PROTECTION BY CO-IMMUNIZATION OF INFLUENZA HEMAGGLUTININ AND TANDEM REPEAT M2e VIRUS-LIKE PARTICLE VACCINES IN AGED MICE

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by

HYUNJUNG CHUNG

Under the Direction of Sang-Moo Kang, PhD

ABSTRACT

Recurrence of distinct strains of seasonal influenza viruses requires the development of universal vaccines. Previous studies have demonstrated the cross-protection effects of influenza virus A-derived extracellular domain of ion channel matrix protein 2 (M2e) vaccines in an adult mouse model. However, M2e vaccine efficacy of wide-range protection against influenza remains largely unknown in aged mice. I hypothesized that co-immunization with M2e virus-like particle (M2e-VLP) vaccine and hemagglutinin-based VLP vaccine (HA-VLP) can provide superior protection against homologous virus and antigenically different strains of influenza viruses in aged (15-18 months old) BALB/c mice compared to the current HA-based vaccine. In this study, I examined a strategy of co-immunization of HA-VLP and M2e-VLP as a potential
approach for effective vaccination for the immunosuppressed and elderly against a wide-range of influenza strains.

INDEX WORDS: Virus-like particle vaccine, Influenza virus, Immunosenescence, Cross-protection
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DEDICATION

I would like to thank my family and friends for their support and endless love.
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LIST OF ABBREVIATIONS

1. VLP : Virus-like particle
2. HA : Influenza Hemagglutinin
3. M2e : Influenza ectodomain of ion channel matrix protein 2
4. PR8-VLP : Hemagglutinin-based A/Puerto Rico/1934/H1N1-derived virus-like particle vaccine
5. M2e-VLP : Tandem repeating influenza viral matrix protein 2 expressed on virus-like particle platform
6. sPR8 : A/Puerto Rico/1934/H1N1-derived split inactivated vaccine
1 INTRODUCTION

1.1 Influenza virus

Influenza virus belongs to the family *Orthomyxoviridae* and possesses eight-segmented, negative sense single stranded RNA. Influenza type A viruses are subcategorized into various subtypes based on distinct combinations of hemagglutinin (HA) and neuraminidase (NA), the two major surface proteins of influenza A viruses [2, 39]. Currently, there are 18 HA (H1 to H18) and 11 NA (N1 to N11) subtypes known to present and to keep mutating in human, swine, and avian hosts [40]. Other than their high rate of antigenic drift from frequent point mutation, influenza’s eight segmented RNA nature can undergo genetic reassortment leading to dramatic genetic changes from co-infection of more than one influenza strain into a host [40]. The two features are the major forces that allows influenza viruses to escape existing immune systems and cause epidemic and pandemic outbreaks in humans acquiring development of cross protective influenza vaccines [33].

1.2 Immunosenescence

Immunosenescence in late ages reduces immune responses upon vaccination leading to lower vaccine efficacy and higher susceptibility to infection and to morbidity [1]. In aged individuals, decreased T-cell proliferation, augmented CD8$^+$ cytotoxic/suppressor cell numbers, and decreased CD4$^+$ T-cell numbers were determined to be main features of immunosenescence. Thus, vaccine formulas that are effective in multiple strains of influenza and overcoming immunosenescence features in elderly are in need for development.
1.3 Current HA-based vaccine and its limitations

Current influenza vaccine formulations are hemagglutinin-based and effective against homologous viral infection upon immunization. As a dominant protein that accounts 30% of total proteins of influenza virions, HA is highly immunogenic in hosts. However, limitations of HA’s variable nature due to antigenic drift and antigenic shift leading to insufficient protection against distinct subtypes of influenza viruses acquired vaccine developments protecting homologous and heterologous influenza strains [2].

1.4 Virus-like particles as vaccine platform and intramuscular immunization routes

Virus-like particles (VLP) have been proposed as a potential human vaccine platform possessing high immunogenicity. Due to its lack of genome material, VLPs are known for their safety with loss of replication ability in hosts. VLPs are currently in phase II human clinical trials and known to have advantages in shortened production time compared to conventional egg-based vaccines. The immunogenicity of conventional split inactivated virus vaccines and HA-based VLP vaccines were tested in young adult and aged mice models. In the ELISA result of this study, egg-derived, HA-based split A/H1N1/PR8/34 vaccine exhibited less systemic vaccine-specific IgG responses compared to A/PR8-derived HA-VLP after prime and boost immunization in both young adult and aged BALB/c mice.

Intramuscular immunization benefits from high blood supply in muscle tissues for systemic immune responses along with reduced local adverse effects [7]. In addition, to mimic the main route for human vaccination, intramuscular injection, the VLPs, in this study were immunized intramuscularly into BALB/c mice with PBS as delivery vehicle.
1.5  M2e-VLP and HA-VLP co-immunization as cross protective vaccine combination candidate

Antigenic proteins highly conserved throughout diverse influenza A viruses are currently targeted and extensively studied as potential candidate for universal vaccines. M2e, ectodomain of M2 ion channel protein of influenza viruses, is one of the antigenic particles expressed on the surface of influenza A viruses that is well-conserved in distinct heterosubtypic strains. M2e is known to provide protection against a wider-range of influenza strains upon vaccination of young adult mice. In previous studies, cross-protection efficacy of M2e-VLP with tandem repeats of influenza A-derived M2e expression against variant strains was found to be superior to HA-based influenza vaccines in young adult models [3]; however, HA-based vaccines were determined to offer more effective protection upon homologous challenge [4,5,6]. In young adult mice, the synergistic effects of HA-based split inactivated virus and M2e-VLP vaccines was determined. The co-immunization of M2e-VLP along with split inactivated vaccines has shown to have adjuvant effects and induced high levels of homologous and heterologous antibody responses [37]. In this study, I hypothesized that intramuscular co-immunization with HA-based VLP, derived from A/PR8/H1N1 virus, and M2e-VLP vaccines induces HA-strain-specific IgG antibodies and protection against both homologous (A/PR8/H1N1) and heterologous (A/WSN/33/H1N1) strains in young adult (6-8 weeks old) and 15-18-month-old BALB/c mouse models.

Protective immune correlates were analyzed after prime and boost vaccination via determination of antibody response levels from immune sera from young adult and aged BALB/c mice. Both of young adult and aged mice were grouped into immunization groups of Naïve, HA-VLP only (1 µg), split PR8 (0.1 µg), HA-VLP (1 µg) + M2e-VLP (10 µg), and split PR8 (1 µg)
+ M2e-VLP (10 µg). With M2e- and HA-VLP combination as main vaccine target antigens, split vaccine platform was also tested for comparative antigenicity analysis of VLP and split vaccines. The mice were immunized twice, prime and boost immunization with two weeks of intervals. Then, the mice were challenged with lethal dosage of heterologous H1N1 influenza 3 weeks after boost immunization. The protection efficacy was determined by body weight loss monitoring (12- or 7-day monitoring post challenge) and day 7 post challenge lung viral titration post homologous or heterologous challenge. Protective cytokine-secreting cell responses in splenocytes, mesenchymal lymph nodes’ memory antibody secreting-cell responses, and hemagglutination inhibition activity levels of immune sera were analyzed. Moreover, cross reactive antibody responses were analyzed using day 7 p.i. immune sera against antigenically distinct influenza strains.

Influenza HA VLP vaccine + M2e-VLP Vaccine

Immunogenecity
- Vaccine antigen specific Ab response
- Isotype-switching Ab

Cellular & cytokine response
- Antibody secreting cell response (in vitro IgG response)
- Cytokine secreting cell response (Interferon-γ)

Protection against Heterologous Challenge
- Body weight loss prevention
- Viral clearance

Figure 1 Diagram of hypothesis
2 EXPERIMENTAL METHODS

2.1 Animals and reagents

Female 6 to 8-week old and 15 to 18-month old BALB/c mice were purchased from The Jackson Laboratory and maintained in the animal facility at Georgia State University (GSU). All mouse experiments followed the approved Georgia State University IACUC protocol (A18001). A/PR8/H1N1 hemagglutinin-based virus-like particle and tandem repeat M2e-VLP were prepared as described in previous study. [7]

2.2 Immunization and virus infection

To investigate the co-immunization effects in cross-protection, in 6 to 8-week old and 15 to 18-month old BALB/c mice were immunized intramuscularly with 0.2 ug of HA-VLP alone, or co-immunized with 10 ug of M2e-VLP. Both prime and boost immunizations were performed with an interval of 2 weeks and immune sera were collected 12 days after each immunization. At 2 weeks after boost immunization, naïve and immunized mice in both age groups were challenged with either of A/PR8/H1N1/1934 or A/WSN/H1N1/1933 viruses. Post-infection, the mice were monitored to determine body weight loss either for 14 days or 7 days for sample collection to determine protection efficacy. Sera, lungs and spleens were harvested after sacrifice for further experiments.

2.3 Antibody Enzyme-linked immunosorbent assay (ELISA)

To determine serum antibody response, the antigens (inactivated H1N1 A/PR8/34, inactivated A/WSN/33, and M2e peptide antigen (GenScript, Piscataway, NJ, USA) were coated onto the Costar ELISA plates overnight at 4°C. The plates were washed with Phosphate Buffered
Saline Tween 20 (PBST) for 3 times and blocked with 1% Bovine Serum Albumin dissolved PBS (1% BSA) at room temperature (RT) for 2 hrs. The plates were washed for 3 times before serially diluted sera samples were applied to the plates and incubated at RT for 2 hrs. After 3-time-washing, secondary antibodies conjugated with horseradish peroxidase (HRP) were applied and incubated at RT for 1 hr. After 3-time-washing, tetramethylbenzidine (TMB) was applied to the wells as a substrate for HRP to detect HRP enzyme conjugate activity and the process was stopped with phosphoric acid. The optical density was measured at 450 nm.

2.4 Lung virus titration

Lung extracts were prepared with a tissue grinder and homogenized in 1.5 ml of PBS per each lung. Lung homogenates were serially diluted in 10-fold and injected into embryonated chicken eggs incubated for 10 days at 36 ºC. The 50% egg infectious dosage (EID$_{50}$) was determined following the Reed and Muench’s method as described [8,9].

2.5 Hemagglutinin inhibition assay (HAI)

To perform HAI assay, immune sera were incubated with receptor destroying enzymes (purchased from Sigma Aldrich) at 37 ºC for 18 hours and 30 minutes at 56 ºC to inactivate complement. After inactivation, immune sera were serially diluted and incubated with 8 HAU of heterologous (A/WSN/33) influenza virus strain in V-bottom microplates for 30 minutes. 0.5% chicken red blood cell was applied to test hemagglutination after 40 minutes. The detection limit of HAI titer was 2 of Log2.
2.6 Cytokine ELISPOT

To detect interferon (IFN)-γ secreting cells from spleens, splenocytes ($5 \times 10^5$ cells/well) were cultured on 96 well plates previously coated with capturing anti-mouse IFN-γ monoclonal antibodies (BD Biosciences, San Diego, CA) in the presence of inactivated A/PR8 or inactivated A/WSN (4 µg/ml) for 3 days. The cytokine spots were developed with streptavidin-labeled alkaline phosphatase (BD Pharmingen, San Diego, CA) and biotinylated mouse IFN-γ antibodies. 3,3’-diaminobenzidine substrate was used to visualize the spots and the spots were counted by an ELISPot reader (BioSys, Miami, FL).

2.7 Data Analysis

Statistical analysis for all data will be done with GraphPad Prism software version 5.01 (GraphPad Software Inc, La Jolla, CA, USA). Data of different groups will be analyzed using one-way ANOVA and Tukey’s multiple comparison tests. $p$-Values < 0.05 will be considered significant.
3 RESULTS

3.1 HA-VLP and M2e-VLP co-immunization enhances cross protectivity in both young adult and aged BALB/c mouse groups.

3.1.1 HA-VLP and M2e-VLP co-immunization induces vaccine-specific antibody levels in young adult and aged mouse under age effects.

Antigen-specific IgG isotype antibody responses upon vaccination were determined in young adult (6-week-old) and aged (15-18-month-old) BALB/c mice (Fig. 2). Mice were grouped into singular or combination vaccination of Naïve, HA-VLP only (1 µg), sPR8 only (0.5 µg), HA-VLP + M2e-VLP (1 µg + 10 µg, respectively), and sPR8 + M2e-VLP (0.5 µg + 10 µg) groups. Each group’s vaccine antigen-specific IgG isotypes were analyzed at 2 weeks post boost immunization. The HA-VLP + M2e-VLP combination groups in both ages showed significantly high level of IgG, IgG1, and IgG2a antibodies specific to both inactivated A/H1N1/Puerto Rico/1934 virus (iPR8) and M2e antigen (Fig. 2). In young adult HA-VLP only group were analyzed to have significant IgG responses but were determined to have insignificant increase in IgG1 and IgG2a antibody responses (Fig A-C). Among HA-VLP only groups, aged mice exhibited slight increase in iPR8-specific IgG but did not induce IgG1 and IgG2a isotype responses in aged mice (Fig. 2D-F).

The identical immunization groups of the two different age groups were analyzed to have similar pattern of vaccine antigen-specific antibody responses. To study age effects to the immune responses to vaccine formulas, differences in antibody level were analyzed between the two age groups using sera ELISA on plates coated with either of iPR8 or M2e antigens (Fig. 2). Aged BALB/c mice immunized with HA-VLP and M2e-VLP vaccines exhibited lower IgG, IgG1, and IgG2a specific to iPR8 compared to young adult mice (Fig. 2A-F). HA-VLP + M2e-
VLP immunization induced significant M2e-specific immune response in both young adult and aged BALB/c, but aged mice showed less IgG, IgG1, and IgG2a levels compared to young adults (Fig. 2G-L). In summation, although young adults had tendency of expressing higher iPR8- and M2e-specific antibody levels compared to aged mice, HA- and M2e-VLP co-immunized aged BALB/c developed significant vaccine antigen-specific antibody responses after boost immunization.
Naïve young adult (6 weeks old) BALB/c mice or aged (18 months old) BALB/c mice (n=5 per group) were prime and boost immunized with HA-VLP (1 µg), sPR8 (0.1 µg), M2e-VLP (10 µg) + HA-VLP (1 µg), or sPR8(0.1 µg) + M2e-VLP (10 µg). At 2 weeks after prime and boost immunization, serum IgG antibody levels were determined by ELISA using the homologous virus (inactivated A/PR8 virus) or M2e antigens. Virus specific serum IgG, IgG1 isotype, IgG2a isotype antibodies in (A-C) young adult or (D-F) aged BALB/c mice. (G-L) M2e-specific serum IgG1 isotype, IgG2a isotype antibodies in (G-I) young adult or (J-L) aged mice. Naïve: No vaccine and no virus, Naïve inf: Unvaccinated mice after infection with virus, HA-VLP: VLPs containing HA only, HA-VLP + M2e-VLP: HA – VLP co-immunized with M2e-VLP.
3.1.2 HA- M2e-VLP co-immunization group confers more effective protection against antigenically heterologous influenza virus strain in both age-groups.

I determined cross-protection efficacy of the vaccine formula by challenging boost immunized mice with either of homologous A/H1N1/PR/8/34 or heterologous A/H1N1/WSN/33 virus (Fig. 3). In preliminary data, as expected, the HA-VLP only mice showed perfect protection against lethal dose of homologous virus while naïve mice lost significant body weight over 25%. Although the challenge virus has the similar hemagglutinin type 1 as the HA-VLP derived from A/H1N1/PR/8/34, the HA-VLP only immunization did not induce protection in both 6-week-old and aged mice determined by severe weight loss post infection (Fig. 3A-B). In contrast, with co-immunization of both HA-VLP and M2e-VLP, young aged BALB/c lost less than 5% and aged mice lost less than 11% of body weight post infection as testimony for wider range of protection than HA-VLP only vaccine. Overall, the HA-VLP and M2e-VLP immunized group of BALB/c mice showed less weight loss indicating better cross protection than the naïve control or HA-VLP only groups.

To further determine protection efficacy, I performed lung viral titration at day 7 post viral challenge (Fig. 3C). The naïve infection group exhibited the highest lung viral titer at $10^8$ EID$_{50}$ (200ul of 1.5 ml lung homogenate in PBS), followed by HA-only group with $10^6$ viral titer level. The HA-VLP + M2e-VLP group showed approximately $10^3$ to $10^4$ lung viral titer load.

Hemagglutination inhibition assay was performed to determine systemic homologous and heterologous virus specific antibody responses in BALB/c mice. The sera were collected at day 7 post infection with A/H1N1/WSN/1933 strain. When the sera from HA-VLP and M2e-VLP groups were incubated with homologous virus, A/PR8, hemagglutination inhibition activity was observed dilution factor of $2^6$ to $2^7$, while HA-VLP only group showed 2- to 4-fold less HAI
activity (Fig. 3D). Sera from HA-VLP + M2e-VLP group showed hemagglutination inhibition activity up to $2^4$ to $2^5$ with 8 hemagglutination unit (8 HAU) of A/WSN (Fig 3E). Other controls like naïve, naïve infected, and HA-VLP only groups, showed $2^1$ to $2^2$ when applied with 8 HAU of the WSN strain. In summary, lung viral titration and HI assays show a correlation with protective efficacy data shown in body weight loss monitor.

Figure 3 Combined M2e- and HA-VLP showed cross-protection preventing body weight loss, clearing lung viral titer, and inducing hemagglutination inhibiting antibody responses. (A-B) Naïve, HA-VLP, M2e VLP + HA VLP immunized young and aged BALB/c mice were challenged I.N. with lethal doses of A/H1N1/WSN/33 and monitored for 14 days and 7 days, respectively. (C) Lung virus titer was analyzed from lung homogenate samples harvested at day 7 p.i.. (D-E) Sera anti-hemagglutination inhibition reactivity against 4 HAU of homologous (A/PR8) and heterologous (A/WSN) viruses was analyzed by HAI assay in aged mice day 7 p.i. with A/WSN. Statistical significance was determined using one-way ANOVA.
3.1.3 HA-VLP and M2e-VLP co-immunization promotes effective antigen-specific cellular immune responses in aged BALB/c.

To further study cellular immune responses after challenge, memory antibody secreting-cell responses have been tested using Day 1 and 5 \textit{in vitro} IgG ELISA. Mesenchymal lymph node (MLN) cells were harvested from at day 7 post challenge and cultured in iPR8-coated plates for either 1 or 5 days (Fig. 4). On day 1 of \textit{in vitro} cultures, the MLN cells from HA-VLP + M2e-VLP group exhibited significantly higher level of iPR8-specific IgG compared to naïve and HA-VLP only groups (Fig. 4A). Moreover, the MLN cells from the combination vaccination group induced higher A/PR8-specific IgG levels compared to Naïve and HA-VLP only groups (Fig. 4B).

After immunization, cytokine secreting-cell responses participates in development of cross protection efficacy mediated by M2e- and HA-VLPs (Fig. 4C). Splenocytes were collected from mice at day 7 post A/WSN challenge and cultured on ELISpot plate coated with IFN-γ-capturing antibody \textit{in vitro} for 3 days. During the 3-day \textit{in vitro} culture, The cells were stimulated with either of iPR8 or iWSN to develop cytokine-secreting cell spots, which can be utilized as indicator of cytokine secreting T cell response for comparative analysis. With iPR8 stimulation, HA-VLP and M2e-VLP co-immunized aged BALB/c showed nearly 4-fold higher levels of IFN-γ secreting splenocytes compared to HA-VLP group and 10-fold higher level compared to naïve infection group. HA-VLP + M2e-VLP group exhibited nearly 4-fold higher levels of IFN-γ compared to HA-VLP only group and 6-fold higher group compared to naïve groups.

In summation, the results suggest that HA-VLP and M2e-VLP co-immunization effectively develops vaccine and heterologous challenge antigen specific antibody- and
protective cytokine-secreting cell responses compared to HA-VLP only immunization in aged mice.

**Figure 4 M2e- and HA-VLP vaccination provokes antibody secreting memory cell responses and protective cytokine secretion responses.**

(A-B) In vitro IgG antibody producing cell responses in draining lymph nodes (MLN) collected day 7 post challenge with A/WSN virus. A/PR8 specific IgG antibody levels were determined by ELISA in culture supernatants after 1 day (A) or 5 days (B) cultures. (C) The spleen cells harvested from vaccinated mice at 7 days after challenge were cultured for 3 days with inactivated A/PR8 (iPR8) or inactivated A/WSN (iWSN) virus as a stimulator. Levels of IFN-gamma cytokine secreting cell spots were measured by counts of spots using DAB solution.

### 3.1.4 HA-VLP- and M2e-VLP-immunized sera possess higher level of antibodies that are specific to wider-range of antigenically distinct virus strains

To further analyze cross protectivity across serologically distinct influenza strains, I performed sera ELISA using 6 different strains of influenza to analyze how each serum has different level of cross-reactive IgG, IgG1, and IgG2a isotypes (Table 1). The sera were collected at day 7 post challenge and applied to ELISA plates coated with either of A/H1N1/PR8, A/H1N1/WSN, A/H1N1/Cal, A/H3N2/Phil, A/H5N1/Viet, or A/H7N9/Shanghai. The sera from HA-VLP and M2e-VLP co-immunization group induced higher total IgG and isotype switching IgG responses compared to naïve, naïve infection (A/WSN infected), and HA-VLP only groups when both homologous and heterologous H1N1 influenza strains were coated. With homologous or heterologous H1N1 antigens were coated, HA-VLP only groups exhibited
slightly higher antibody responses compared to naïve and naïve infection groups. In addition, M2e-VLP and HA-VLP groups exhibited significantly higher antibody levels compared to the other groups when distinct influenza strains with H3N2, H5N1, and H7N9 were used as coating antigens. With the different subtypes of influenza strains, HA-VLP only group showed similar level of antibody levels as naïve infection group.

Hemagglutination inhibition assay (HAI assay) was performed to determine the M2e- and Ha-VLP groups can induce HA inhibiting serum antibodies in aged mice. While sera from HA-only group showed similar level of HA inhibiting activity, the combined vaccine group’s sera exhibited high level of HA inhibition activity. In summation, combined M2e- and HA-VLP vaccines provoked higher antibody responses specific to wider range of influenza strains compared to single HA-VLP group.

Table 1 M2e- and HA-VLP combination induced serum antibody responses specific to wide range of influenza A strains.

Sera were collected from immunized aged mice at day 7 p.i. with heterologous A/WSN. Serum IgG1, IgG1 isotype, IgG2a isotype antibody responses specific to homologous, heterologous, and heterosubtypic influenza strains were determined by ELISA with optical density at 450 nm. The immune sera were serially diluted and the ELISA result at 1,000x sera dilution are shown. A/PR8: A/PR8/1934 H1N1, A/WSN: A/WSN/1933 H1N1, A/Cal: A/Cal/2009 H1N1, A/Phil: A/Phil/1982 H3N2, A/Viet/2004 H5N1, and A/Shanghai/2013 H7N9.
**Figure 5** M2e- and HA-VLP vaccination provokes HA inhibiting antibody responses specific to diverse heterosubtypic strains of influenza A strains.

Sera anti-hemagglutination inhibition reactivities against 4 HAU of heterologous (A/Cali) and heterosubtypic (A/Phil, A/Viet, A/Shanghai) viruses were analyzed by HAI assay using chicken red blood cells in aged mice sera day 7 p.i. with A/WSN. A/Cali: A/Cal/2009 H1N1, A/Phil: A/Phil/1982 H3N2, A/Viet/2004 H5N1, and A/Shanghai/2013
4 CONCLUSIONS

4.1 Cross protective influenza vaccines targeting age groups with immunosenescence

Current influenza vaccines take advantages in providing protection against influenza virus strains with HA antigenic strain matching with the vaccines [31]. The main reason is that hemagglutinins (HA) are under frequent changes due to rapid genetic mutation rates leading to virus’s escape from current immune systems [25,30]. Annual updates are required for vaccines due to its limitation in narrow range of targeted influenza strains [30,31]. Current vaccines are not suitable to confer protection against potential pandemic influenza strains with unique HA antigenicity [30,31]. Therefore, selected highly conserved viral antigens are considered to be candidates for universal or cross protective influenza vaccines. Moreover, immunosenescence necessitate for aged individuals a vaccination strategy to overcome their naturally degenerative immunity and provoke immune responses protective to diverse influenza strains including the ones with higher chances of epidemics or pandemics. In this study, an influenza antigen, M2e, as candidates for possible cross protection, and A/PR8 H1N1-derived HA-VLP, as candidate for homologous protection against H1N1 strains, were investigated as effective homologous and heterologous vaccine developments targeting elderly.

4.2 Co-immunization-induced immunity for cross protection in aged mice

Among highly conserved influenza antigens, the ectodomain of ion channel protein of influenza virus has been extensively studied as potential target for universal vaccines [19,33]. In previous studies, it has been found that M2e vaccine-induced M2e-specific antibodies protect hosts via decreasing body weight loss and lung viral titration against heterosubtypic influenza A virus challenges [19,37]. However, M2e-specific antibodies only were insufficient for perfect
protection against heterosubtypic infections leading to signs of moderate pathology [30]. Potential of M2e and HA combination as a cross protection vaccine candidate for elderly is rooted on the previous findings that M2e vaccination can lead to better cross protection than HA-based vaccination, while HA-specific immune responses can more effectively induce homologous protection compared M2e-vaccination [19,37]. It has been studied that, in young adult mice, combination of split inactivated vaccines (HA-based) and M2e-VLP vaccines showed enhanced heterologous protection retaining HA-based vaccine’s homologous protection compared to single immunized vaccine groups [19,37].

In this study, I investigated how M2e-VLP and HA-VLP co-immunization contributes to cross protection in aged mice in comparison with HA-VLP and split inactivated virus (as control) vaccines. HA-VLP vaccines were determined to induce antigen-specific antibodies than split inactivated vaccines. M2e-VLP and HA-VLP co-immunization induced higher levels of antibodies specific to HA or M2e compared to single HA-VLP vaccinated group in aged mice. When compared with young adult age groups, aged group showed limited antibody responses after vaccination. However, when combined M2e- and HA-VLP were immunized, it rescued and exhibited significantly higher antibody levels compared Naïve, HA-VLP only, sPR8 only, sPR8 and M2e-VLP only groups. In addition, combined vaccination led aged hosts to enhanced protection efficacy against heterologous virus strains. Thus, M2e- and HA-VLP combined vaccination was effective in provoking immune responses and protection against homologous and heterosubtypic influenza A strains in aged mice.
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