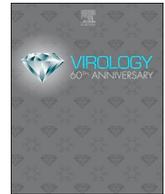




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Enhancing the cross protective efficacy of live attenuated influenza virus vaccine by supplemented vaccination with M2 ectodomain virus-like particles

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ABSTRACT

Current influenza vaccines including live attenuated influenza virus (LAIV) provide suboptimal protection against drift and potential pandemic strains. We hypothesized that supplementing LAIV with a highly conserved antigenic target M2 ectodomain (M2e) would confer cross-protection by inducing humoral and cellular immune responses to conserved antigenic targets. Intranasal vaccination with LAIV (A/Netherlands/602/09, H1N1) supplemented with tandem repeat M2e containing virus-like particles (M2e5x VLP) induced M2e- and virus-specific antibodies. Upon heterosubtypic virus challenge, M2e5x VLP-supplemented LAIV vaccination of mice induced significantly improved cross protection by preventing weight loss and lowering lung viral titers. Further mechanistic studies on heterosubtypic immunity suggest that T cell responses to M2e and nucleoprotein as well as systemic and mucosal antibodies to M2e and viruses might be contributing to cross protection. Therefore, this study demonstrates a novel vaccination strategy to improve the cross protective efficacy of LAIV by supplementing with a conserved M2e antigenic target.

1. Introduction

Influenza virus is responsible for yearly epidemics of respiratory viral diseases in humans, with substantial medical and economic burdens, causing approximately 250,000–500,000 annual deaths worldwide (Osterholm, 2005; Viboud et al., 2010). The challenging difficulty in preventing influenza is the antigenic diversity of virus. Influenza A virus has 18 subtypes (H1–H18) of hemagglutinin (HA) and 11 subtypes (N1–N11) of neuraminidase (NA). The current vaccine formulations based on HA immunity do not provide sufficient broad protection against antigenically distinct strains. Influenza virus continues to introduce antigenic changes in HA and NA resulting in antigenic drifted mutants or shifted mutations such as the emergence of the 2009 pandemic H1N1 virus with triple reassortments (Hancock et al., 2009; Smith et al., 2009). Strain-specific vaccination is relatively effective for influenza prevention and epidemic control only when vaccine strains

closely match the circulating viruses. Inactivated influenza and live-attenuated influenza virus (LAIV, FluMist based on the cold-adapted A/Ann Arbor/6/60 donor virus) vaccines are most commonly used for vaccinating humans and animals.

The U.S. Flu Vaccine Effectiveness Network has estimated the overall vaccine effectiveness between 10% and 60% for influenza seasons from 2005 to 2018 (CDC). The vaccine efficacy is highly variable depending on the antigenic closeness between the vaccines and circulating strains. During the 2014–2015 season, the overall vaccine effectiveness was estimated to be only 6% against the H3N2 component because of drifting mutations in circulating H3N2 strains (Zimmerman et al., 2016). Early clinical studies demonstrated that LAIV (FluMist) has comparable or better efficacy in children 2–7 years of age, compared to inactivated virus vaccines (Belshe, 2004; Belshe et al., 2008, 1998). Also, a study of systemic review reports that the efficacy of LAIV was moderate in children after vaccination during the two consecutive

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seasons (Caspard et al., 2016). However, 2014–2015 U.S. vaccine effectiveness data indicated relatively very poor or no efficacy of LAIV (Shim et al., 2016). Overall, the efficacy of LAIV is low to moderate and should be improved for better protection against homologous and heterologous viruses.

In contrast to highly variable HA proteins, conserved domains have been explored as a “universal” antigenic target to develop cross protective influenza vaccines. Influenza virus M2 protein has an extracellular conserved domain (M2e) which is highly conserved among influenza A viruses (Lee et al., 2015; Liu et al., 2005). Also, HA fusion stalk domains were investigated as a potential target for universal vaccines against influenza (Margine et al., 2013; Steel et al., 2010). In a previous study, we constructed a tandem repeat of heterologous M2e (M2e5x) in a membrane-anchored form on enveloped virus-like particles (M2e5x VLP) (Kim et al., 2013). Intramuscular or intranasal immunization of mice with M2e5x VLP conferred a range of cross protection (Kim et al., 2013; Lee et al., 2018). Despite the merits of broadening the breadth of cross protection, the efficacy of M2e or stalk domain vaccines is low due to non-neutralizing immunity compared to homologous protection by neutralizing HA-based vaccines (Jegerlehner et al., 2004; Lee et al., 2016a). Thus, providing strain-specific influenza vaccines with cross-protective epitopes would broaden the breadth of cross protection in addition to vaccine-matched homologous protection.

In this study, we investigated whether a strategy of supplementing LAIV with M2e5x VLP could enhance the efficacy of cross protection. M2e5x VLP-supplemented LAIV was found to be effective in inducing M2e immunity and conferring cross protection as well as virus-specific immune responses after intranasal vaccination of mice. Also, the adjuvant effects of M2e5x VLP on enhancing the immunogenicity of LAIV and T cell immune responses were observed and discussed.

2. Materials and methods

2.1. Viruses, live attenuated influenza vaccine, and M2e5x VLP

Influenza A viruses A/California/04/2009 (2009 pandemic H1N1, a gift from Dr. Richard Webby), A/Philippines/2/1982 (A/Phil, H3N2), and rgH5N1 were propagated in embryonated chicken eggs. The rgH5N1 virus is a reassortant containing H5 HA with the deleted polybasic cleavage site and N1 NA derived from A/Vietnam/1203/2004 (H5N1) and the backbone genes from A/PR/8/34 virus (Song et al., 2010). To use as an ELISA coating antigen, A/California/04/2009 virus was inactivated by treatment with formalin at a final concentration of 1:4000 (v/v) as described previously (Quan et al., 2008). The LAIV vaccine used in this study is an attenuated 2:6 reassortant containing H1 HA and N1 NA genes from A/Netherlands/602/09 (H1N1) and the internal genes from the attenuated A/turkey/Ohio/313053/04, and its attenuated phenotypes were previously characterized (Pena et al., 2011). M2e5x VLP containing a tandem 5 repeats of M2e (M2e5x) was produced in insect cells using the baculovirus (rBV) protein expression system and purified as previously described (Kim et al., 2013). M2e5x is composed of human (2 ×, SLLTEVETPIRNEWGSRSN), swine (1 ×, SLLTEVETPTRSEWESRSS), Avian 1 (1 ×, SLLTEVETPTRNEWESRSS), and Avian 2 (1 ×, SLLTEVETLTRNGWGCRCS) influenza viruses (Kim et al., 2013). A recommended level for recombinant subunit vaccines was reported to be 20 EU/ml (Brito and Singh, 2011), and the endotoxin levels of purified 5xM2e VLP vaccine were in a range of far below the acceptable limit (< 1.2 endotoxin units (EU) /15 µg M2e5x VLP/0.1 ml) as determined by Chromogenic LAL endotoxin assay kit (Cat# L00350, GenScript).

2.2. Immunization and viral infection

BALB/c mice (6–8 weeks old, Harlan Laboratories) were intranasally (i.n.) immunized with an attenuated pandemic LAIV vaccine (1 × 10⁵ of the 50% tissue culture infectious dose per mouse) alone or

in combination with M2e5x VLP [LAIV + M2e5xVLP (15 µg)] at weeks 0 and 4. To determine the efficacy of protection, vaccinated mice were anesthetized by isoflurane (Baxter, Deerfield, IL) and i.n. infected with rgH5N1, A/PR8, or A/Phil (H3N2) influenza virus at 6 weeks after boost immunization at a lethal dose as indicated in the figure legends. Some vaccinated mice were intraperitoneally (i.p.) treated with CD4 (GK1.5) or CD8 (2.43) depletion antibodies 2 days before and 1 day after challenge with A/Phil (H3N2). Depletion of CD4 or CD8 was confirmed (over 99% depleted, data not shown) by flow cytometry analysis as previously presented (Ko et al., 2016). Infected mice were daily monitored for body weight changes and survival rates or euthanized to collect samples. Animal experiments were approved under the guidelines by the Georgia State University Institutional Animal Care and Use protocol.

2.3. ELISA

The antibody titers specific for M2 ectodomain or influenza virus were determined by enzyme-linked immunosorbent assay (ELISA) using synthetic M2e peptides or inactivated A/California/04/2009 virus (iCal) as a coating antigen. To determine antibody production *in vitro*, the cells from spleen or bone marrow were harvested 6 days post influenza virus infection. The cells were cultured in the presence of M2 peptides or iCal and incubated for 1 or 5 days at 37 °C. Levels of antigen specific antibodies in culture supernatants were determined by ELISA.

2.4. Lung viral titers

Lung homogenates were used to determine lung viral titers by using the embryonated chicken eggs as growth substrates. Viral titers were determined by hemagglutination assay as described (Lee et al., 2016a, 2016b).

2.5. Cytokine assays

The levels of inflammatory cytokines in bronchoalveolar lavage fluids (BALF) and lung extracts were analyzed by ELISAs, performed as previously described (Lee et al., 2016a, 2016b). Briefly, Ready-Set-Go TNF-α, and IFN-γ kits (eBioscience, San Diego, CA) were used to determine the levels of inflammatory cytokines 6 days post infection.

2.6. Flow cytometry and intracellular cytokine staining analysis

Bronchoalveolar lavage (BAL) fluids were obtained by infusing 1 ml of phosphate-buffered saline (PBS) into the trachea using a 25-gauge catheter (Exelint International Co., Los Angeles, CA) to collect non-adherent cells in the airways. The lung tissues were homogenized and spun on 44/67% Percoll gradients at 2800 rpm for 15 min. Cell bands were harvested and washed with PBS. BAL and lung cells were stained with phenotypic marker monoclonal antibodies (mAb) specific for CD45, CD11b, CD11c, MHCII, F4/80, CD103, B220, and Ly6c (eBioscience or BD Pharmingen, San Diego, CA). For intracellular cytokine staining, enriched lymphocytes were *in vitro* stimulated with the synthetic M2e peptides or NP_{147–155} H-2K^d (TYQRTRALV) (Deliyannis et al., 2006) (5 µg/ml) in the presence of Brefeldin A (20 µg/ml) for 5 h at 37 °C incubator. Cytokine producing lymphocytes were then fixed/permeabilized using BD Cytofix/Cytoperm™ Plus kit. The samples were analyzed on a Becton-Dickinson LSR-II/Fortessa flow cytometer (BD, San Diego, CA) and analyzed by Flowjo software (Tree Star Inc.).

2.7. Statistics

All data were presented as means ± SEM (standard error of mean). To determine the statistical significance, an unpaired two-tailed Student's *t*-test or one-way ANOVA was used to compare the groups. Prism software (GraphPad software Inc., San Diego, CA) was used for

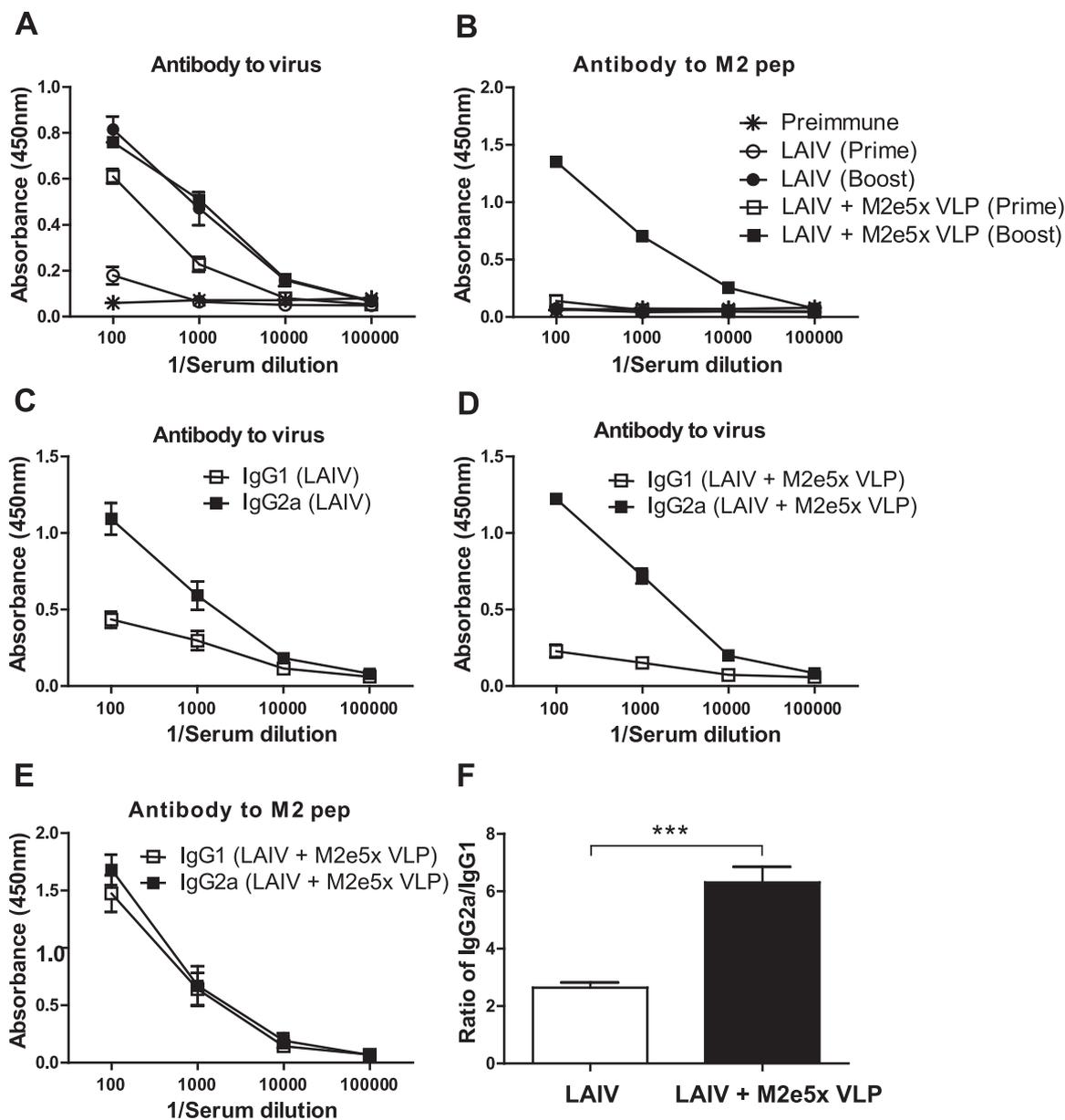


Fig. 1. LAIV Vaccination supplemented with M2e5x VLP enhances virus-specific IgG2a and M2e-specific IgG antibody responses. Naïve BALB/c mice ($n = 15$) were intranasally immunized with an attenuated pandemic A/Netherlands/602/09 (LAIV) alone or LAIV supplemented with M2e5x VLPs (15 $\mu\text{g}/\text{mouse}$) at week 0 and boosted at week 4. Antigen-specific serum antibody levels at 3 weeks after prime and boost were measured by ELISA. (A) IgG antibodies specific for the vaccine virus A/California/04/09 (H1N1 2009 pandemic, panH1N1). (B) M2e specific IgG antibodies. (C) LAIV vaccination-induced IgG isotypes to virus. (D) LAIV + M2e5xVLP vaccination-induced IgG isotypes to virus. (E) LAIV + M2e5xVLP vaccination-induced M2e specific IgG isotype antibodies. (F) Ratio of IgG2a/IgG1 isotype antibodies to panH1N1 virus in the LAIV or LAIV + M2e5x VLP groups. Statistical significance was determined using an unpaired two-tailed Student's *t*-test. Error bars are means \pm SEM of concentration or ratios from individual animals. ***, $P < 0.001$.

all data analysis. P values are indicated by an asterisk (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

3. Results

3.1. LAIV with M2e5x VLP supplementation enhances virus-specific IgG2a and M2e-specific IgG antibody responses

To investigate whether supplementing LAIV with tandem repeat M2e VLP vaccine (M2e5x VLP) would enhance broad cross-protection against heterosubtypic influenza viruses, naïve BALB/c mice ($n = 15$) were intranasally immunized with A/Netherlands/602/09 (H1N1) LAIV alone or supplemented with M2e5x VLP (LAIV + M2e5x VLP). The blood samples were collected from LAIV alone or LAIV + M2e5x VLP

immune mice and antigen-specific antibodies for M2e and H1N1 virus were determined by ELISA. H1N1 virus-specific antibodies were observed at lower levels in the LAIV alone group than those in the M2e5x VLP supplemented group (LAIV + M2e5x VLP) after prime immunization (Fig. 1A). Comparable amounts of antibodies to H1N1 virus were induced after boost immunization with LAIV alone or M2e5x VLP supplemented groups (Fig. 1A). Antibodies specific for M2e were at a low level after prime but significantly increased to high levels by over 1000 folds in the LAIV + M2e5x VLP group after boost immunization (Fig. 1B). LAIV alone immune mice did not induce M2e specific antibodies at a detectable level.

We determined a pattern of vaccine-specific IgG antibody isotype levels in sera after LAIV or LAIV + M2e5x VLP vaccination. The increased IgG antibodies specific for virus in the LAIV + M2e5x VLP prime

group were found to be a predominantly IgG2a isotype. LAIV and M2e5x VLP supplemented vaccination induced higher levels of IgG2a isotype antibodies to H1N1 virus than those of IgG1 antibodies (Fig. 1C and D). As a result, M2e5x VLP-supplemented LAIV vaccination resulted in a 3-fold increase in the IgG2a/IgG1 ratio compared to those in LAIV alone vaccination (Fig. 1F). In addition, similar amounts of M2e specific IgG2a and IgG1 antibodies were observed in the LAIV + M2e5x VLP group (Fig. 1E). Also, we determined serum IgG directed against rgH5N1 and A/Phil (H3N2) virus in sera from the M2e5x VLP, LAIV, and LAIV + M2e-VLPs groups and found similarly low levels of serum IgG antibodies against these viruses between the two groups (Supplemental Fig. S1). These results suggest that supplementing a LAIV vaccine with M2e5x VLPs induces higher levels of IgG2a isotype antibody, a T helper type 1 (Th1) immune response to H1N1 virus and M2e specific antibodies compared to LAIV alone vaccination.

3.2. M2e5x VLP-supplemented LAIV confers enhanced cross-protection

We investigated whether M2e5x VLP-supplemented LAIV vaccination would enhance the efficacy of cross protection. To determine the efficacy of cross protection, the groups of mice vaccinated with LAIV alone or LAIV + M2e5x VLP were challenged with a reassortant A/Vietnam/1203/2004 (rgH5N1 with poly basic residues deleted) virus at 6 weeks after boost (Fig. 2). Mice that were vaccinated with LAIV + M2e5x VLP displayed no weight loss until day 6 post infection (Fig. 2A). In contrast, LAIV alone vaccinated mice showed a significant loss (approximately 15%) in body weight at day 6 after virus infection whereas naïve mice exhibited over 20% weight loss by day 6 post-infection. To compare the efficacy of cross-protection, the lung viral loads were determined at 3- and 6-day post-infection (dpi). At 3 days after infection, mice with LAIV + M2e5x VLPs showed a reduced virus titer by a magnitude of 2 log₁₀ compared to naïve and LAIV groups (Fig. 2A). The group of mice vaccinated with LAIV + M2e5x VLPs showed approximately ~ 100-fold and 160-fold lower level of lung viral titers compared to those in LAIV alone and naïve mouse groups 6 days after challenge, respectively (Fig. 2B). Interferon-gamma (IFN- γ) cytokine was detected at higher levels in the bronchoalveolar lavage (BAL) fluids and lungs of unimmunized and LAIV alone groups compared to those in the LAIV + M2e5x VLP group 3 and 6dpi (Fig. 2C). Also, high levels of tumor necrosis factor (TNF) were observed in the naïve and LAIV BAL samples than those in the LAIV + M2e5x VLP group. Naïve lung samples showed higher levels of TNF than those in the supplemented and LAIV groups at day 6 after infection (Fig. 2C).

In an independent experiment, we determined whether the addition of M2e-VLPs to LAIV (LAIV + M2e5x VLP) would also increase the protection against a high dose homologous virus challenge (A/Cal 2009 H1N1). As shown in body weight change monitoring data (Supplemental Fig. S2), the LAIV + M2e5x VLP displayed less weight loss than the LAIV alone group, suggesting increased homologous protection by LAIV + M2e5x VLP vaccination. In comparison with M2e5x VLP alone, the efficacy of M2e5x VLP alone group displayed less weight loss against rgH5N1 virus challenge than the naïve control mice (Supplemental Fig. S3). More importantly, the efficacy of LAIV + M2e5xVLP was higher than either vaccine alone as determined by weight changes and lung viral titers after rgH5N1 virus challenge. Overall, these data support that supplemented LAIV + M2e5xVLP vaccination can confer enhanced cross-protection as evidenced by better control of early lung viral loads and preventing the induction of proinflammatory cytokines in the BAL and lungs compared to LAIV alone vaccine after heterosubtypic virus challenge.

3.3. LAIV + M2e5x VLP vaccination prevents cell infiltration into the lungs due to heterosubtypic virus challenge

To better assess cross protective efficacy, inflammatory innate immune cell types were analyzed in the BAL and lungs day 6 post

challenge with rgH5N1 virus. The LAIV + M2e5x VLP group showed significant reduction in cell numbers of inflammatory monocytes (CD11b⁺Ly6c^{hi}F4/80⁺) and neutrophils (CD11b⁺Ly6c⁺F4/80⁺) in the BAL (airways) and lungs compared to the naïve group (Fig. 3A). As compared with LAIV alone mice, LAIV + M2e5x VLP immune mice displayed 10 to 50-fold reduction in monocytes and 5–6 fold lower in neutrophils in the BAL and lungs day 6 post rgH5N1 virus infection (Fig. 3A).

A previous study showed that influenza virus infection is correlated with respiratory DC migration to the lung draining lymph nodes (Legge and Braciale, 2003). LAIV alone or LAIV + M2e5x VLP immune mice revealed lower numbers of plasmacytoid DCs (pDCs, B220⁺CD11c⁺MHCII^{hi}), CD103⁺ DCs (CD103⁺CD11c⁺MHCII^{hi}CD11b⁻) and CD11b⁺ DCs (CD11b⁺CD11c⁺MHCII^{hi}CD103⁻) compared to those in naïve mice after rgH5N1 virus infection (Fig. 3B). These results suggest that LAIV + M2e5x VLP is more effective in preventing innate immune cell infiltration than LAIV alone after heterosubtypic virus infection.

3.4. LAIV + M2e5x VLP vaccination enhances antibody-secreting cells and mucosal antibody responses specific for virus and M2e

We determined whether LAIV + M2e5x VLP vaccination would enhance virus and M2e specific mucosal antibodies and antibody-secreting cell responses. Consistent with serum antibody, significant levels of M2e-specific IgG and IgA antibodies were observed in BAL samples from the LAIV + M2e5x VLP group but not from LAIV alone or naïve mice at day 6 post infection (Fig. 4A). IgG and IgA antibodies specific for H1N1 virus were also induced at the highest levels in the BAL from the LAIV + M2e5x VLP group although statistical significance between LAIV and LAIV + M2e5x VLP was not apparent (Fig. 4A).

LAIV + M2e5x VLP prime vaccination increased antibody levels to H1N1 virus (Fig. 1). Next, to determine whether M2e5x VLP supplementation would influence B cell activation and humoral memory responses, germinal center B cells were analyzed using a GL7 marker (Pasare and Medzhitov, 2005). Higher numbers of GL7⁺ germinal center B cells (CD19⁺B220⁺) were found in LAIV + M2e5x VLP immune mice than those in naïve and LAIV immune mice (Supplemental Fig. S4). The cells from spleens and bone marrow were harvested and cultured in 96 well plates in the presence of H1N1 virus or M2e peptides to analyze *in vitro* antibody production. We found that the higher amounts of H1N1-specific IgG antibodies in the spleen and bone marrow from LAIV + M2e5x VLP group than those in LAIV alone and naïve mice for 1 day or 5 days *in vitro* cultures (Fig. 4B). Only the LAIV + M2e5x VLP group resulted in the production of M2e-specific antibodies. These results support that LAIV + M2e5x VLP vaccination efficiently induces antigen-specific mucosal antibodies as well as B cells differentiating into plasma cells secreting anti-H1N1 and -M2e antibodies.

3.5. Immune sera from LAIV + M2e5x VLP vaccination confer cross protection

An *in vivo* protective assay was performed to determine the roles of immune sera in conferring cross protection. The rgH5N1 or A/PR8 (H1N1) virus was mixed with immune sera from LAIV alone or LAIV + M2e5x VLP immune mice and used to infect naïve mice. Immune sera from LAIV alone or naïve mice were not able to confer protection against rgH5N1 or A/PR8 (H1N1) virus infection of naïve mice as evidenced by severe weight loss of over 20% and no surviving mice (Fig. 5). In contrast, all naïve mice that infected with a mix of virus and LAIV + M2e5x VLP immune sera were protected and survived although they exhibited moderate weight loss (an average of 8%) against rgH5N1 virus and substantial weight loss against A/PR8 virus (Fig. 5). Therefore, these data provide evidence that immune sera of LAIV + M2e5x VLP immune mice provide enhanced cross protection to naïve mice but not LAIV immune sera.

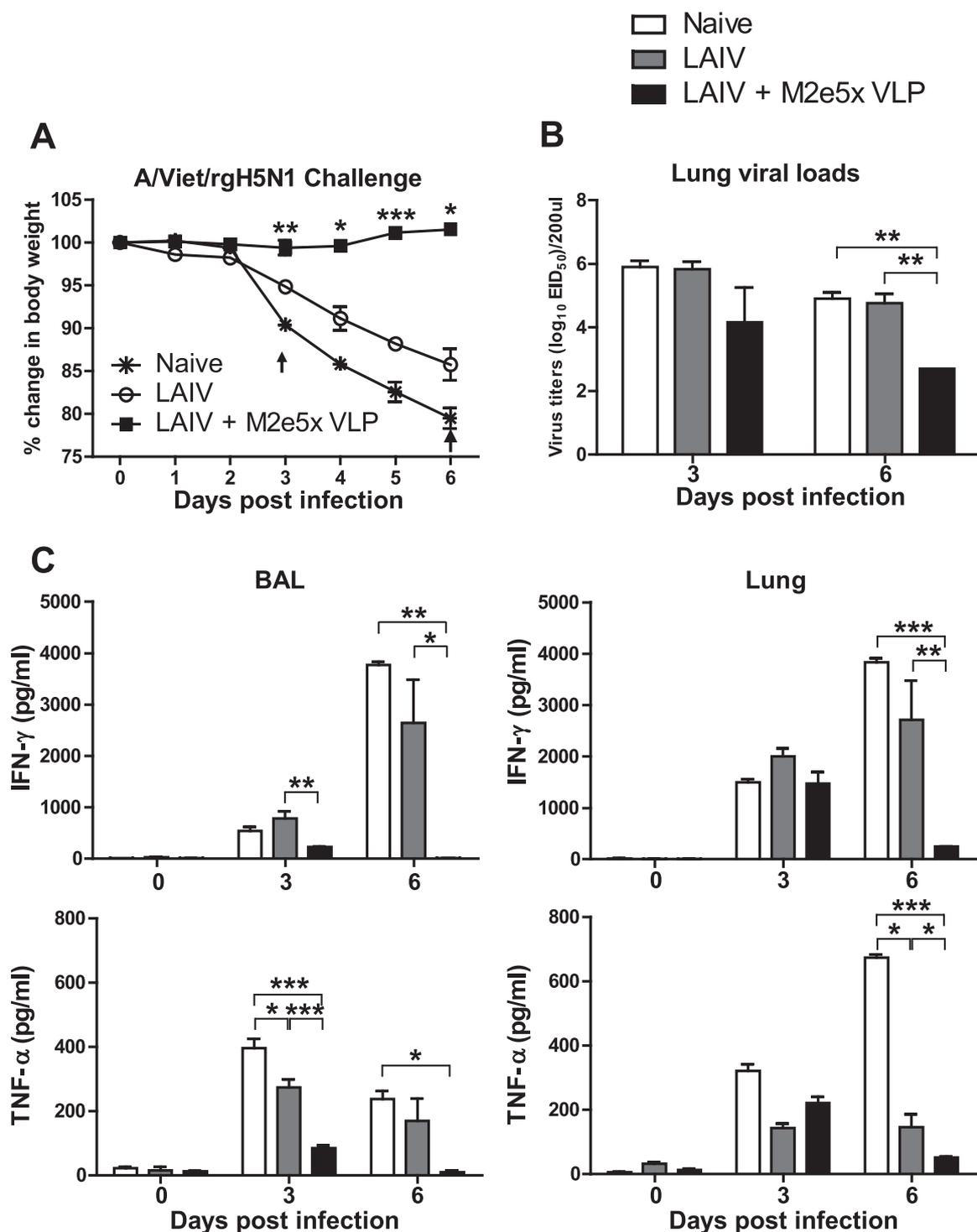


Fig. 2. M2e5x VLP supplemented LAIV confers enhanced cross protection against heterosubtypic influenza virus. The groups of mice (n = 10 out of 15 mice vaccinated) vaccinated with LAIV alone or LAIV + M2e5x VLP were infected with a lethal dose of reassortant A/Vietnam/1203/2004 virus (rgH5N1, 6xLD₅₀) 6 weeks after boost vaccination. (A) Changes in body weight were monitored for 6 days. (B-C) The BAL fluids and lung homogenates from individual mice were collected at 3- and 6-day post infection (dpi) as indicated by an arrow symbol. (B) Lung viral titers by an egg inoculation assay 3 and 6 dpi. (C) Levels of IFN- γ and TNF cytokines in the BAL and lungs by a cytokine ELISA assay. Statistical significance was determined using the one-way ANOVA. Error bars are means \pm SEM of concentration or ratios from individual animals. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

3.6. LAIV + M2e5x VLP vaccination induces antigen specific IFN- γ secreting T cell responses

Antigen-specific memory T cell responses were determined in LAIV + M2e5x VLP immune mice before and after challenge with rgH5N1 virus at 6 weeks after boost (Fig. 6). Cells from the BAL and lungs were

collected at 0, 3 and 6 dpi to determine IFN- γ producing cellular responses by an intracellular cytokine staining assay (Supplementary Fig. S5A, B). Higher numbers of IFN- γ producing M2e-specific CD4⁺ T cells in the LAIV + M2e5x VLP group were found in the BAL (2-fold increase) and lungs (5-fold increase) at day 0 than those in naïve or LAIV alone immune groups (Fig. 6A), indicating that LAIV + M2e5x VLP vaccination

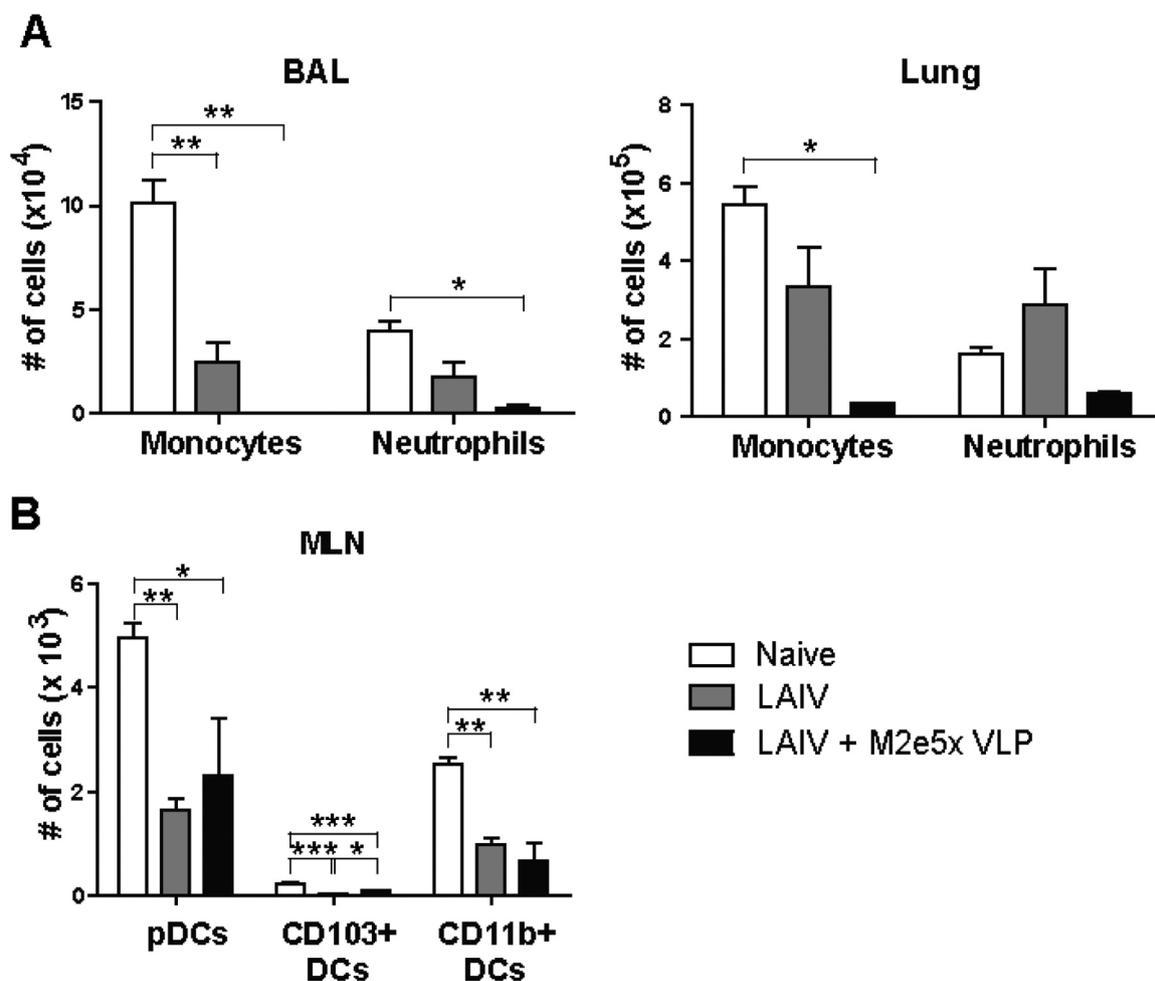


Fig. 3. Enhanced protection by LAIV + M2e5x VLP reduces immune cell recruitment to the lungs and draining lymph nodes upon virus challenge. The cells from the airways and lungs, and MLN of individual mice ($n = 5$ out of 10 mice vaccinated and challenged per group) were collected 6 days after rgH5N1 (6xLD₅₀) virus infection. Immune cells were analyzed using flow cytometry specific markers such as CD45, CD11c, CD11b, CD103, B220, MHCII, Ly6c and F4/80. (A) Cell numbers of monocytes (CD11b⁺CD11c⁺Ly6c^{hi}F4/80⁺) and neutrophils (CD11b⁺CD11c⁺Ly6c⁺F4/80⁺) in the BAL and lungs as determined by flow cytometry. (B) Dendritic cell (DC) subsets including plasmacytoid DCs (pDCs, B220⁺CD11c⁺MHCII^{hi}F4/80⁺CD45⁺), CD103⁺ DCs (CD103⁺CD11c⁺MHCII^{hi}F4/80⁺CD45⁺), and CD11b⁺ DCs (CD11b⁺CD11c⁺MHCII^{hi}F4/80⁺CD45⁺) in the mediastinal lymph nodes (MLN) as determined by flow cytometry. Statistical significance was determined using the one-way ANOVA. Error bars are means \pm SEM of concentration or ratios from individual animals. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

induced the generation of M2e-specific memory CD4⁺ T cells. At 3 or 6 dpi, M2e-specific CD4⁺ T cells producing IFN- γ were further increased in the BAL (3 dpi: \sim 19-fold increase; 6 dpi: \sim 7-fold increase) and lungs (3 dpi: \sim 43-fold increase; 6 dpi: \sim 2.8-fold increase) from the LAIV + M2e5x VLP group compared to those from the naïve or LAIV alone groups (Fig. 6A).

Higher numbers of NP-specific CD8⁺ T cells secreting IFN- γ were observed in the BAL and lungs of mice vaccinated with LAIV + M2e5x VLP compared to those in the naïve or LAIV alone group before infection and 3 dpi (Fig. 6B and Supplementary Fig. S5B). LAIV alone immune mice showed a substantial increase in IFN- γ producing NP-specific CD8⁺ T cells compared to those in naïve mice at 6dpi. LAIV + M2e5x VLP immune mice revealed approximately 4-fold and 8-fold higher NP-specific CD8⁺ T cells in the BAL and lungs respectively, compared to those in LAIV alone mice (Fig. 6B).

3.7. LAIV + M2e5x VLP vaccination induces cross-protective T cell responses

To better determine the cross-protective roles of CD4⁺ and CD8⁺ T cells from LAIV + M2e5x VLP immune mice in contributing to hetero-subtypic protection, mice were treated with PBS, CD4, or CD8 depletion antibodies before challenge with A/Philippines/2/82 (5xLD₅₀, H3N2)

virus (Fig. 7). Mice administrated with PBS, CD4 or CD8 monoclonal antibodies showed a moderate loss of body weight (approximately 10%) until 5 dpi, but after 6 dpi PBS-treated mice showed a better recovery in body weight (Fig. 7A). Meanwhile, naïve mice showed a more substantial body weight loss up to 20% at 7 dpi. The highest levels of lung virus titers were observed in unimmunized naïve mice with infection. The PBS control mice immunized with LAIV + M2e5x VLP showed approximately 2.5-fold and 15-fold lower lung viral titers compared to those in CD4- and CD8-depleted mice prior to virus challenge, respectively (Fig. 7B). These data suggest that LAIV + M2e5x VLP vaccination induces a cross protective CD8⁺ T cell response, contributing to lowering lung viral loads and enhanced hetero-subtypic cross protection. CD4 depletion in the LAIV + M2e5xVLP group resulted in a pattern of displaying more weight loss and higher lung viral titers compared to the PBS control group although the differences were not statistically significant.

To further examine the roles of T cells in cross protection, LAIV alone vaccinated mice were treated with CD4⁺ or CD8⁺ T cell depleting antibodies and then challenged with a sub-lethal dose of A/Philippines/2/82 (0.5xLD₅₀, H3N2) virus (Supplementary Fig. S6). There was a more weight loss and high lung viral loads in the LAIV group with CD4⁺ or CD8⁺ T cell depletion compared to those in the control LAIV group without T cell depletion. These results suggest the

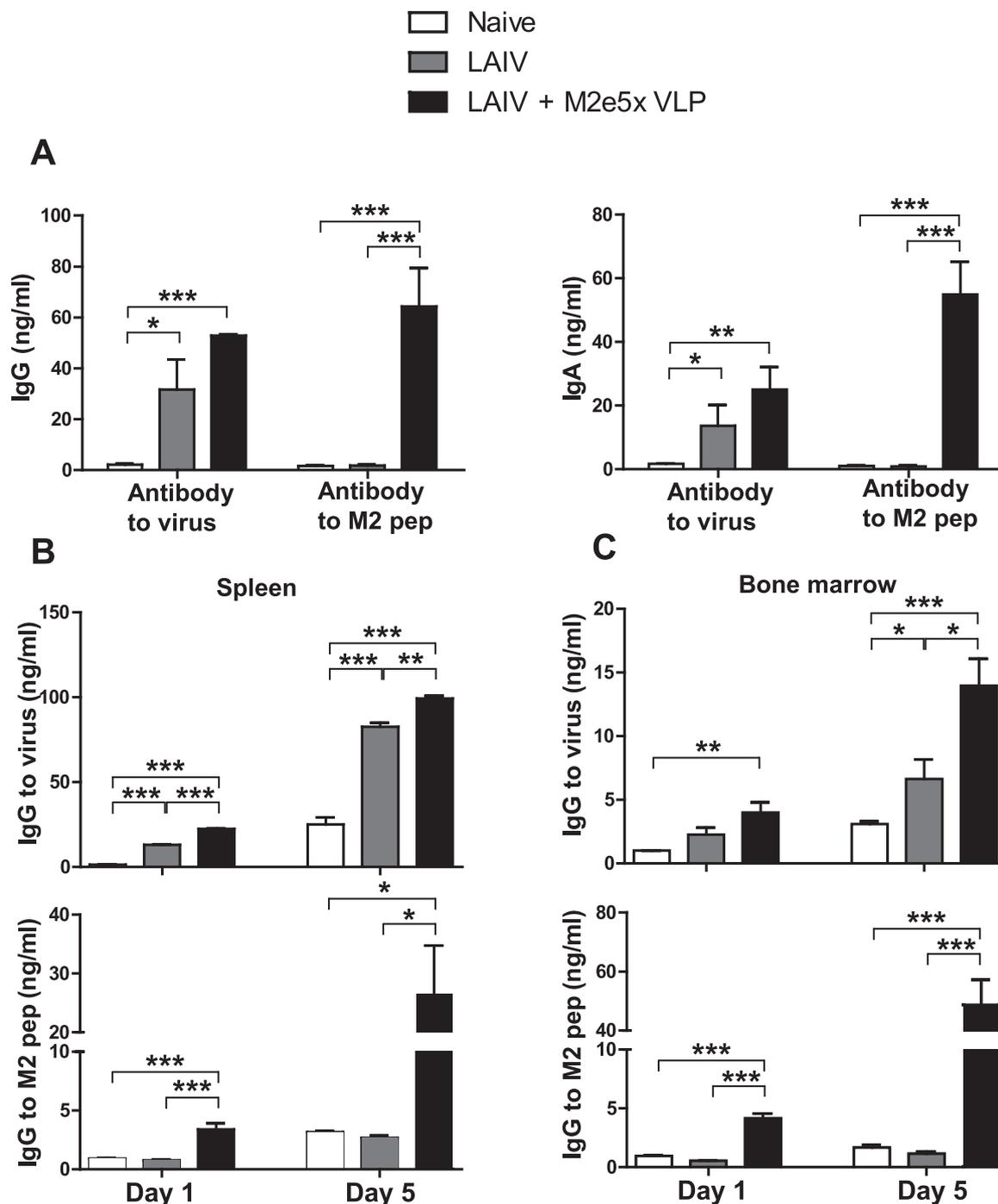


Fig. 4. LAIV + M2e5x VLP enhances antibody-secreting cells and mucosal antibody responses. The BAL fluids from LAIV alone or LAIV + M2e5x VLP vaccinated mice ($n = 5$) were collected 6 days post rgH5N1 ($6 \times LD_{50}$) virus infection. (A) Levels of IgG and IgA antibodies to M2e peptide or panH1N1 virus in the BAL fluids. (B–C) Antigen specific IgG antibody secreting cell responses *in vitro*. The cells from the spleens (B) and bone marrows (C) were harvested 6 dpi and *in vitro* cultured for 1 day or 5 days to detect IgG antibodies specific to human M2 peptide or panH1N1 virus. Statistical significance was determined using the one-way ANOVA. Error bars are means \pm SEM of concentration or ratios from individual animals. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

roles of $CD4^+$ T cells in cross protection, although the magnitudes of impacts as a result of depleting $CD4^+$ T cells are different.

4. Discussion

The CDC did not recommend the Flumist LAIV for the 2017–2018 influenza season because of its low efficacy against H1N1 influenza virus (Grohskopf et al., 2017). After 2 years of discontinuation, the CDC vaccine advisory group approved the LAIV restoring in the vaccine lineup for the 2018–19 flu season to the US market, considering the

potential merits of taking non-needle vaccines in school age children. It is highly significant to improve the efficacy by vaccination with LAIV via an intranasal route. The dose of LAIV vaccine used in this study was sufficient for conferring protection against homologous virus (A/California/04/09) (O et al., 2014; Pena et al., 2011). Consistent with the results in this study, LAIV virus vaccination strategy based on HA immunity might induce partial cross protection but would not be effective in providing significant protection against the viruses with HA strains antigenically different from the vaccine strain. A low to moderate level of M2e specific antibodies was induced in sera of mice that were three

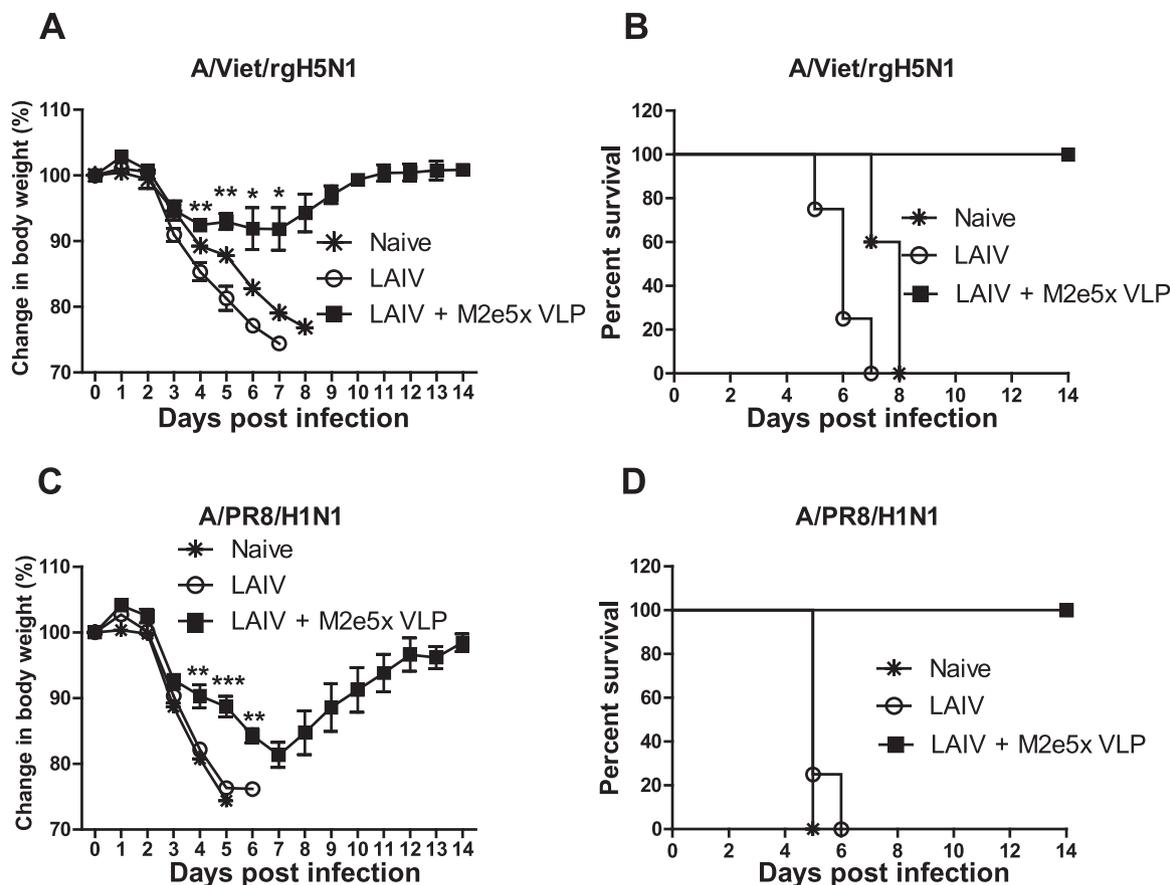


Fig. 5. Immune sera from LAIV + M2e5x VLP vaccination confer cross protection in naive mice. Naive BALB/c mice ($n = 4$) were infected with A/Vietnam/rgH5N1 virus ($5xLD_{50}$) (A and B) or A/PR8/H1N1 virus ($5xLD_{50}$) (C and D) which were pre-mixed with sera from naive, LAIV, or LAIV + M2e5x VLP vaccinated mice. (A and C) Changes in body weight and (B and D) survival rates were monitored for 14 days. Statistical significance was determined using an unpaired two-tailed Student's *t*-test. Error bars are means \pm SEM of concentration or ratios from individual animals. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

times repeatedly infected with influenza virus (Feng et al., 2006). Although human sera after natural influenza virus infection showed a 4-fold increase in M2e-specific antibodies, this level is still at a low and of short duration (Feng et al., 2006). The levels of anti-M2 antibodies increased with age in some populations (Zhong et al., 2014). Current vaccination and even virus infection would not be sufficient for inducing M2e immunity. Therefore, we focused on improving cross protection by supplementing LAIV with M2e5x VLP. We found that M2e5x VLP supplementation exhibited multiple effects on enhancing cross protection of LAIV vaccine. Inclusion of M2e5x VLP in the LAIV vaccination was highly effective in inducing M2e humoral and cellular immunity, and in significantly enhancing cross protection.

Anti-M2e antibodies are likely involved in inhibiting virus budding or killing infected cells, mediated *via* natural killer cells or macrophages through antibody-dependent cell-mediated cytotoxicity, Fc opsonization complement activation, which is dependent on Fc γ receptors *in vivo* (El Bakkouri et al., 2011; Kim et al., 2014; Kolpe et al., 2018; Lee et al., 2014). Broadly neutralizing anti-influenza antibodies through binding to the HA stalk and head domains were shown to require the engagement of Fc receptors for *in vivo* protection (DiLillo et al., 2016, 2014). Depletion of macrophages also resulted in reducing cross protection in mice with M2 immunity (Song et al., 2011b). Non-neutralizing immunity such as against M2e is weaker despite its covering wider breadth of protection than strain-specific neutralizing immunity against HA (Jegerlehner et al., 2004; Lee et al., 2016a). A strategy of supplementing strain specific HA-based vaccines with provision of cross protective antigens would provide a promising approach to protect against a wider range of viruses.

In addition to inducing M2e specific immunity, we found that M2e5x VLP might exert adjuvant effects on increasing virus reactive

IgG antibodies, recall memory B cell responses secreting IgG antibodies specific for vaccine virus, modulating immune responses toward Th1 immunity, and cross protection. This study presents evidence that humoral immune responses have contributed to enhancing cross protection by vaccination with LAIV + M2e5x VLP. Sera of LAIV + M2e5x VLP vaccination conferred significant cross protection in naive mice whereas LAIV only sera did not. High levels of M2e specific IgG and IgA antibodies were induced in the BAL fluids but not detected in the LAIV group at 6 days after challenge. Virus-specific and M2e-specific IgG recall B cells differentiating into IgG secreting plasma cells were also generated at higher levels in the LAIV + M2e5x VLP group than those in the LAIV group.

As shown by intracellular cytokine staining, M2e-specific CD4⁺ T cells secreting IFN- γ were significantly higher in mice with LAIV + M2e5x VLP vaccination compared to LAIV only vaccination. It is worth noting that NP-specific CD8⁺ T cell responses were induced in mice with LAIV + M2e5x VLP vaccination upon challenge. Supporting the important contribution of T cells, depletion of CD8⁺ T cells prior to virus challenge resulted in reducing the efficacy of protection. Previous studies reported the significant roles of CD4⁺ T cells specific for M2e epitopes in providing protection in mice (Eliasson et al., 2018; Kim et al., 2014). This study suggests that the presence of M2e5xVLP and LAIV-induced HA antibodies contributes to cross protection even in the absence of induced CD4⁺ T cells in naive mice. Thus, it might be possible that the impact of CD4 depletion is likely to be compromised in the presence of cross protective M2e5xVLP and LAIV-induced antibodies. Nonetheless, CD4 depletion in LAIV + M2e5xVLP vaccination displayed more weight loss and higher lung viral titers compared to the PBS control although the differences were not statistically significant

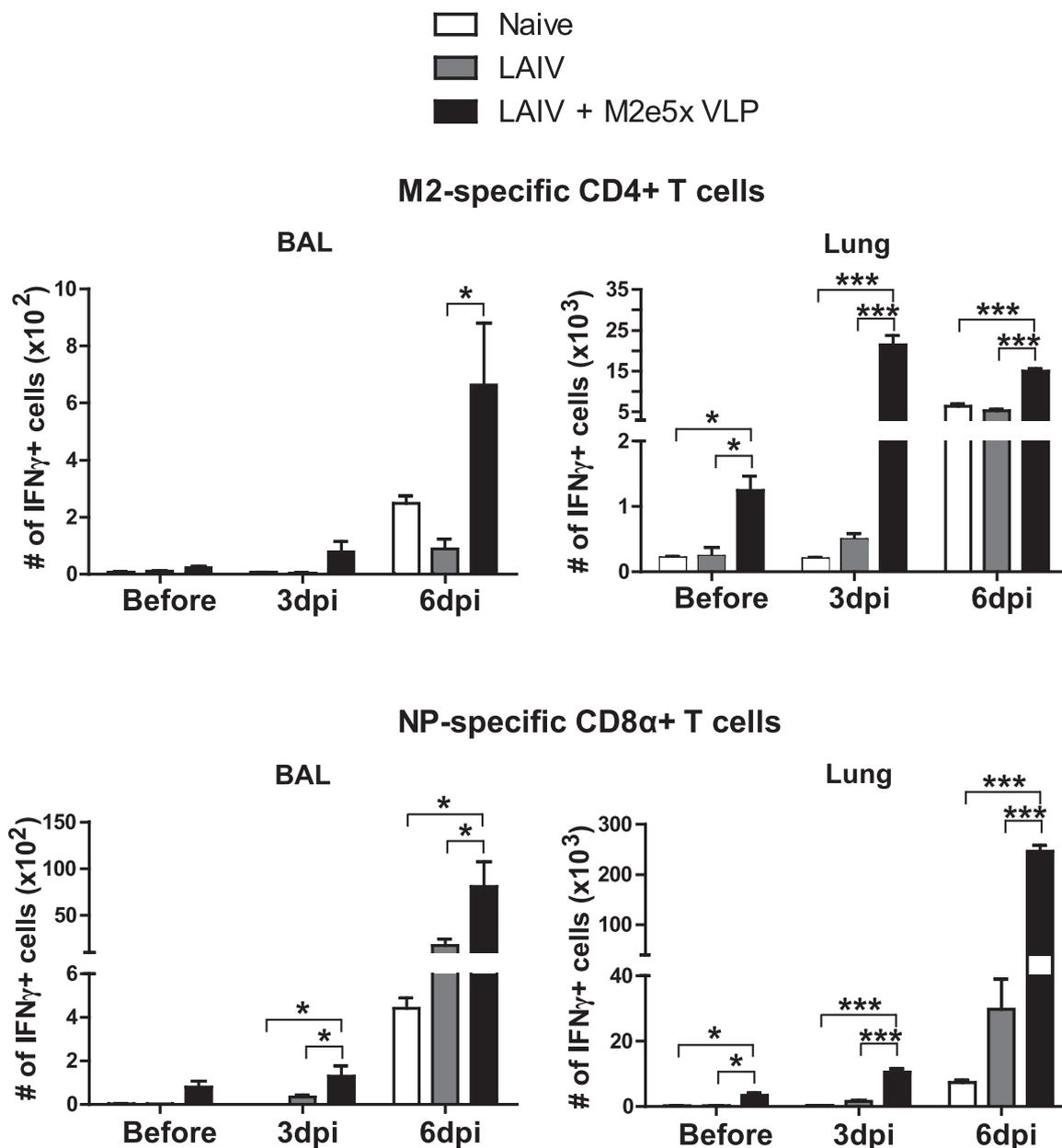


Fig. 6. LAIV + M2e5x VLP vaccination enhances M2- and NP-specific T cell responses at the mucosal sites of virus infection. Immune cells were harvested from BAL and lungs of mice 3 or 6 dpi with A/Vietnam/rgH5N1 (6xLD₅₀). Antigen-specific T cell responses were analyzed by intracellular cytokine staining flow cytometry analysis of BAL and lung cells after stimulation with M2₂₋₂₄ (A) or NP₁₄₇₋₁₅₅ (B) peptides. Statistical significance was determined using the one-way ANOVA. Error bars are means ± SEM of concentration or ratios from individual animals. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

after high dose challenge. CD4 depletion in LAIV alone vaccination resulted in more prominent weight loss and moderately high lung viral titers after sublethal dose challenge, supporting the roles of CD4⁺ T cells in cross protection.

The underlying mechanism through which M2e5x VLP exerts its adjuvant behavior remains to be determined. Phospholipid-based liposomes were reported to adjuvant diphtheria toxoid inducing increased antibody titers in mice after co-immunization (Allison and Gregoriadis, 1974). Liposome-based adjuvants that were formulated *in vitro* to carry subunit vaccines and immunostimulatory molecules were considered as a versatile approach to enhance immune responses to vaccine antigens (Schmidt et al., 2016). Influenza VLPs produced in insect cells by the rBV expression system were shown to contain baculovirus (BV)-derived capsid and envelope proteins (Song et al., 2011a). Similarly, M2e5x VLP as a particulate nature composed of insect cell-derived lipid bilayers containing M2e5x proteins in a

membrane-anchored form might have BV components. Mannose-binding residues in BV gp64 were proposed to interact with the mannose receptor lectin proteins on macrophages and DCs (Abe et al., 2003), possibly facilitating the uptake of M2e5x VLP and activating DCs. Live but not inactivated BV was reported to act as a B and T cell adjuvant for monomeric and VLP protein antigens when co-delivered in mice (Abe et al., 2009; Heinimäki et al., 2017). Th1 type adjuvant effects of 5xM2e VLP were also observed with inactivated split influenza virus vaccination of mice when co-delivered intramuscularly, whereas adjuvant effects by control VLP without M2e5x were significantly reduced (Kim et al., 2013). In addition to particulate nature of M2e5x VLP, it is possible that residual BV in the VLP preparations might be contributing to Th1 adjuvant effects. BV does not replicate in mammalian cells (Brusca et al., 1986; Tjia et al., 1983). The level of endotoxin in the M2e5x VLP preparations was below the limit of detection (Lee et al., 2018). Influenza VLP vaccines produced in insect cells have

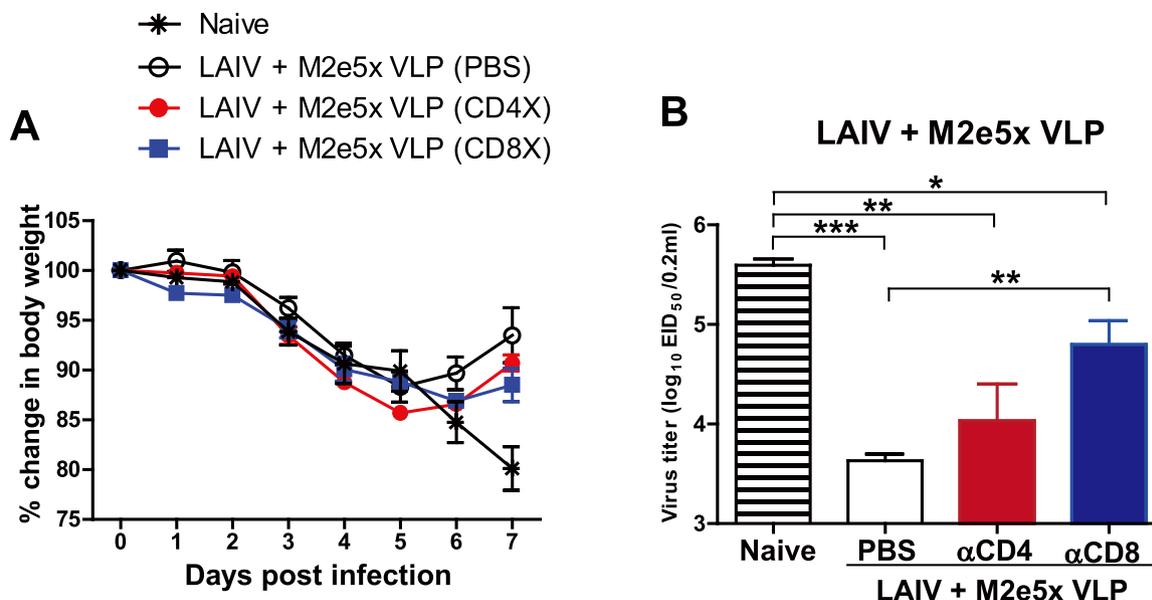


Fig. 7. Depletion of T cells reduces the efficacy of cross protection by LAIV + M2e5x VLP vaccination. LAIV + M2e5x VLP vaccinated mice ($n = 4$) were intraperitoneally administered anti-CD4 (GK1.5) or anti-CD8 (2.43) monoclonal antibodies to deplete CD4⁺ T cells and CD8⁺ T cells prior to challenge with a lethal dose of A/Philippines/2/82 (H3N2) (5xLD₅₀). PBS was used as a control. (A) Body weight changes. (B) Lung viral titers as determined by an egg inoculation assay at day 7 post infection. Statistical significance was determined using an unpaired two-tailed Student's *t*-test. Error bars are means \pm SEM of concentration or ratios from individual animals. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

been demonstrated to be safe and effective in multiple clinical trials (Fries et al., 2013; Khurana et al., 2011; Lopez-Macias, 2012; Lopez-Macias et al., 2011).

In summary, we investigated the impact of supplemented vaccination with M2e5x VLP on improving the cross protective efficacy of LAIV. Supplemented (LAIV + M2e5x VLP) vaccination moderately modulated the immune responses toward IgG2a isotype (Th1 type) antibodies specific for virus as well as M2e-specific antibody responses. Significantly enhanced cross protection against rgH5N1 virus was observed with LAIV + M2e5x VLP vaccination, which is evidenced by less weight loss, and lower levels of lung viral titers and inflammatory cells and cytokines. M2e and virus specific antibody secreting cell responses were highly induced in spleen and bone marrow cells from the group with M2e5x VLP supplemented vaccination. Both humoral and T cell responses were induced at higher levels in LAIV + M2e5x VLP vaccinated mice displaying cross protection. This study suggests that cross protective efficacy of LAIV could be significantly enhanced by supplemented vaccination with tandem repeat M2 ectodomains VLP.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.virol.2019.01.017](https://doi.org/10.1016/j.virol.2019.01.017).

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