Original Article

Virus-like particle vaccine by intranasal vaccination elicits protective immunity against respiratory syncytial viral infection in mice

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Abstract

Respiratory syncytial virus (RSV) is a leading cause of lower respiratory infection in infants and children, but there is still no licensed vaccine available. In this report, we developed virus-like particle (VLP) vaccines based on the Bac-to-Bac baculovirus expression system, consisting of an influenza virus matrix (M1) protein and the RSV fusion protein (F) or glycoprotein (G). These RSV VLPs were identified by western blot analysis and electron microscopy. Female BALB/c mice immunized intranasally (i.n.) with RSV-F VLPs, RSV-G VLPs, or both showed viral-specific antibody responses against RSV. Total IgG, IgG1, IgG2a, and mucosal IgA were detected in mice with RSV-F plus RSV-G VLPs, revealing potent cellular and mucosal immune responses. Moreover, we found that these mixed RSV VLPs conferred enhanced protection against live RSV challenges, showing significant decreases in lung viral replication and obvious attenuation of histopathological changes associated with viral infections. These results demonstrate that RSV-F plus RSV-G VLPs by intranasal vaccination is a promising vaccine candidate that warrants further evaluation using cotton rat and primate models.

Key words: respiratory syncytial virus, virus-like particles, intranasal vaccination, fusion protein, glycoprotein

Introduction

Respiratory syncytial virus (RSV) is an important pathogen of lower respiratory infection in infants and children, and it is responsible for nearly 160,000 deaths annually worldwide [1]. Following primary RSV infection, which generally occurs before 2 years of age, immunity to RSV remains incomplete, and frequent re-infections occur throughout life, with the most severe infections occurring at extreme ages and among immune-compromised individuals [2]. Despite decades of effort towards developing an RSV vaccine, there is still no licensed vaccine available. To this end, new strategies for RSV vaccines are urgently required.

RSV is a negative-strand non-segmented RNA virus that consists of two subtypes, A and B, and 10 genes encoding 11 proteins. The viral envelop bears the major antigens: the fusion protein F and the glycoprotein G. The F protein is highly conserved among RSV isolates. Antibodies induced by the F protein showed efficient
protection against both RSV subtypes A and B [3]. The G protein is heavily glycosylated and variable in sequence between the two subtypes [4]. These two proteins are the most important targets of RSV candidate vaccines for the induction of neutralizing and protective antibodies [5,6].

Virus-like particles (VLPs) are a novel approach for viral vaccines containing viral antigen proteins that form a virus-like structure but lack the viral genome [7,8]. They do not have replication ability. Previous studies showed Newcastle Disease Virus (NDV) RSV VLPs could induce effective systemic and mucosal immune response [9,10]. In addition, recombinant baculoviruses (rBVs) expressing RSV VLPs containing either F or G could evoke the neutralizing antibody and Th1/Th2-balanced immune response, but not mucosal immunity, thereby conferring protection against RSV challenge in the mouse model [11]. Furthermore, F or G nanoparticle, a combination of VLP or plasmid DNA may be a potential anti-RSV prophylactic vaccine which can induce balanced innate and adaptive immune responses and long-term protections [12,13].

In this study, we described a VLP vaccine candidate that consists of the RSV F or G protein and influenza M1 protein based on the Bac-to-Bac baculovirus expression system. Subsequently, BALB/c mice were immunized intranasally (i.n.) with RSV-F VLPs, RSV-G VLPs, or both. The antibody titer, cytokine response, and protective efficacy against live RSV were evaluated in vivo.

Materials and Methods

Ethics statement

Female BALB/c mice of 4–6 weeks age (Animal Experimental Center, Beijing, China) were used in this study. Groups of mice under sodium pentobarbital anesthesia (60–80 mg/kg) were immunized and challenged. All animal experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of Beijing Institute of Microbiology and Epidemiology. Meanwhile, this study was specifically approved by the IACUC and ethics committee of Beijing Institute of Microbiology and Epidemiology under the number ID: SYXK2012-005.

Virus and cells

The RSV subgroup (a wild-type strain) was obtained from the American Type Culture Collection (ATCC, Manassas, USA). Virus was grown in human laryngeal epithelial (HEp-2) cells (ATCC) cultured in Minimum Eagle’s Medium and 40% F-12 medium (GIBCO, Scotland, USA), with 10% fetal bovine serum (FBS). Spodoptera frugiperda Sf9 cells (ATCC) used for protein expression were maintained in suspension in serum-free SF900II medium (GIBCO, Scotland, USA), with 10% fetal bovine serum. Sf9 cell cultures were infected with rBV P3 stock-infected Sf9 cells in flasks at 27°C. Sf9 cell cultures were infected with rBV P4 stock during the mid-logarithmic phase of growth at cell densities of ~2 × 10^6 cells/ml with an MOI of 2. After 3 days, cell culture supernatants were collected by centrifugation at 6000 g for 25 min at 2–8°C. These VLPs in the supernatants were concentrated at 80,000 g. The concentrate was re-suspended in PBS. The VLPs were laid on top of a discontinuous sucrose gradient composed of 2 ml layers of 20%, 40%, 50%, and 60% sucrose. The VLP band found in the 40%–50% sucrose gradient layers were collected, diluted with PBS, concentrated at 28,000 g for 2 h. Finally, these VLPs were re-suspended in PBS and stored at −80°C until further use.

Construction of recombinant plasmid pFastBac Dual-M1-F and pFastBac Dual-M1-G

The RSV A2 strain F and G genes and the influenza virus A/Ann Arbor/6/60 (H2N2) M1 gene were optimized using the Vector NTI Advance (http://www.thermofisher.com/vectorniti-software/vector-nti-advance-software) and the optimized sequence was synthesized by Shanghai Sangon Company (Shanghai, China). The RSV F or G full-length sequence was cloned into pFastBac Dual vector (Invitrogen, Carlsbad, USA) pH promoter with BssHII/Xhol sites, resulting in recombinant plasmid pFastBac Dual-F or pFastBac Dual-G. Meanwhile, the influenza M1 gene was also cloned into pFastBac Dual vector p10 promoter with SphlinNheI sites, resulting in F or G sequence being cloned into pFastBac Dual-M1 to construct pFastBac Dual-M1-F or pFastBac Dual-M1-G, respectively.

Generation of recombinant baculoviruses

Recombinant plasmid pFastBac Dual-M1-F and pFastBac Dual-M1-G were transformed into DH10Bac Escherichia coli. Then blue/white selection was used to identify colonies containing the recombinant bacmid-M1-F and bacmid-M1-G, respectively. The recombinant bacmid DNA was solely transfected into Sf9 cells using Cellfentin II Reagent (Invitrogen) to produce recombinant baculoviruses. Once the cells appeared infected, the viruses were harvested from the cell culture medium. The P1 viral stocks were used to re-infuse cells to generate a high-titer P2 and P3 stock.

Expression of recombinant VLPs

Our RSV VLPs were generated from rBV P3 stock-infected Sf9 cells, in which the RSV F or G gene and influenza M1 gene were contained. Serum-free SF900II medium was used for suspending Sf9 cells in flasks at 27°C. Sf9 cell cultures were infected with rBV P4 stock during the mid-logarithmic phase of growth at cell densities of ~2 × 10^6 cells/ml with an MOI of 2. After 3 days, cell culture supernatants were collected by centrifugation at 6000 g for 25 min at 2–8°C. These VLPs in the supernatants were concentrated at 28,000 g. The concentrate was re-suspended in PBS. The VLPs were laid on top of a discontinuous sucrose gradient composed of 2 ml layers of 20%, 40%, 50%, and 60% sucrose. The VLP band found in the 40%–50% sucrose gradient layers were collected, diluted with PBS, concentrated at 28,000 g for 2 h. Finally, these VLPs were re-suspended in PBS and stored at −80°C until further use.

Characterization of VLPs

RSV-F or RSV-G VLPs were identified by western blot analysis and electron microscopy analysis. Purified RSV VLPs were used in these experiments. The VLPs and an equal volume of 2x Loading buffer were mixed and boiled at 100°C for 5 min. And then the samples were run in 12% reducing SDS-PAGE and transferred to the PVDF membrane. Polyclonal rabbit anti-Flu M1 antibody (Abcam, Cambridge, UK) was used to detect the levels of Flu M1 protein of a major 25 kDa polypeptide, and monoclonal anti-RSV F and G antibodies (Sino Biological Inc., Beijing, China) was used to detect the expressions of RSV-F and RSV-G proteins.

In another experiment, these VLPs were subject to negative staining following by transmission electron microscopy for observing the viral morphology and size determinations. Purified RSV VLPs were adsorbed by floatation for 5 min on freshly discharged 400-mesh carbon-coated copper grid, negatively stained with 1% phosphotungstic acid, and visualized using a transmission electron microscope operating at 120 kV at 100,000× magnification.

Immunization and challenge in BALB/c mice

Groups of BALB/c mice under sodium pentobarbital anesthesia (60–80 mg/kg) were immunized i.n. twice with 20 µg of total RSV F or G VLPs at 2 weeks interval. For the RSV F plus RSV G VLP groups, mice were given 10 µg of F VLPs and 10 µg of G VLPs in the same regimen. Blood samples were collected before immunization and on Days 14 and 28 after prime. After 14 days of boost, nasal lavages and bronchoalveolar lavage fluid (BALF) were collected to detect mucosal secretory IgA (sIgA) titers. For viral challenge,
immunized mice under sodium pentobarbital anesthesia were infected i.n. with live RSV A2 virus (1.5 × 10⁶ pfu) in 30 µl of PBS 2 weeks after the second immunization. The health and survival rates of mice were monitored daily until 14 days post-challenge, and changes in body weight were also recorded. At the end of the experiments, all animals were humanely euthanized by cervical dislocation and then carefully observed and palpated to make sure that there was no respiration and heart beat.

**RSV-specific antibody response**

The levels of RSV A2 virus-specific antibodies (IgG, IgG1, and IgG2a) in serum and slgA in mucosal lavages (nasal, lung BALF) were determined using an enzyme-linked immunosorbent assay (ELISA). Detailed protocols were performed as previously described [10]. In this experiment, 96-well plates were coated with 5 µg/ml inactivated RSV A2 virus antigens and each sample was measured in duplicate.

**Cellular immunity to VLPs**

Two weeks after boost, spleens were collected from mice and prepared as homogenates. Spleen cells were collected using lymphocyte separation medium (DAKEWEI, Beijing, China) and were added into wells (1–2 × 10⁵ cells/well) of the ELISPOT plates (Millipore, Billerica, USA) coated with anti-mouse IFN-γ or anti-mouse IL-4 antibodies, following by stimulation with inactivated RSV viral antigens (5 µg/ml) for 24–48 h. After stimulation (IFN-γ: 24 h; IL-4: 48 h), plates were washed and incubated with biotinylated anti-mouse antibody (Cellular Technology, Kennesaw, USA) followed by incubation with streptavidin-HRP. Plates were scanned and counted using an automated ELISPOT reader system (Cellular Technology). Results were calculated and expressed as spot forming cells per 10⁵ spleen cells.

**Protection of mice from RSV infection after VLP vaccination**

Two weeks after the second immunization, all groups of mice were sodium pentobarbital anesthetized and challenged i.n. with 1.5 × 10⁶ pfu live RSV A2 virus for evaluating protective immunity. Mice were monitored for weight changes throughout a 14-day observation period. At Day 4 post-challenge, individual lungs were collected for pathological examination and virus load. For virus load experiments, the individual lungs were prepared as homogenates. Briefly, lungs were homogenized (w/v = 1:15) on ice and tissue debris was pelleted by centrifugation at 5000 g for 10 min at 4°C and supernatants were immediately diluted serially with FBS-free medium. HEp-2 cells at 80% confluency in a 24-well plate were incubated with serial 10-fold dilutions for 1 h at 37°C. Then the medium was replaced by 2% FBS growth medium containing 3% agar. At Day 6 post-infection,

![Figure 1. Construct generation](https://academic.oup.com/abbs/article-abstract/49/1/74/2681822)
plaque formation was counted after staining with neutral red solution. RSV titers of the nasal turbinates and lungs were determined using the 50% tissue reduction assay with HEp-2 cells.

Statistical analysis
The data were analyzed by ANOVA using GraphPad Prism5 software and Tukey’s Sidak’s multiple comparisons test. All experiments were performed in triplicate. P < 0.05 indicated statistically significant difference.

Results
Generation of constructs
RSV F or G full-length sequence was inserted under the pH promoter, and the influenza M1 sequence was inserted under the p10 promoter in the pFastBac Dual vector to construct pFastBac Dual-M1-F/G (Fig. 1A,B). The sizes and positions of the RSV F and G genes and influenza M1 gene in the pFastBac Dual vector were confirmed by enzyme digestion, and the nucleotide sequences were confirmed by DNA sequencing (data not shown). Recombinant bacmids contained the F+ M1 or G+M1 gene according to PCR, respectively (Fig. 1C,D). These results showed that these plasmids contained the genes of interest.

Characteristics of VLPs
RSV F or G VLPs were produced as described in the Materials and Methods. The expression of RSV VLP proteins was identified by western blot analysis using anti-RSV or anti-Flu M1 polyclonal antibodies. As seen in Fig. 2A,B, all three proteins showed stable expression in VLPs in a dose-dependent manner. Expression increased with an increase in RSV VLP concentration from 5 to 10 µg. Meanwhile, wild-type RSV A2 and A/PR/8/34 viruses were used as controls. Notably, the 70-kDa RSV F and 75-kDa RSV G proteins were detectable in the RSV A2 lane, while the 25-kDa M1 protein was detectable in the influenza PR8 lane. Based on these data, we deduced that G protein is the glycosylation status and F protein is

Figure 2. Identification of recombinant RSV VLPs
(A,B) RSV VLPs produced by Sf9 cells were purified from culture supernatants using sucrose gradient ultracentrifugation and confirmed by SDS-PAGE (A) and western blot analysis (B). (C,D) RSV VLPs were observed by electron microscopy. Viral particle size is shown. Over 70% of the VLP viral particles were 60–80 nm in diameter.
the size of un-cleaved F (not processed F). In addition, these VLPs were examined by electron microscopy. RSV-F VLPs showed an obvious peak in the viral size distribution at 60–80 nm, and RSV-G VLPs were similar but were more heterogeneous in size (Fig. 2C,D). Morphology and sizes of the RSV VLPs were consistent with that of the wild-type RSV.

**Serum antibody immunity to VLPs**

Groups of female BALB/c mice were inoculated i.n. with RSV-F and/or RSV-G VLPs to evaluate immunogenicity. Each group of mice was immunized i.n. with RSV-F and RSV-G VLPs on Days 0 and 14, respectively (Fig. 3A). The levels of RSV-specific total IgG, IgG1, and IgG2a antibody in serum of immunized mice were determined using ELISA. As shown in Fig. 3B–D, RSV-F plus RSV-G VLPs induced significantly stronger antibody responses, and the titers of serum IgG were ~103.6 on Day 14 after the boost, indicating the maturation of virus-specific antibodies. Meanwhile, both antigens induced higher IgG1 and IgG2a titers than in the PBS control. Notably, RSV-F and/or RSV-G VLPs induced much higher IgG2a than IgG1 titers. The IgG1 and IgG2a titers of RSV-F plus RSV-G VLP-vaccinated mice were 101.20 ± 0.02 and 102.80 ± 0.01, respectively. These results indicate that vaccination with RSV-F plus RSV-G VLPs elicits IgG2a-dominant responses.

**Mucosal responses after immunization with VLPs**

To evaluate whether RSV-F and/or RSV-G VLPs can induce potent sIgA antibody responses in the mucosa, RSV-specific sIgA antibodies were measured in nasal and lung lavage fluids by ELISA. As shown

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**Figure 3. Experimental schedule and RSV A2-specific antibody responses to RSV VLPs**

Groups of mice were primed with 20 µg RSV-F and/or RSV-G VLP vaccines or PBS i.n. at the start of the vaccination protocol. Two weeks later, an i.n. boost immunization with the same antigen was administered. Two weeks post-boost, mice were killed to analyze the immune response. The remaining mice were challenged with 1.5 × 10^6 pfu RSV. Four days after challenge, mice were killed for viral titer and lung pathological assays. In the remaining mice, body weight, and survival were monitored daily for at least 14 days (A). Serum samples were collected from each group (n = 10) 2 weeks after the prime and boost, and were serially diluted in triplicate for ELISA. Total IgG (B), IgG2a (C), and IgG1 (D) were determined. Results are expressed as means ± SD. ***P < 0.001.
in Fig. 4, at Day 14 after the boost, higher sIgA titers were detected in nasal and lung lavage fluids of RSV-F and/or RSV-G VLP-vaccinated mice, whereas no antibodies were detected in the PBS group. The sIgA titer in nasal and lung lavage fluids of the RSV-F plus RSV-G VLP-vaccinated group was ~100 and 200 times higher than the control groups, suggesting that intranasal immunization of mice with the RSV-F plus RSV-G VLP vaccine induces a strong mucosal antibody response.

Cellular immune responses after immunization with VLPs
To further assess the ability of RSV VLP proteins to elicit a cellular immune response, the number of IFN-γ- and IL-4-producing cells was determined by ELISPOT. At 2 weeks after boost, mouse spleens were collected and prepared as homogenates. Spleen cells were subject to RSV antigen stimulation for 24–48 h. As shown in Fig. 5, the RSV-F and/or RSV-G VLP groups had significantly higher levels of IL-4-producing T cells than the PBS control group. Similarly, it was found that RSV-F and/or RSV-G VLPs induced significantly more IFN-γ-producing T cells than the PBS control. Of note, the number of IFN-γ-secreting cells was relatively higher than the number of IL-4-secreting cells in the RSV-F plus RSV-G VLP-vaccinated group, indicating that potent cellular immune responses were induced by RSV-F plus RSV-G VLPs.

Protective efficacy against live RSV A2 viral challenge infection
To evaluate the protective efficacy of the RSV-F and/or RSV-G VLP vaccine against live RSV, mice were challenged with 1.5 × 10⁴ pfu live RSV A2 virus 2 weeks after the last immunization. As shown in Fig. 6A, the body weights of RSV-F VLP- and PBS-vaccinated mice decreased rapidly on Day 6 and recovered quickly by Day 7 post-challenge. And the F+G VLP group was found to keep on increasing body weight after 7 days post-infection. The RSV-F plus RSV-G VLP group showed an obvious increase in body weight on Day 7 post-infection. We demonstrated that RSV-F/G VLPs could slow down weight loss.

Meanwhile, lung viral loads in the mice were determined 4 days post-challenge. The recovery of infectious virus from lung and nasal tissues was shown in Fig. 6B,C. A total of 1.5 × 10⁴ pfu of infectious viruses were recovered from lung tissues of mice in the control group challenged with the wild-type RSV, but 0.2 × 10⁴ pfu of RSV was recovered in mice immunized with RSV-F plus G VLPs. Meanwhile, 0.4 × 10⁴ pfu of infectious viruses were recovered in mice immunized with RSV-F or RSV-G VLP alone. Similarly, 1.0 × 10⁴ pfu of RSV were detected in the nasal tissues of non-immunized mice infected with RSV. In the vaccinated groups, viral titers in nasal tissues were significantly reduced. These data revealed that vaccination with RSV-F plus RSV-G VLPs effectively inhibited viral replication in the lung and nasal tissues of mice.

For histological examination, lung tissues were collected 4 days after RSV A2 virus challenge, and the results of HE staining were shown in Fig. 7. The non-immunized mice challenged with RSV A2 virus showed thickening of the alveolar wall and infiltration of inflammatory cells. Mice immunized with RSV-F plus G VLPs showed very mild inflammation compared with immunization with RSV-F or RSV-G VLPs alone. Histological alterations in the lung correlated well with the viral loads from lung tissues, showing that the inoculated RSV was cleared in mice immunized with RSV VLPs.

Discussion
Decades of attempts have been made to develop effective RSV vaccines, but there is still no licensed vaccine at present. Previous studies indicate that RSV F and G proteins are the best targets for generating neutralizing and protective antibodies [14–16]. Recently, Sang-Moo Kang group reported that F VLP can induce protection without causing pulmonary
RSV diseases by inducing RSV neutralizing antibodies, as well as by modulating specific subsets of DC cells and CD8 T-cell immunity [6]. Lee et al. [17] demonstrated that the mixed RSV VLP F and VLP G by the intramuscular route provided a high level of protection against RSV without vaccine-induced immune-pathology, and RSV G VLP vaccine caused a certain level of histopathology compared with F VLP vaccines. In addition, Haynes’s group revealed that G glycoprotein is vital in the enhanced inflammatory response to FI-RSV vaccination [18]. Herein, potent cellular, humoral, and mucosal immune responses were induced by intranasal immunization using VLPs containing the RSV F or G protein. We found that the VLP vaccine candidates elicit serum and mucosal RSV-specific antibody and may also be responsible for the significant increase in cytokine levels.

VLPs are a novel type of viral vaccine known to be safe in the vaccinated population using convenient purification protocols. VLP vaccines elicit strong immune responses including cellular, humoral, and mucosal immunities. They have been successfully applied in rotavirus [19], human papilloma virus (HPV) [20], and hepatitis B virus [21]. Notably, the HPV vaccine produced by using the Bac-to-Bac system has had great success in the clinic, providing a novel strategy for RSV vaccine development. The precursor of RSV F, F0, is cleaved by Furin protease, yielding F1 and F2, respectively. The Furin cleavage is required for the membrane fusion, but not virus infection. As shown in Fig. 2, the RSV F protein in VLP is nearly 70 kDa. We speculated that majority of F protein on the VLP surface might be in a pre-fusion form, which is represented on the native virus.

The Bac-to-Bac expression system still has some problems such as incomplete glycosylation. In our studies, RSV G protein was found to have less glycosylation in VLP than that in native virus. In addition, the recombinant VLPs contained the host protein such as gp64 [10]. But the envelope protein gp64 was not detected in either RSV-F or RSV-G VLP (data not shown). Recombinant VLPs are produced in serum-free medium and not from mammalian cells. They may be safer for use because VLPs produced by rBV are free of viruses and oncogenic substances that may exist in mammalian cells and serum [22,23].

The RSV is more pleomorphic than influenza virus [11] and RSV M protein is very difficult in VLP packages without NP [5]. Here, the influenza virus M protein was used as a core protein in our RSV VLPs. We successfully obtained the viral morphology VLPs with size range from 60 to 80 nm which are smaller than the native RSV. It is noticeable that these VLPs were in smaller in size because the recombinant VLPs contained flu M and RSV F or G, rather than the whole RSV protein components. As shown in Fig. 2, the F protein concentration in F VLPs was equal to the G protein concentration in...
Additionally, we found that VLP F+G induced higher levels of serum nAb, mucosal IgA, and cellular immune response than VLP F or VLP G alone by intranasal administration. As shown in Fig. 6A, the body weight by F+G VLP group mice kept increasing for 7 days posted RSV challenge. These results are consistent with an earlier work reporting that mixed RSV-F+G VLPs had better immunity and protection than RSV-F or RSV-G VLPs alone [17].

Previous studies showed that the RSV-G VLPs may be more subtype-specific [23], while the RSV-F VLPs are thought to provide protection from both RSV A and B subclasses [24,25]. Dr Schmidt and our group reported an RSV VLP vaccine candidate using a chimeric protein in which the ectodomain was originated from the RSV G protein, and the cytoplasmic and transmembrane domains and the M protein were derived from NDV. These VLPs consist of the full sequences of RSV F and G proteins which contain all neutralizing epitopes and T-cell epitopes. They can elicit a much stronger and more effective immune response than that of the epitope vaccine candidates [9,10]. In 2011, Quan et al. [11] have explored these immune responses using the pFastBacI plasmid as a vector. In contrast to their study, we used the pFastBac Dual vector with two promoters in our experiments; this vector can carry two foreign genes and avoid the co-transfection of two bacmids. More importantly, Dual vector produces both influenza M1 and RSV G or RSV F simultaneously in the same culture bottle.

Intranasal injection has been shown to be an effective approach that can elicit safe and effective immune responses in humans. For example, intranasal trivalent, cold adapted, attenuated influenza (FluMist) was approved by Food and Drug Administration (FDA) in 2003, showing better immune responses and protection in clinical trials [26]. Similarly, Dubovsky’s group developed a live attenuated intranasal vaccine candidate, MED1-559, for the prevention of RSV-induced respiratory illness [27]. In lines with these data, our studies showed that intranasal immunization enhances mucosal antibody production, improves human immune system responses, and is as effective as intramuscular vaccination while reducing the antigen dosage. Meanwhile, we found that IgG antibody response exist in lung extract (data not shown). These data further support that mixed RSV VLP vaccine shows better responses in lung virus titer and pulmonary pathology compared with RSV F or G VLP alone. Of course, the regimen of prime-boost need to be further investigated in order to obtain optimal antibody responses. In addition, our RSV-F plus RSV-G VLP vaccine could induce IgG2a isotype dominant responses together with very low levels of IgG1 isotype responses through intranasal vaccination. We also found that there is a closely correlation between RSV neutralizing activity and lung viral clearance (data not shown) by determining the RSV neutralizing titers in immunized sera against wild-type virus. These findings are encouraging because enhanced lung pathological damage after vaccination is closely related to Th2 (IgG1) allergy-like or Th2- associated responses.

In conclusion, our mix of VLPs is more immunogenic and effective than RSV-F or G VLP alone and does not cause any enhanced RSV diseases. Since RSV proteins in VLPs are presented in a repetitive virus-like structure, they may be highly immunogenic and induce effective humoral, cellular, and mucosal immune responses. These findings also suggest that i.n. vaccination is a safe, effective approach to develop RSV VLP vaccines. We conclude that intranasal vaccination of RSV G plus RSV F VLPs are potential RSV vaccine strategies worthy to be further evaluated in primate models, such as cotton rat and monkey.

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