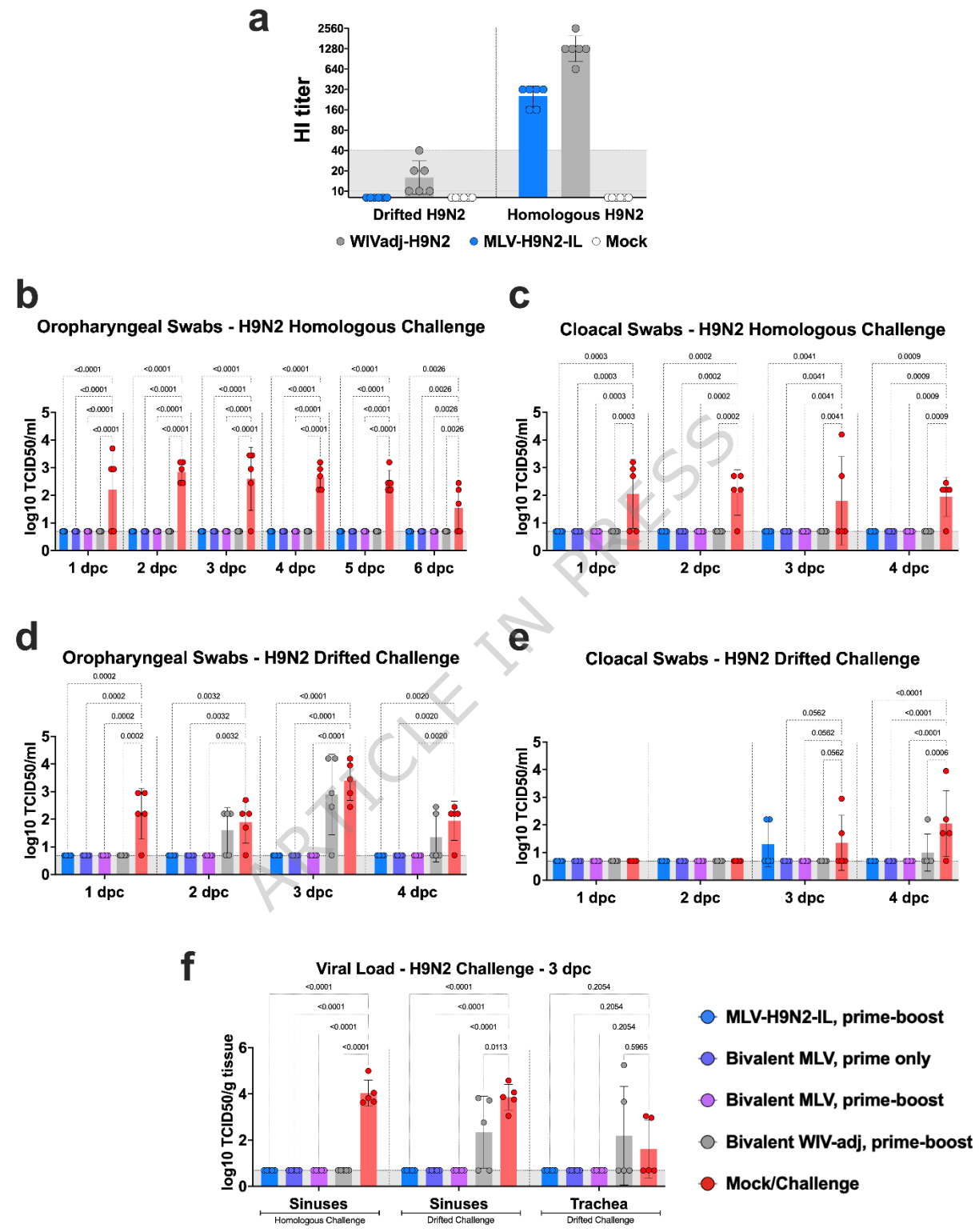
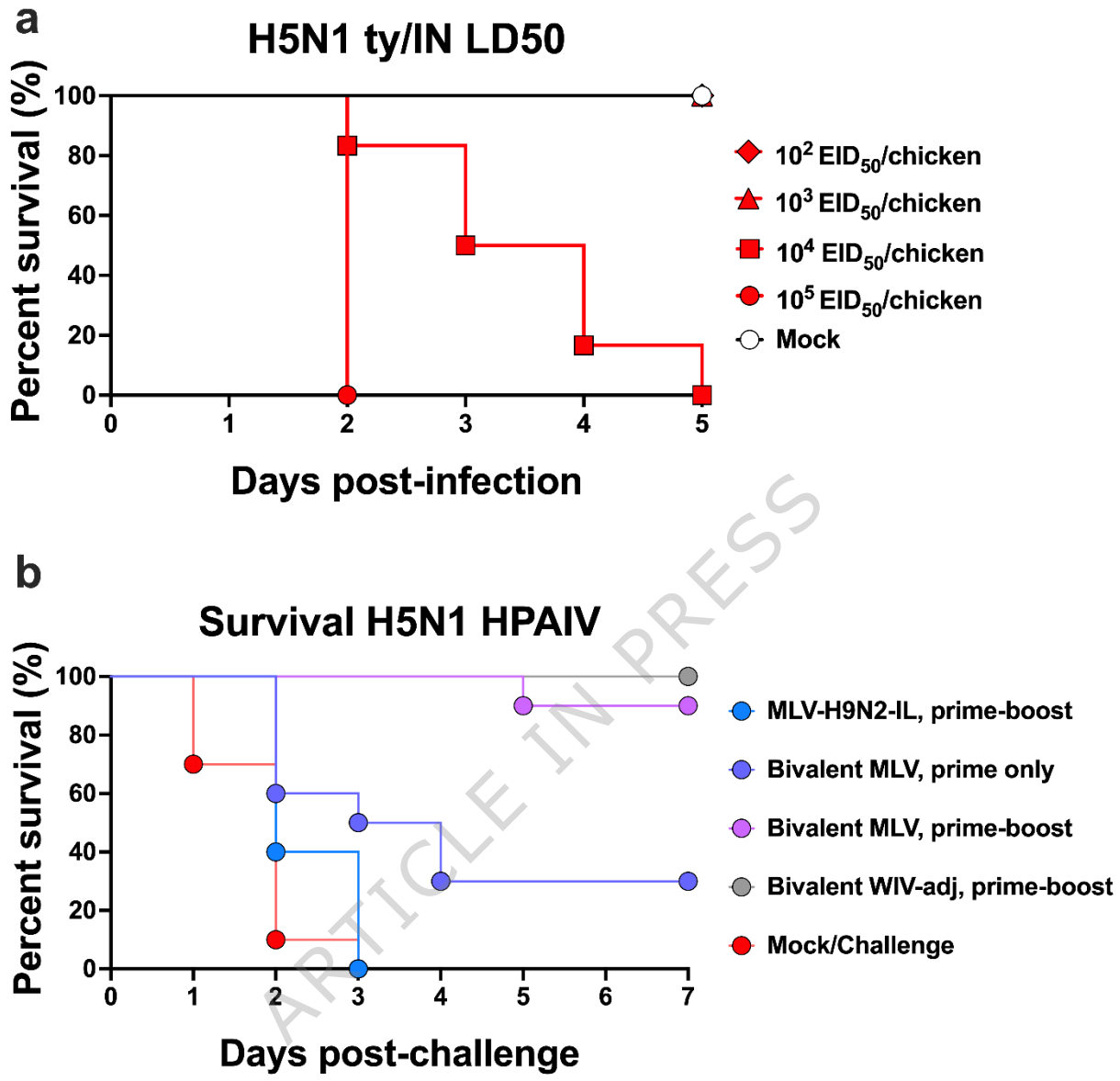


Figure 4. Bivalent MLV vaccination provides strong protection against homologous and antigenically drifted H9N2 challenges. (a) To demonstrate the absence of cross-reaction between the vaccine strain (ck/EGY) and the antigenically drifted challenge virus (ck/SX), HI assays were performed. Homologous sera derived from vaccinated chickens were tested against both the drifted strain and the homologous vaccine strain. When homologous sera were used against the antigenically drifted virus (indicated as "drifted" under the left graph), no significant cross-reaction was observed. Conversely, high HI titers were evident when the same homologous sera were tested against the homologous vaccine strain (indicated as "homologous" under the right graph). On day 28 post-prime, chickens were challenged with either the homologous H9N2 or antigenically drifted H9N2 strains at $1x10^8$ EID_{50}/chicken via oculo-nasal-tracheal-cloacal routes. Oropharyngeal **(b)** and cloacal **(c)** swabs (n=5/timepoint) were collected from 1 to 6 days post-challenge following homologous H9N2 challenge. Oropharyngeal **(d)** and cloacal **(e)** swabs (n=5/timepoint) were collected from 1 to 6 days post-challenge following antigenically drifted H9N2 challenge. **(f)** Sinuses and trachea were collected for both viruses at 3 days post-challenge. All samples were subjected to virus titration, and titers are shown as the mean ± SD Log_{10} $TCID_{50}$/mL (swabs) or mean ± SD Log_{10} $TCID_{50}$/g tissue (tissues). The limit of detection (LOD) is shown at 0.6999 TCID50 /mL. An ordinary two-way ANOVA was performed to calculate P values, followed by Tukey's multiple comparison tests. Only statistically significant differences are shown.



680

681 **Figure 5. Low antibody titers against H5 still provide protection after H5N1 challenge. (a)** A
682 lethal dose study using the prototypical clade 2.3.4.4b H5N1 strain, A/turkey/Indiana/22-003707-
683 003/2022 (H5N1) was performed. Chickens were infected with different doses of the virus (10^5
684 to 10^2 EID_{50}/chicken) and monitored for 5 days. Based on the proportion of deaths, the
685 calculated LD50 was 3162 EID_{50}/ml, or 3.162 x 10^3 EID_{50}/ml. **(b)** On day 28 post-prime, chickens
686 were challenged via the oculo-nasal-tracheal-cloacal routes with 50 chicken LD50 (1.58 x 10^5
687 EID_{50} per chicken) of the H5N1 strain, and survival was calculated for each group.

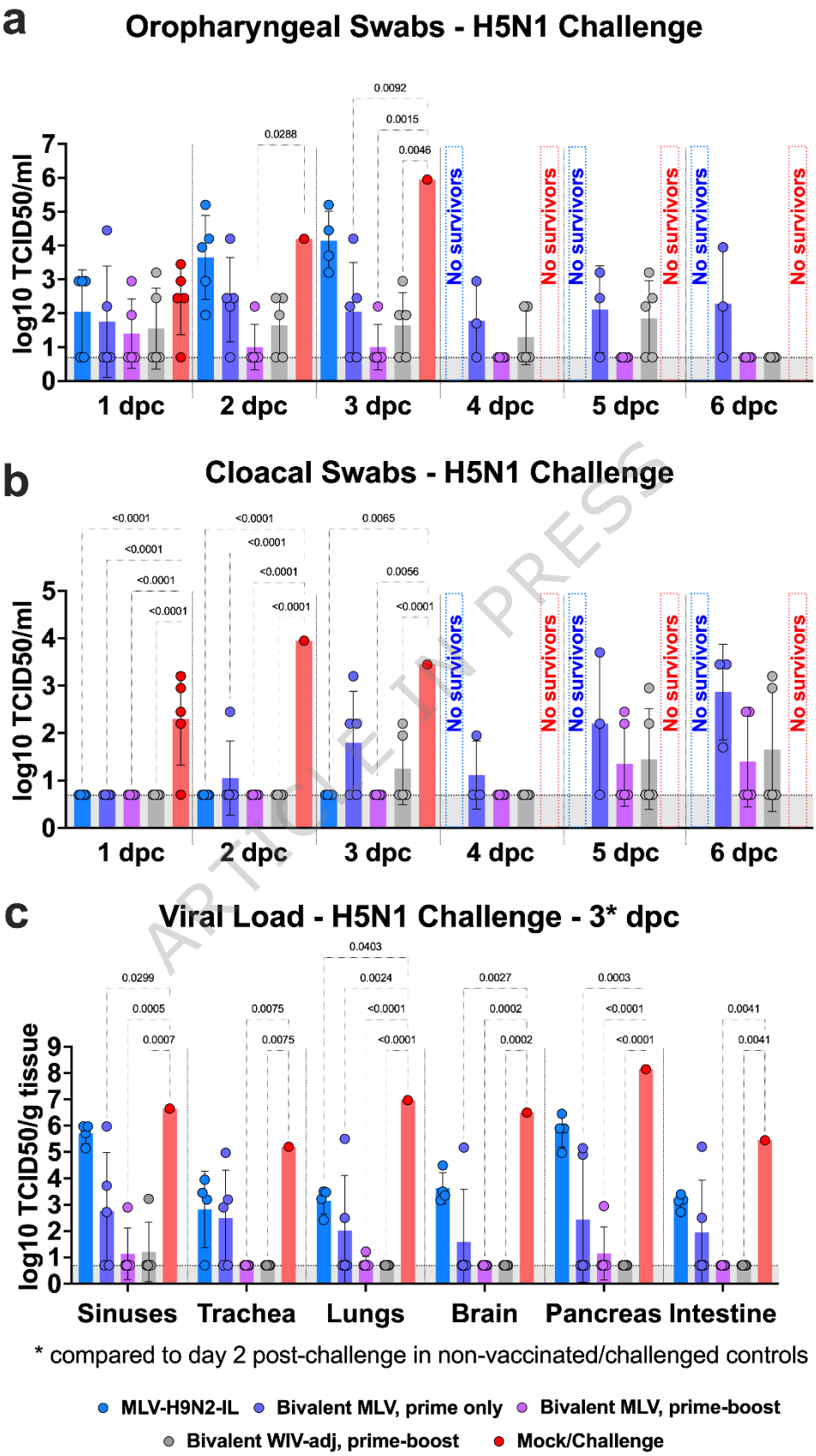


Figure 6. Viral loads after H5N1 challenge indicate protection for the bivalent MLV, prime-boost group. Oropharyngeal **(a)** and cloacal **(b)** swabs (n=5/timepoint) were collected from 1 to 6 days post-challenge following H5N1 challenge. Please note that some datapoints are missing, as animals from some groups quickly succumbed to the disease. **(c)** Sinuses, trachea, lungs, brain, pancreas, and intestine were collected at 3 days post-challenge. All samples were subjected to virus titration and titers are shown as the mean ± SD Log_{10} $TCID_{50}$/mL (swabs) or mean ± SD Log_{10} $TCID_{50}$/g tissue (tissues). The limit of detection (LOD) is shown at 0.6999 TCID50 /mL. Ordinary one-way ANOVA, followed by Tukey's multiple comparison tests, was used to calculate P values. A mixed-effects model was employed for analyses with missing data. Only statistical differences are shown.