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Highly immunogenic influenza virus-like particles containing B-cell-activating factor (BAFF) for multi-subtype vaccine development

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ABSTRACT

Virus-like particle (VLP) technology is an attractive platform for the development of seasonal and pandemic influenza vaccines. Influenza VLPs can be obtained by the overexpression of HA, M1, NA, and/or M2 viral proteins in insect, mammalian, or plant cells. In this study, we reported to obtain highly immunogenic influenza VLPs by molecular incorporation with B-cell-activating factor (BAFF) or proliferation-inducing ligand (APRIL). Since BAFF and APRIL act as homotrimers to interact with their receptors, we engineered the VLPs by direct fusion of BAFF or APRIL to the transmembrane anchored domain of H5HA gene. Results showed that immunizations with the HA-transmembrane anchored BAFF- or APRIL-VLPs only formulated in alum but not MPL adjuvant elicited significantly higher IgG titers in sera. However, only the BAFF-VLPs formulated in alum adjuvant elicited more broadly neutralizing antibodies against the homologous and two heterologous H5N1 clade/subclade viruses and conferred protective immunity against live virus challenges. As the multi-subtype influenza vaccines containing a variety of HA subtypes can confer broader protective immunity, we also obtained multi-subtype H5H7 BAFF-VLPs and H1H5H7 BAFF-VLPs and demonstrated that these multi-subtype BAFF-VLPs were able to induce the production of neutralizing antibodies against multiple HA subtypes. Our findings provided useful information for the development of highly immunogenic, multi-subtype influenza VLP vaccines.

1. Introduction

Virus-like particle (VLP) technology is an attractive platform for the development of seasonal and pandemic influenza vaccines. Influenza VLPs are non-infectious nanoparticles that do not contain genomic RNAs but contain the native HA and NA oligomeric structures that support maximum vaccine immunogenicity without compromising safety or tolerability (Haynes, 2009; Lopez-Macias, 2012). Influenza VLPs can be obtained by the overexpression of HA, M1, NA, and/or M2 viral proteins in insect cells (Bright et al., 2008; Quan et al., 2007; Ross et al., 2009; Wei et al., 2011), mammalian cells (Giles et al., 2012; Giles and Ross, 2011; Wu et al., 2012), or plant cells (D'Aoust et al., 2010; Landry et al., 2010), and have been demonstrated to elicit protective immune responses following either single- or double-dose intramuscular VLPs injections in mice or ferrets (Ross et al., 2009; Hossain et al., 2011; Wei et al., 2011; Lin et al., 2013). Successes have been also reported with intranasal VLPs immunizations in mice or ferrets (Hossain et al., 2011; Perrone et al., 2009; Wang et al., 2010). Since simultaneous delivery of antigens and adjuvants to the same antigen-presenting cells can further enhance antigen presentation and the elicitation of stronger adaptive immune responses (Beljanski et al., 2015), we reported that influenza VLPs can be genetically incorporated with the flagellin FliC (*flic* gene of *Salmonella typhimurium*) or GM-CSF to enhance the VLP immunogenicity (Wei et al., 2011; Liu et al., 2016).

B-cell-activating factor (BAFF, BLyS) and a proliferation-inducing ligand (APRIL) are both members of the TNF superfamily of cytokines. BAFF and APRIL are important B-cell factors involved in the survival and differentiation of antigen-activated B cells and antibody-secreting cells (ASCs) (Bossen and Schneider, 2006). Both BAFF and APRIL act as homotrimers that interact with homotrimeric receptors, although BAFF has been known to be active as either a hetero- or homotrimer and can aggregate into 60-mer particles (Bossen and Schneider, 2006). In

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addition, BAFF-APRIL can form single-chain heteromers at varying stoichiometric ratios and exhibit distinct receptor-binding properties; however, the BAFF-APRIL heteromers are less active than BAFF (Schuepbach-Mallepell et al., 2015). The use of BAFF and APRIL as molecular adjuvants has been reported for HIV-1 vaccine design either using soluble envelope trimers (Melchers et al., 2012) or DNA vaccines (Gupta et al., 2015).

In the present study, we obtained the wild-type (WT), BAFF- or APRIL-containing H5N1 VLPs (BAFF-VLPs, APRIL-VLPs) in a baculovirus-insect cell expression system. BALB/c mice were immunized with two doses of H5N1 VLPs without or with the addition of alum or MPL adjuvant. The titers of H5HA-specific IgG, IgG subtypes, virus neutralization (NT), NA-inhibiting (NI), and anti-M2 antibodies in sera were determined, as well as the number of ASCs in the spleen and their protection against live virus challenges. Finally, the multi-subtype BAFF-VLPs were successfully constructed to contain H1, H5, and H7 for influenza vaccine development.

2. Materials and methods

2.1. VLPs construction, production, purification, and characterization

The pFastBac dual expression vectors encoding the HA gene of A/Thailand/1(KAN-1)/2004(H5N1) (H5 clade 1), the NA gene of A/Viet Nam/1203/2004(H5N1) (H5 clade 1), and the M1 and M2 genes of A/WSN/1933(H1N1) were constructed for VLP expression as previously described (Wei et al., 2011; Liu et al., 2016). The mouse genes of APRIL (accession number: AAG22534.1) and BAFF (accession number: AAD22475.1) were synthesized and separately cloned and fused to the transmembrane anchored domain of the HA gene. Recombinant baculoviruses expressing HA/M1, M2/NA, BAFF-HAtm/M1, or APRIL-HAtm/M1 were obtained and the VLPs were harvested from Sf9 cells co-infected with baculoviruses as previously reported (Wei et al., 2011; Liu et al., 2016). Culture supernatants were filtered using a 100 kDa vivaflow filter membrane (Sartorius) and pelleted under 20% sucrose gradient sedimentation (33,000 rpm) for 4 h at 4 °C (0–60% w/v sucrose gradient sedimentation), 4 °C (Hitachi RPS40ST rotor). Fractions were characterized by Western blotting using 12% SDS polyacrylamide gel (SDS-PAGE) analysis with anti-HA, anti-NA, anti-M1, and anti-M2 antibodies (Genetex). For Transmission electron microscopy (TEM) analysis, 4 µl of purified VLPs were deposited on a carbon-vaporized 200-mesh copper grid, stained with 2% uranyl acetate solution, air-dried, and observed using a JEM-1400 TEM microscope.

2.2. Red blood cell (RBC) agglutination assay

Purified VLPs of 50 µl (3.5 µg total proteins) were 2-fold serially diluted and 50 µl of 0.5% turkey RBCs were added to each well of microtiter plates. After 30 min of incubation at room temperature, the results were recorded. The HA unit was determined by the reciprocal of the last dilution containing non-agglutinated RBCs.

2.3. Mouse immunizations

Groups of female BALB/c mice (6-to-8 weeks old) were purchased from the Taiwan National Laboratory Animal Center. Five mice per group were immunized with 0.5 µg HA content of purified VLPs mixed with 300 µg alum phosphate (Brenntag Biosector A/S) or 30 µg MPL (InvivoGen) adjuvant. Immunizations were conducted by intramuscular injection over a three-week interval. All procedures involving animals were conducted in accordance with guidelines established by the Laboratory Animal Center of National Tsing Hua University (NTHU). Animal use protocols were reviewed and approved by the NTHU Institutional Animal Care and Use Committee (IACUC). Mice that survived the immunization experiments were sacrificed using carbon dioxide (CO_2) to ameliorate suffering.

2.4. ELISA assay

H5HA proteins (KAN-1) (2 µg/ml) or 1 µg M2 ectodomain (M2e) peptide (MSLLTEVETPIRNEWGCRCNDSSD) in 0.05 M carbonate buffer were coated on 96-well plates overnight at 4 °C. After washed with PBST (PBS containing 0.05% Tween-20), the plates were blocked with blocking buffer (1% bovine serum albumin (BSA) in PBS) for 1–2 h at room temperature. After washed with PBST three times, two-fold serially diluted sera in dilution buffer (1% BSA and 0.05% Tween-20 in PBS) were added to 96-well plates, incubated for 1 h. After washed with PBST three times, anti-mouse IgG, IgG1 or IgG2a HRP-conjugated antibodies (1:10000) were added and incubated for 1 h. After three times PBST washes, the samples were incubated with TMB substrate (3, 3, 5, 5-tetramethylbenzidine) (BioLegend) in the dark for 15 min, and then the reaction was stopped by adding 2N H_2SO_4. The values of OD 450 nm were measured by a spectrophotometer. The end-point titers were defined as the serum dilution point that produced an OD_{450} value ≥ 0.2.

2.5. H5N1 and H7N9 pseudotyped particles (H5N1pp and H7N9pp) NT assay

HEK293T cells were co-transfected with pNL-Luc-E$^-$R$^-$, pcDNA3.1(+) expressing the HA gene of A/Thailand/1(KAN-1)/2004(H5N1) (H5 clade 1), A/Indonesia/5/2005(H5N1) (H5 clade 2.1.3.2), or A/Anhui/1/2005(H5N1) (H5 clade 2.3.4) plus pcDNA3.1 expressing the NA gene of the A/Viet Nam/1203/2004(H5N1) (H5 clade 1) strain; or the HA gene of A/Shanghai/02/2013 (H7N9) plus pcDNA3.1 expression the NA gene of A/Shanghai/02/2013 (H7N9). MDCK cells were inoculated with the diluted sera and H5N1pp or H7N9pp to measure the luciferase activity using neolite luciferase substrate (PerkinElmer) as previously reported (Wei et al., 2011; Liu et al., 2016). NT antibodies were quantified as reduced luciferase expression level following H5pp or H7pp transduction in MDCK cells. NT IC50 values were determined as the serum dilution required to obtain 50% reduction in relative light unit compared to control wells with H5N1pp or H7N9pp alone.

2.6. pH1N1 virus plaque NT assay

MDCK cells were grown in 6-well plates. Two-fold serially diluted immunized sera were mixed with equal volume of 100 PFU pH1N1 (A/California/04/2009) viruses in MEM-α containing 0.5 µg/ml TPCK-trypsin (Sigma- Aldrich). Plaques were counted and the reduction of plaque numbers was measured for neutralization activity.

2.7. NA enzyme activity and NI assay

The enzyme-linked lectin assay (Lambre et al., 1990) was used to determine NA enzyme activity of purified VLPs. 100 µl of 50 µg/ml fetuin (Sigma) were coated in 96-well plates overnight. Purified VLPs were serially diluted and reacted with 100 µl/well peroxidase conjugate lectin from *Arachis hypogaea* (peanut). The NA enzyme activity was determined by adding TMB substrate and read with an ELISA reader at an OD of 450 nm. For NI titers, serially diluted serum samples incubated with equal volumes of 1 µg H5N1 VLPs were added to fetuin-coated plates, and then reacted with peroxidase-labeled peanut agglutinin (Sigma). Corresponding IC50 values were defined as the reciprocal serum dilution inhibiting 50% of viral NA enzyme activity.

2.8. Detection of ASCs in spleen

Multiscreen 96-well filtration plates were coated with 1 µg H5HA proteins overnight. Plates were blocked with complete RPMI-1640 (10% FBS, 1x P/S, 1x Sodium pyruvate, 1x NEAA and 100 µM β-ME). Splenocytes were added to 96-well plates (5×10^5 per well) incubated

for 48 h and reacted with HRP-conjugated anti-mouse IgG antibodies. AEC substrate (Sigma-Aldrich) was added and stopped with ddH_2O.

2.9. Protection against live virus challenges

Three weeks following the second-dose immunization of these VLP-immunized mice, all groups were intranasally challenged with 10 LD_{50} of the NIBRG-14 vaccine strain (NIBSC code: 07/252) which is a re-assortant virus prepared by reverse genetics from A/Vietnam/1194/2004 (H5N1) virus, in which the polybasic HA cleavage site has been excised, and A/PR/8/34(H1N1) virus. PBS-immunized mice were used as a mock control. The volume of NIBRG-14 viruses delivered intranasally was 25 µl per mouse. We used intraperitoneal injection with 100 µl of Zoletil 50 (Virbac) at the dose of 25 mg/kg during murine inoculation. Survival rates and body weights will be recorded daily for 14 days. Experimental procedures were reviewed and approved by the IACUC of Academia Sinica, Taiwan. According to IACUC guidelines, a weight loss of 25% or more was established as an end-point.

2.10. Statistical analyses

All results were analyzed using one-way analysis of variance (ANOVA) by the GraphPad Prism v6.01 software. Statistical significance in all results was expressed as the following: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. All experiments were performed at least twice.

3. Results

3.1. Expression and characterization of H5N1 WT-VLPs, BAFF-VLPs, and APRIL-VLPs

Mouse BAFF or APRIL cDNAs were directly fused to the transmembrane-anchored domain of the gene encoding H5HA (KAN-1) and cloned into the pFastBac dual vector (Fig. 1A). H5N1 WT-VLPs, BAFF-VLPs, APRIL-VLPs were obtained from the culture supernatants of Sf9 insect cells co-infected with the following combinations of recombinant bacuoviruses: (i) Bac-HA-M1 and Bac-M2-NA for H5N1 WT-VLPs, (ii) Bac-HA-M1, Bac-M2-NA and Bac-APRIL/HAtm-M1 for H5N1-APRIL-VLPs, (iii) Bac-HA-M1,Bac-M2-NA and Bac-BAFF/HAtm-M1 for H5N1-BAFF-VLPs. These VLPs were further characterized by sucrose gradient analysis and examined by Western blotting for the presence of four viral proteins (HA, NA, M1, and M2) or the fusion proteins of BAFF/HAtm-M1 or APRIL/HAtm-M1 in sucrose gradient fractions (Fig. 1B–D) and by TEM visualization (Fig. 1E). To test and visualize the influenza HA-induced agglutination of red blood cells (RBCs), serial dilutions of H5N1 WT VLPs, BAFF-VLPs, and APRIL-VLPs were mixed with turkey RBCs. Results indicated that a 2^8 HA unit for H5N1 WT-VLPs, a 2^7 HA unit for H5N1-BAFF-VLPs, and a 2^6 HA unit for H5N1-APRIL-VLPs (Fig. 1F). On the other hand, the NA enzymatic activities for these VLPs were measured using fetuin-based assay since the NA cleaves the terminal sialic acids from glycan moieties (Lambre et al., 1990). Results demonstrated that H5N1 WT VLPs, BAFF-VLPs, and APRIL-VLPs retained a similar range of influenza viral NA enzymatic activity (Fig. 1G).

3.2. H5HA-specific IgG, IgG1, and IgG2a antibodies and ASCs elicited by H5N1 WT-VLPs, BAFF-VLPs, and APRIL-VLPs immunizations

Groups of BALB/c mice were intramuscularly immunized with two doses of WT-VLPs, APRIL-VLPs or BAFF-VLPs, each containing 0.5 µg of HA per dose without or with MPL or alum adjuvant (Fig. 2A). Mouse sera were collected at two weeks following the second immunization. Results indicated that mice immunized with WT-VLPs, APRIL-VLPs or BAFF-VLPs with the addition of MPL or alum adjuvant produced higher titers of H5HA-specific total IgG antibodies than that of VLPs without adjuvant (Fig. 2B). Immunizations with APRIL-VLPs and BAFF-VLPs with alum adjuvant significantly increased antibody titers than WT-VLPs with alum adjuvant, and induced significantly higher titers than WT-VLPs, APRIL-VLPs or BAFF-VLPs with the use of MPL adjuvant (Fig. 2B). Mice immunized with BAFF-VLPs plus alum adjuvant showed the highest IgG titers among all the immunized groups (Fig. 2B). For H5HA-specific IgG1 titers in sera, the mice immunized with WT-VLPs, APRIL-VLPs and BAFF-VLPs with alum adjuvant showed highest titers than those immunized with VLPs and MPL adjuvant, indicating a preferring Th2-screwed cellular response by the use of alum adjuvant (Fig. 2C). However, mice immunized with BAFF-VLPs with alum or MPL adjuvant showed higher IgG2a titers than those of the groups immunized with WT-VLPs and APRIL-VLPs with or without MPL adjuvant or alum (Fig. 2D). As a result, immunizations with BAFF-VLPs with alum adjuvant were found to induce the highest titers of H5HA-specific IgG, IgG1 and IgG2a antibodies. To determine the numbers of H5HA-specific ASCs elicited by VLP immunization, splenoctyes were collected 3 weeks after the second dose immunizations and subsequently stimulated with H5HA proteins for ELISPOT staining IgG-secreting cells. Results indicated that the numbers of ASCs from the immunized groups APRIL-VLPs and BAFF-VLPs with alum adjuvant were significantly higher than those obtained in other immunized groups (Fig. 2E). Therefore, immunizations with BAFF-VLPs and APRIL-VLPs with alum adjuvant induced more potent B-cell responses in the spleen.

3.3. NT, NI, and anti-M2 antibodies elicited by H5N1 WT-VLPs, BAFF-VLPs, and APRIL-VLPs immunizations

H5N1pp assays were performed to measure neutralizing antibodies against various H5N1 clade/subclade viruses. Serum neutralization curves for WT-VLPs, APRIL-VLPs and BAFF-VLPs without or with the addition of alum or MPL adjuvant showed that antibody neutralization all increased in a in dose-dependent manners against the homologous strain KAN-1 (clade 1), and three heterologous Indonesia (clade 2.1.3.2), and Anhui (clade 2.3.4) (data not shown). Their corresponding half-maximal (50%) inhibitory concentrations (IC50s) were calculated based on these neutralization curves. The BAFF-VLPs with alum group showed almost one-to two-log higher IC50s against the homologous KAN-1 (clade 1) (Fig. 3A) and two heterologous Indonesia (clade 2.3.1.2) (Fig. 3B) and Anhui (clade 2.3.4) (Fig. 3C) when compared to those of the other treatment groups. We also measured the titers of NI antibodies in sera against H5N1 VLPs. Results showed that immunizations with BAFF-VLPs plus MPL or alum adjuvants elicited significantly higher NI titers than immunizations with WT-VLPs and APRIL-VLPs with MPL or alum adjuvants (Fig. 3D). Only immunizations with BAFF-VLPs and APRIL-VLPs formulated with alum adjuvants elicited increased M2e-specific IgG titers (Fig. 3D).

3.4. Protective immunity elicited by H5N1 WT-VLPs, BAFF-VLPs, and APRIL-VLPs immunizations

Protective immunity in the immunized mice was determined by challenges with 10 MLD50 H5N1 viruses (NIBRG-14) 3 weeks following the second-dose immunizations. Survival rate and body weight loss of mice were recorded daily for 14 days. Results indicated that only immunization with BAFF VLPs with alum adjuvant conferred complete protection against live virus challenges with a 100% survival rate (Fig. 4A) and recovery of weight loss to approximately 90% (Fig. 4B). No protection was observed for all immunization groups, including the WT-VLPs, and APRIL-VLPs, BAFF-VLPs with MPL adjuvant, WT-VLPs and APRIL-VLPS with alum adjuvant, and the PBS-immunized groups (Fig. 4).

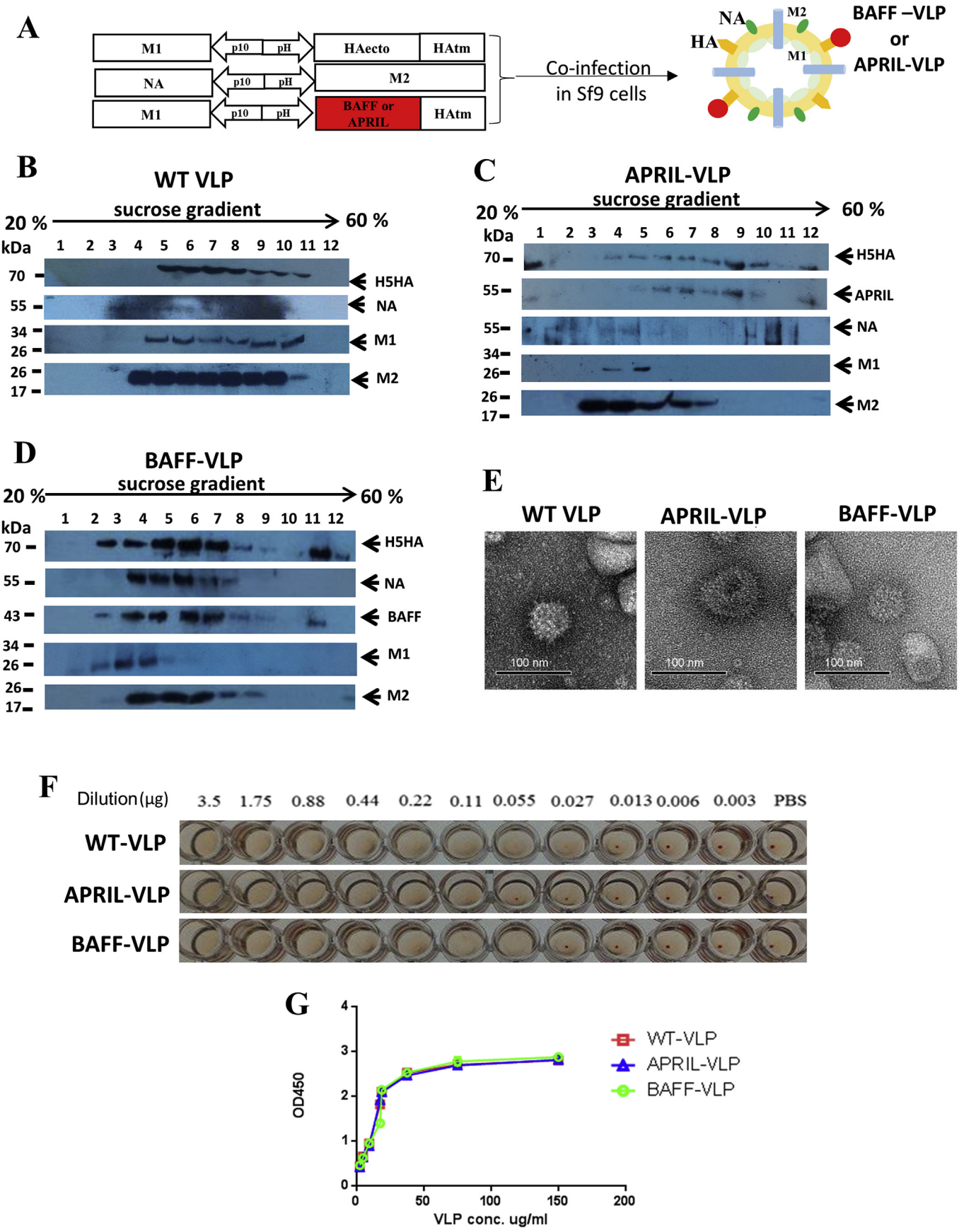

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Fig. 1. Construction and characterization of H5N1 WT VLPs, BAFF-VLPs and APRIL-VLPs. (A) Baculovirus expression with dual promoters (pH: polyhedron; p10: p10) for co-infection in Sf9 cells for H5N1 BAFF-VLPs and APRIL-VLPs production. Supernatants were harvested, purified from sucrose gradient sedimentation, and resolved in SDS-PAGE gels reacted with anti-H5HA, anti-M1, anti-NA, or anti-M2 antibodies for **(B)** H5N1 WT-VLPs, **(C)** H5N1 APRIL-VLPs, and (**D**) H5N1 BAFF-VLPs. (**E)** Purified particle images of H5N1 WT-VLPs, APRIL-VLPs, and BAFF-VLPs analyzed by TEM; **(F)** Purified particles were serially diluted and mixed with turkey RBCs for 30 min and visualized for RBC agglutination; **(G)** NA enzymatic activity determined using fetuin-based assay for the purified WT-VLPs, APRIL-VLPs, and BAFF-VLPs.

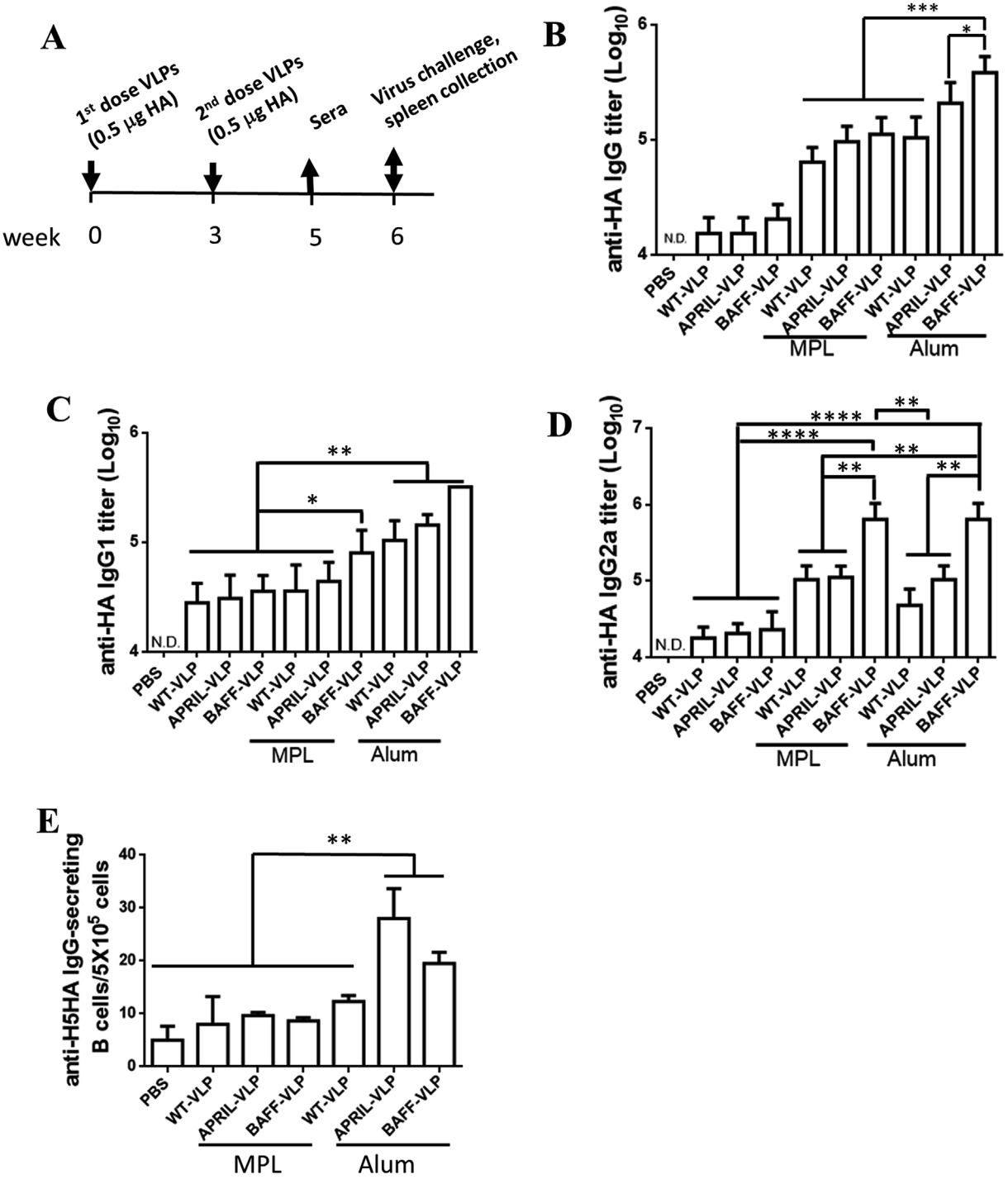


Fig. 2. H5HA-specific antibodies elicited by two-dose H5N1 WT-VLPs, BAFF-VLPs, and APRIL-VLPs immunizations without or with the use of MPL or Alum adjuvants. (A) Two-dose immunization regiments, **(B)** IgG titers in sera, **(C)** IgG1 titers in sera, **(D)** IgG2a titers in sera, **(E)** IgG-secreting ASCs in spleen. Results were analyzed using One-way ANOVA among each group (except PBS). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. N.D. for not detectable.

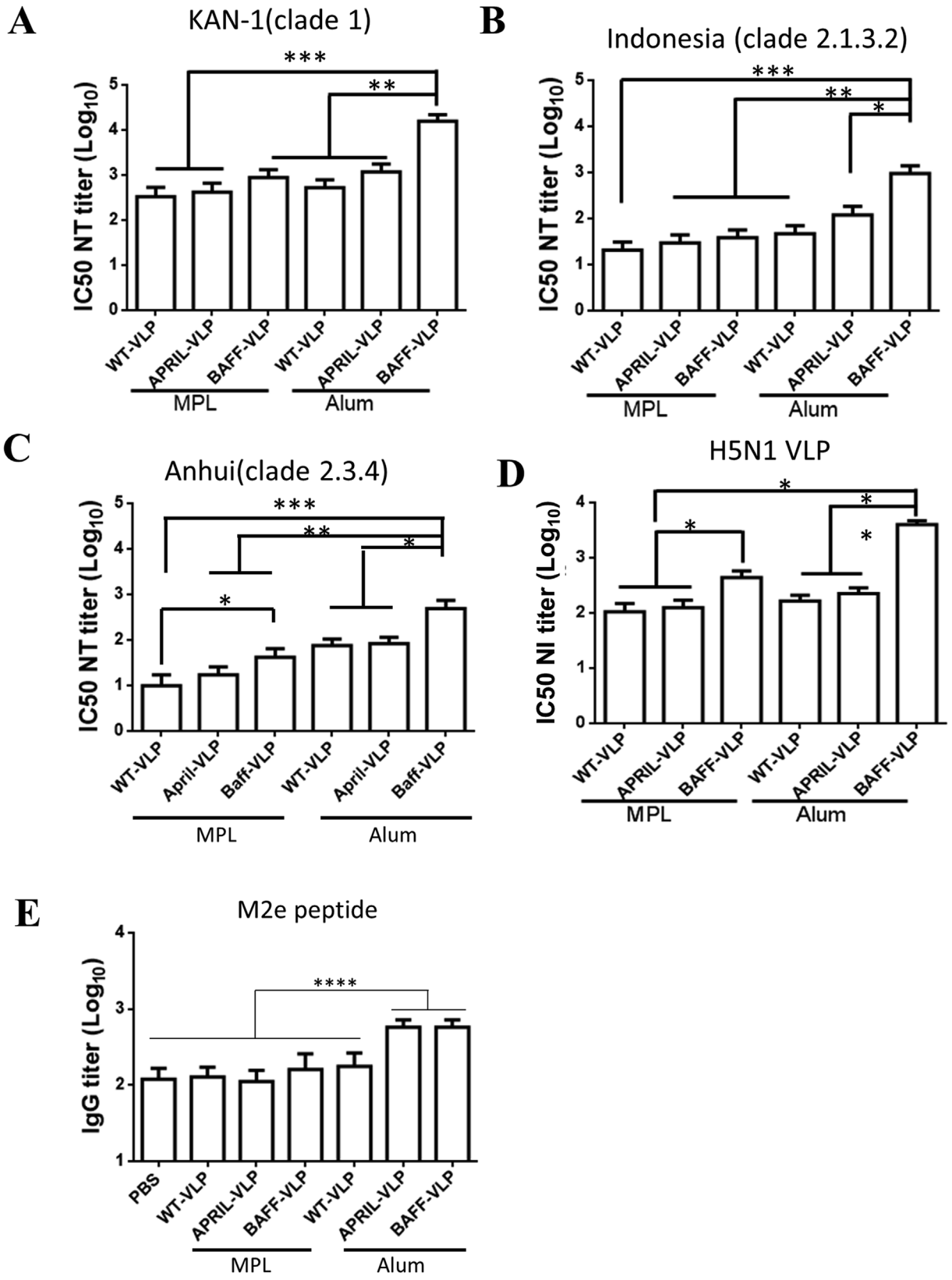


Fig. 3. Neutralization and NI antibodies elicited by two-dose H5N1 WT-VLPs, BAFF-VLPs, and APRIL-VLPs immunizations with the use of MPL or Alum adjuvants. Neutralizing antibody titers are shown as NT IC50 values against **(A)** the homologous strain KAN-1 (clade 1), and two heterologous strains: **(B)** Indonesia (clade 2.1.3.2) and **(C)** Anhui (clade 2.3.4). **(D)** NI antibodies are shown as NI IC50 values against H5N1 strain. Results were analyzed using One-way ANOVA among each group. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

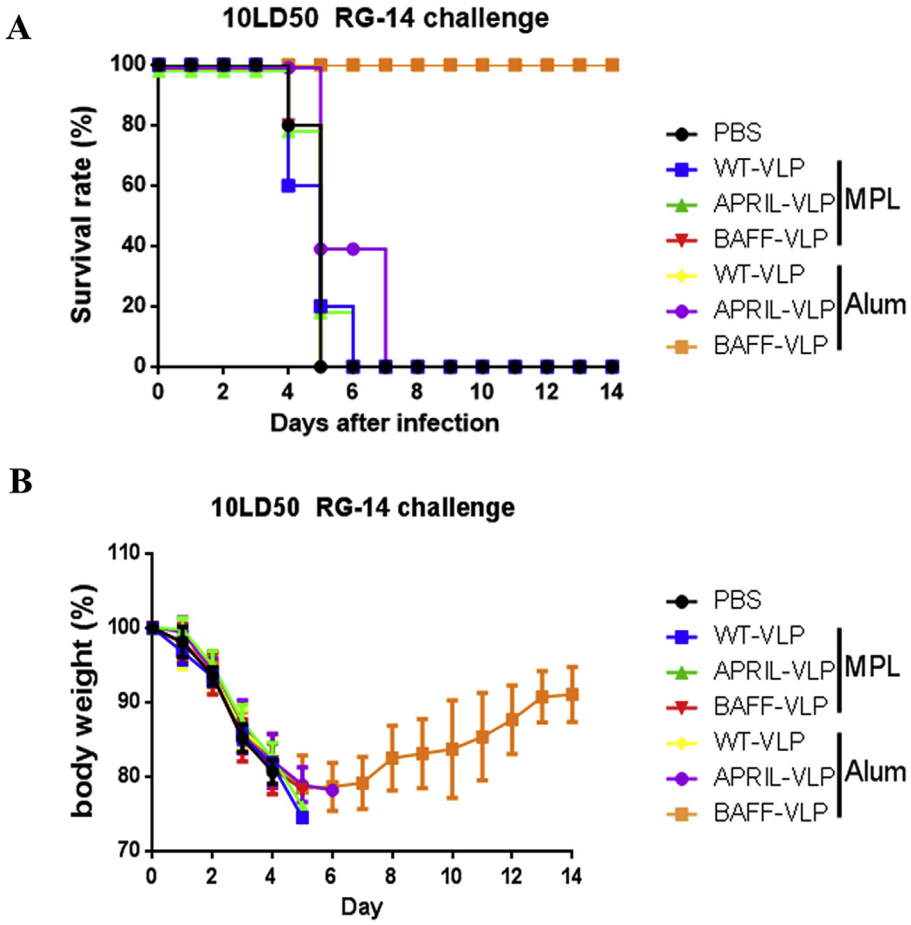


Fig. 4. Protective immunity elicited by H5N1 WT-VLPs, BAFF-VLPs, and APRIL-VLPs immunizations. Three weeks following the second-dose immunization of these VLP-immunized mice, all groups were intranasally challenged with 10 LD_{50} of the H5N1 (NIBRG-14) viruses. **(A)** Survival rate and **(B)** body weight loss were monitored for 14 days. Mice whose body weights fell below 75% of their initial weights were sacrificed.

3.5. Long-term sustained immunity from H5HA-specific IgG and NT antibodies

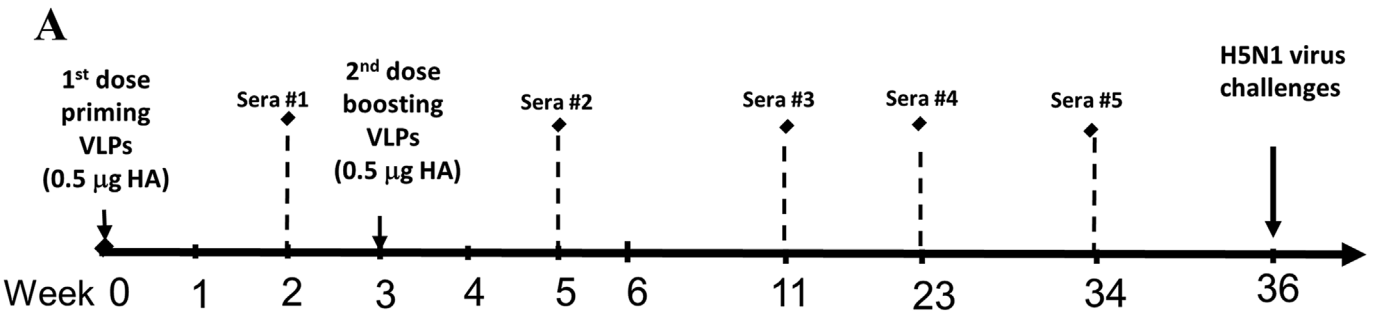
To determine the long-term immune responses elicited by immunization with WT-VLPs, APRIL-VLPs or BAFF-VLPs, we measured the titers of H5HA-specific total IgG and neutralizing antibodies up to 34 weeks following the first priming immunizations. Sera were collected at 2 weeks (sera #1), 5 weeks (sera #2), 11 weeks (sera #3), 23 weeks (sera #4), and 34 weeks (sera #5) following the first priming immunizations (Fig. 5A). Results indicated that the IgG titers for the mice immunized with BAFF VLPs with alum adjuvant collected during the last four time points (sera #2–5) were all significantly higher than those obtained from the other immunized groups (Fig. 5B). In addition, we measured the neutralizing antibody titers of sera #5 at 34 weeks after priming immunization. Results showed that immunization with BAFF VLPs containing alum adjuvant induced significantly higher neutralizing antibody titers (IC50s) than the immunization with WT-VLPs plus alum and WT-VLPs, APRIL-VLPs, and BAFF-VLPS plus MPL adjuvant against the homologous strain KAN-1 (clade 1) (Fig. 5C) and two heterologous clades, namely, the Indonesia (clade 2.1.3.2) (Fig. 5D) and Anhui (clade 2.3.4) clades (Fig. 5E). To further determine the long-term sustained protective immunity by WT-VLPs, APRIL-VLPs or BAFF-VLPs, immunized mice at 36 weeks after the priming immunizations were challenged with 10 LD50 H5N1 viruses (NIBRG-14). Results indicated that the survival rates were 40% for the BAFF-VLPs plus alum group and 20% for the APRIL-VLPs plus alum group, but no protection was observed for all other immunized groups (Fig. 6A). Recovery from body weight loss was only observed for BAFF-VLPs and APRIL-VLPS containing alum adjuvant (Fig. 6B).

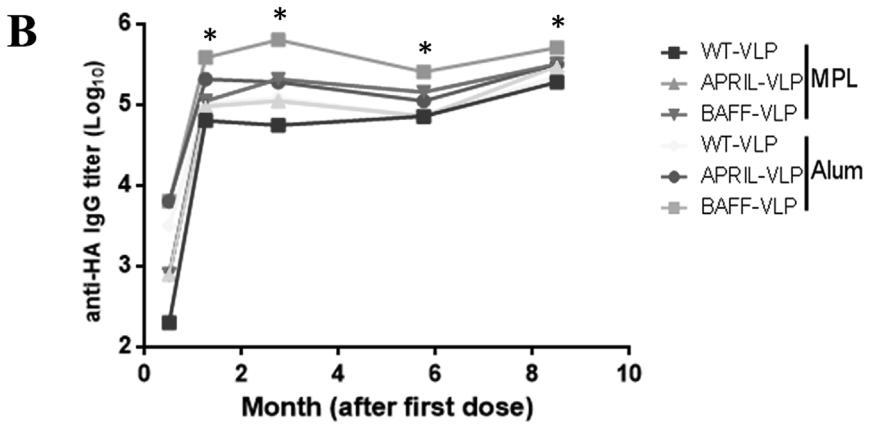
3.6. Construction and characterization of bi-subtype (H5H7), or tri-subtype (H1H5H7) BAFF VLPs

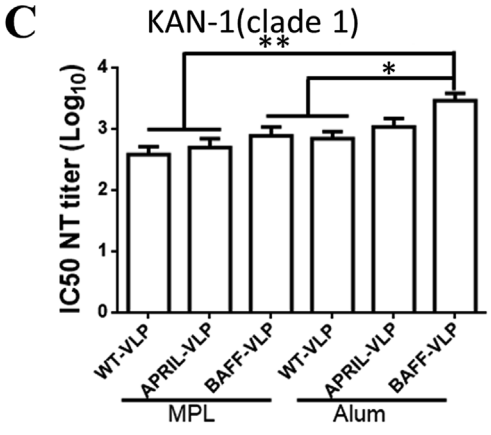
Bi-subtype (H5H7) and tri-subtype (H1H5H7) BAFF-VLPs were constructed. Sf9 insect cells were co-infected with specific sets of recombinant baculoviruses for VLPs production (Fig. 7A). The purified VLPs were characterized by sucrose gradient sedimentation and western blot analysis for bi-subtype (H5H7) BALF-VLPs (Fig. 7B) and tri-subtype (H1H5H7) BAFF-VLPs (Fig. 7C). TEM analysis revealed that the VLPs were spherical and pleomorphic particles (Fig. 7D and E). Immunization results indicated that two multi-subtype BALF-VLPs showed similar IC50s against H5N1 KAN strain for H5, H5H7, and H1H5H7 BAFF-VLPs (Fig. 7F). The bi-subtype (H5, H7) BAFF-VLPs and tri-subtype (H1H5H7) BAFF VLPs showed approximately one-log higher IC50s against H7N9 virus when compared to those of the mono-subtype (H5) BAFF-VLPs (Fig. 7G). The IC50 of the tri-subtype (H1H5H7) BAFF VLPs was significantly higher than that of the mono-subtype (H5) and bi-subtype (H5H7) BAFF-VLPs against pH1N1 viruses (Fig. 7H).

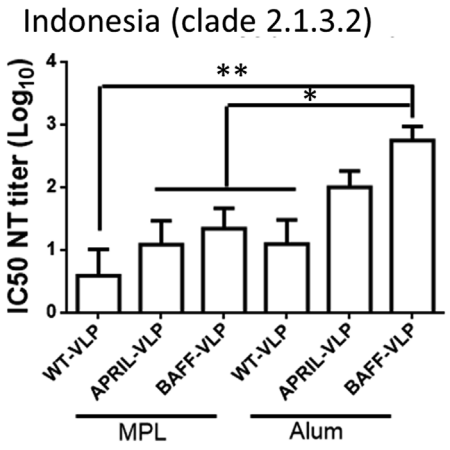
4. Discussion

VLPs can be genetically engineered by the incorporation with molecular adjuvants, such as TLR ligands and cytokines, into the VLPs to enhance their immunogenicities. In this study, we reported a new design to obtain highly immunogenic influenza VLPs by molecular incorporation with BAFF or APRIL, which are B-cell growth factors. BAFF and APRIL act as homotrimers to interact with their receptors (Bossen and Schneider, 2006). We engineered the VLPs by direct fusion of BAFF or APRIL to the transmembrane anchored domain of H5HA gene. The trimeric conformation of BAFF and APRIL incorporation provided by the HA-transmembrane anchor are important, since we failed in









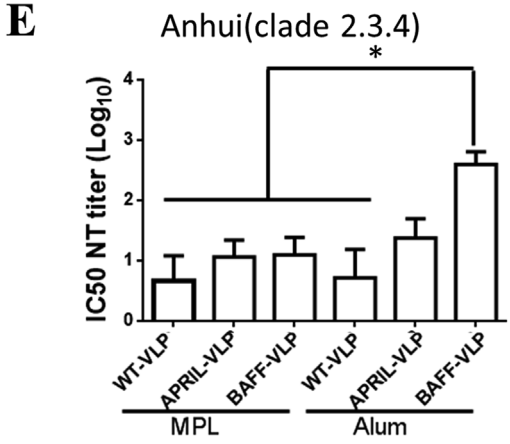


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Fig. 5. Long-term sustained immunity for H5HA-specific IgG and neutralizing antibodies elicited by two-dose H5N1 WT-VLPs, BAFF-VLPs, and APRIL-VLPs immunizations. (A) Sera collected at 2 weeks (sera #1), 5 weeks (sera #2), 11 weeks (sera #3), 23 weeks (sera #4), and 34 weeks (sera #5) following the first-dose immunizations, **(B)** H5HA-specific total IgG titers determined up to 7–8 months following priming immunizations. Neutralizing antibodies in sera #5 (34 weeks following the first-dose immunization) against **(C)** the homologous strain KAN-1 (clade 1), and two heterologous strains: **(D)** Indonesia (clade 2.1.3.2) and **(E)** Anhui (clade 2.3.4). Results were analyzed using One-way ANOVA among each group. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

inducing anti-influenza immunity by the engineered VLPs using M2 fusion to BAFF or APRIL (data not shown). Our results showed that immunizations with the HA-transmembrane anchored BAFF- or APRIL-VLPs elicited significantly higher IgG titers but only the BAFF-VLPs induced more potent and broadly neutralizing antibodies against H5N1 clades and conferred protective immunity against live virus challenges.

Our results demonstrated that the use of alum or MPL adjuvant in VLP formulations is still required to induce effective anti-influenza immunogenicity. Alum is an adjuvant that is known to cause cell death to release numerous molecules that act as damage-associated molecular patterns to alert the innate immune systems (Maricha et al., 2011); alum can also directly activate NLRP3 inflammasomes (Ruwona et al., 2016). MPL is a synthetic TLR4 ligand that can trigger innate immune responses (Ireton and Reed, 2013). Our findings showed that the IgG titers of BAFF- or APRIL-VLPs formulated in MPL or alum adjuvant were at least one-log higher compared to those without adjuvant. Furthermore, our results indicated that BAFF- or APRIL-VLPs formulated with alum instead of MPL adjuvant induced higher titers of IgG1 subtype antibodies and thus more potent Th2 response. By contrast, BAFF-VLPs either formulated with alum or MPL adjuvant induced higher levels of IgG2a antibodies, which favor Th1 cellular responses.

Our findings here regarding efficacy of BAFF verse APRIL, and alum verse MPL adjuvant in elicitation of protective antibodies, demonstrated that only the BAFF-VLPs formulated in alum adjuvant elicited more potent and broadly neutralizing antibodies, higher numbers of ASCs in spleen, and conferred better protection against live virus challenges. Our results are somewhat contradictory to other reports that the APRIL-containing HIV-vaccine was superior to the BAFF-containing HIV vaccine to augment antibody responses with neutralizing capabilities (Melchers et al., 2012; Gupta et al., 2015). It was recently reported that the BAFF-containing recombinant rabies vaccine can rapidly generate antibody-secreting plasma cells through the extrafollicular and not the germinal center B cell pathways (Haley et al., 2017). In contrast, the APRIL-containing recombinant rabies vaccine was found to be dispensable for the long-live antibody-secreting plasma cells and was unrelated to the TACI-associated signaling (Haley et al., 2017). How BAFF and APRIL elicit strong antibody responses by either targeting to B cells by B-cell receptor binding, or targeting to other antigen-presenting cells such as macrophages and dendritic cells requires further investigation, as well as the assessment of potentially undesired autoimmune responses induced by BAFF and APRIL immunizations (Sakai and Akkoyunlu, 2017).

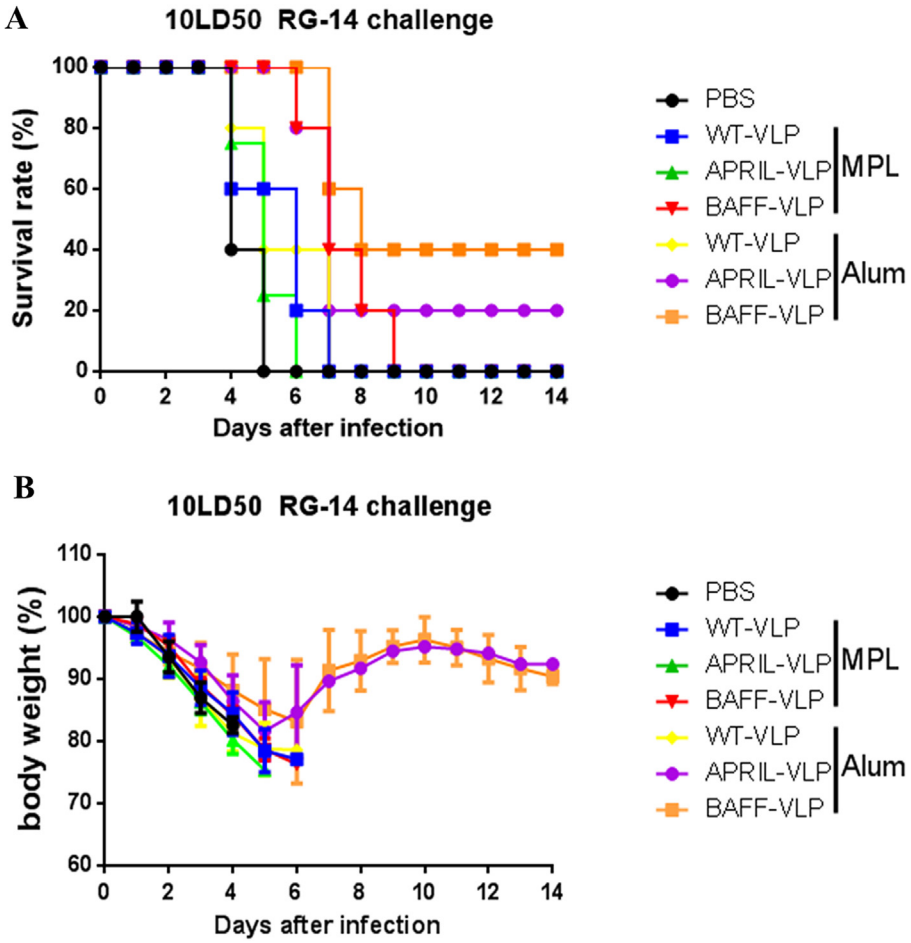

Fig. 6. Long-term protective immunity elicited by two-dose H5N1 WT-VLPs, BAFF-VLPs, and APRIL-VLPs immunizations. Thirty-six weeks following the VLPs immunizations, all groups were intranasally challenged with 10 LD_{50} of the H5N1 (NIBRG-14). **(A)** Survival rate and **(B)** body weight loss were monitored for 14 days. Mice whose body weights fell below 75% of their initial weights were sacrificed.

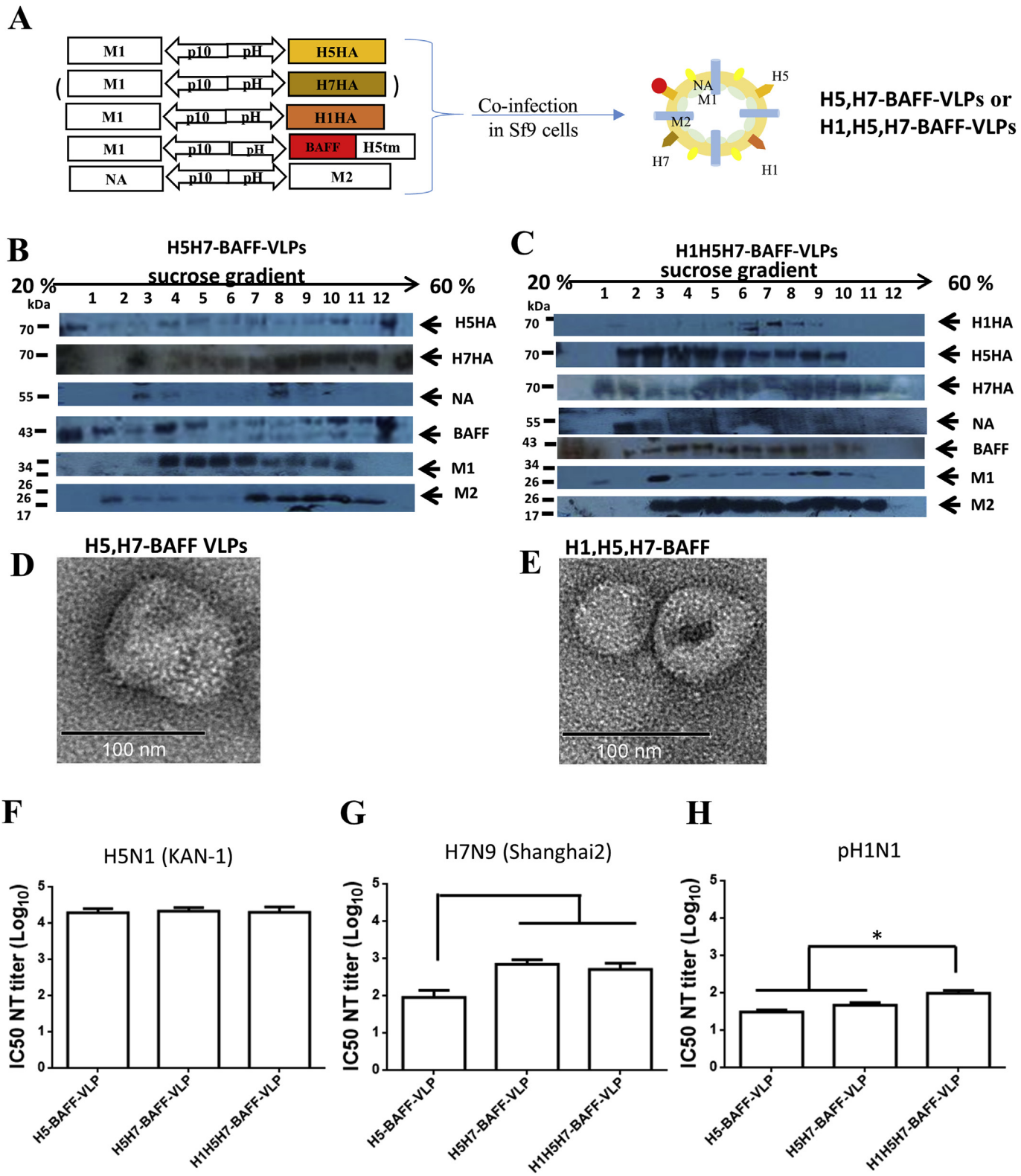

Fig. 7. Construction and characterization of bi-subtype (H5H7), or tri-subtype (H1H5H7) BAFF VLPs. (A) Baculovirus expression for bi-subtype (H5H7) and tri-subtype (H1H5H7) BAFF-VLPs. Supernatants were harvested, purified from sucrose gradient sedimentation, and resolved in SDS-PAGE gels reacted with anti-H5HA, anti-M1, anti-NA, or anti-M2 antibodies for **(B)** bi-subtype (H5H7) BAFF VLPs expressing six proteins (H5HA, H7HA, M1, NA, M2, and BAFF). **(C)** Tri-subtype (H1H5H7) BAFF-VLPs expressing seven proteins (H1HA, H5HA, H7HA, M1, NA, M2 and BAFF). Particle images of **(D)** bi-subtype (H5H7) and **(E)** tri-subtype (H1H5H7) BAFF-VLPs were demonstrated by TEM analysis. Neutralizing antibody titers as NT IC50 values against **(F)** H5N1pp (KAN-1), **(G)** H7N9pp and **(H)** pH1N1 (CA/09) virus. Results were analyzed using One-way ANOVA among each group. *, $p < 0.05$.

In the present study, we only conducted the investigation on alum or MPL adjuvant for BAFF- and APRIL-containing VLPs. As the combination of alum and MPL adjuvant (i.e. AS04) has been used for licensed vaccines against human papillomavirus and hepatitis B virus (Del Giudice et al., 2018), BAFF-VLPs formulated with the combination of alum and MPL adjuvants may further improve its anti-influenza immunity as a recent report for T cell-dependent influenza vaccine with alum and MPL combination adjuvant was shown to effectively alter IgG isotype-switched antibodies for anti-influenza protection (Ko et al., 2017). In addition, we also successfully obtained multi-subtype H5H7, and H1H5H7 BALF-VLPs in this study. Our present findings may provide useful information for the development of highly immunogenic, multi-subtype influenza VLP vaccines.

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.02.004.

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