



# Parainfluenza virus 5 (PIV5) amplifying virus-like particles expressing respiratory syncytial virus (RSV) antigens protect mice against RSV infection



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## ABSTRACT

Respiratory syncytial virus (RSV) is the leading cause of bronchiolitis and pneumonia in children under one year of age. In addition to causing severe respiratory diseases in children, it is also a major cause of morbidity and mortality among the elderly and immunocompromised individuals. RSV is the most common cause of lower respiratory tract infections, yet there are currently no licensed vaccines. A parainfluenza virus 5 (PIV5)-based amplifying virus-like particle (AVLP), which enables the use of PIV5 RNA transcription/replication machinery to express gene of interest, has recently been developed. We evaluated the PIV5-based AVLP system as a vaccine platform for RSV by incorporating the fusion protein (F) gene and the transcription factor protein (M2-1) gene of RSV into the PIV5-AVLP backbone (AVLP-F and AVLP-M2-1, respectively). Mice immunized with a single dose of the AVLP-F or AVLP-M2-1 developed RSV-F or RSV-M2-1-specific immune responses, respectively. Both vaccine candidates elicited antigen-specific cell-mediated responses at levels comparable to or higher than an RSV infection. Most importantly, each vaccine was able to induce protection against RSV A2 challenge in the mouse model. These results indicate the potential of the PIV5-based AVLP system as a platform for vaccines against RSV infection.

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## 1. Introduction

Respiratory syncytial virus (RSV) is the most common cause of lower respiratory tract infection in infants [1,2]. Besides affecting children, RSV can also cause severe respiratory complications in both the elderly and immunocompromised individuals [3]. Even though RSV is one of the leading causes of bronchiolitis [4] and pneumonia [5] in infants, there is currently no licensed vaccine to prevent RSV infection. A formalin-inactivated RSV (FI-RSV) vaccine underwent clinical trials in infants in the 1960s, but this vaccine not only failed to protect against RSV infection, but it also caused enhanced disease in vaccinated children upon natural infection, resulting in two deaths [6]. There have been numerous attempts to develop an RSV vaccine, but none of them have resulted in a licensed vaccine.

Among the different vaccine platforms there are live-attenuated, whole virus-inactivated, particle-based, subunit, nucleic acid, and viral vector-based vaccines [7]. One of the platforms that has been explored in the recent years is particle-based or virus-like particle-based vaccines (VLPs). VLPs are multi-protein, self-assembled structures that closely resemble the virus from which they are derived [8]. Unlike live viruses, VLPs are non-replicating and non-infectious [8], making them a safer alternative to live-attenuated vaccines, especially when the target population is infants less than 1 year of age. The increasing interest in using VLPs as a vaccine platform has been influenced by the success of VLP-based vaccines for human papilloma virus (HPV) [9].

RSV VLP-based vaccines have been protective against RSV challenge. Newcastle disease virus (NDV), adenoviral vectors, insect expression systems, and mammalian expression systems have been used to produce VLPs [10–14]. One of the major challenges of using VLPs as a vaccine platform is to produce large quantities of VLPs, and VLP vaccines often require large doses to be efficacious. To address this problem, we have generated a parainfluenza virus 5 (PIV5)-based amplifying VLP (PIV5-AVLP) platform. Unlike

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traditional VLPs, AVLPs contain the RNA transcription/replication machinery of PIV5, which allows for continuous amplification of foreign genes in the target cells. This unique ability makes it possible to achieve protective immune responses with lower doses, compared to regular VLPs. In addition, the absence of the PIV5 structural proteins in the AVLPL genome prevents the production and spread of infectious progeny from AVLPL-infected cells, making the PIV5-AVLP platform as safe as traditional VLPs.

The PIV5-AVLP system takes advantage of PIV5's ability to infect many mammalian cell types with little cytopathic effect. PIV5 only requires three viral proteins (M, NP, and HN or F protein) for virus-like particle (VLP) formation [15,16]. Since AVLPLs contain PIV5 F and HN, they have similar cell tropism to PIV5, making it possible for the AVLPL to infect many cell types after vaccination and allowing the synthesis of the desired antigen. The development of the PIV5-based AVLPL vaccine platform provides an alternative to live-virus vaccines. One of the two major protective antigens for RSV is the fusion protein (F). Neutralizing antibodies against F have been shown prevent RSV infection [17,18]. Since neutralizing antibodies against RSV F prevent RSV infection, we developed an AVLPL-based vaccine expressing RSV F and tested its immunogenicity and protective efficacy in mice. Besides inducing the production of protective neutralizing antibodies, RSV can stimulate CD8<sup>+</sup> CTL responses that are important in viral clearance [19,20]. The dominant CTL epitope is mapped to RSV M2-1, and this epitope is responsible for approximately 40% of the primary CTL response [20]. In this work, we determined if AVLPLs expressing RSV F or RSV M2-1 would confer antigen-specific immune responses and protect against RSV infection in mice.

## 2. Materials and methods

### 2.1. Cells and vaccines

Dulbecco's modified Eagle medium (DMEM) containing 10% tryptose phosphate broth (TPB), 5% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 µg/mL streptomycin (1% P/S; Mediatech Inc., Manassas, VA) was used to maintain BHK21 cells.

To construct the vaccine plasmids, the enhanced green fluorescent protein (EGFP) gene in the AE31 plasmid, containing the PIV5 NP, V/P, and L genes in addition to a Hygromycin B resistance gene, was replaced with the RSV F or RSV M2-1 gene. Generation of stable cell lines and production of AVLPLs was done as described before [21]. The titers of AVLPL vaccines were determined as described before [21]. The number of infected cells was used to define the number of amplifying particles (AP) per milliliter. PIV5-RSV-F (SH/HN) vaccine was constructed, propagated, and titrated as described before [22]. RSV A2 was grown in Vero cells as previously described [23].

### 2.2. Western blotting (WB)

To determine protein incorporation into AVLPLs, concentrated AVLPL-F and AVLPL-M2-1 particles, RSV A2, and PIV5 were diluted at a 1:1 ratio with 2X Laemmli Sample Buffer (Bio-Rad) with 2-mercaptoethanol, and heated at 95 °C for 5 min. Samples were then resolved on a 10% or 15% acrylamide gel by SDS-PAGE and transferred to Amersham Hybond LFP PVDF membranes (GE). The membranes were incubated with mouse anti-RSV F (RSV5A6), mouse anti-RSV M2-1 (RSV5H5), or mouse anti-PIV5 P/V (Pk) antibodies (1:1000 dilution) followed by incubation with Cy3-conjugated goat anti-mouse IgG (Jackson ImmunoResearch). The blots were visualized on the Typhoon FLA 7000 (GE Healthcare Life Sciences).

### 2.3. Immunofluorescence assay (IFA)

BHK21 cells seeded on 18 mm cover slips were infected with AVLPL-F, AVLPL-M2-1, or RSV A2. RSV A2 was used as a positive control. After 48 h, the cells fixed with 60%/40% acetone/methanol. Protein expression was detected using mouse anti-RSV F [RSV3216(B016)], mouse anti-RSV M2-1 (RSV5H5), and mouse anti-PIV5 P/V (Pk) antibodies (1:500 dilution). Pictures were taken with a Nikon A1R confocal microscope.

### 2.4. Transmission electron microscopy (TEM)

A formvar, carbon-coated 300-mesh grid was floated onto a 40 µL drop of AVLPL-F, AVLPL-M2-1, or PIV5 for 30 min. The grid was removed and excess sample was drained off with the edge of filter paper, and floated onto a drop of 3% aqueous phosphotungstic acid, pH 6.8–7.0 for 1 min. After draining excess stain from grid, the grid was allowed to dry on filter paper prior to viewing with a JEOL JEM-1011 Transmission Electron Microscope at 80 kV (Jeol USA, Inc., Peabody, MA). Representative digital images were taken with an XR80M Wide-Angle Multi-Discipline Mid-Mount CCD Camera from Advanced Microscopy Techniques (AMT, Woburn, MA) at a 30,000× magnification.

### 2.5. Immunization of mice

All animal experiments were performed in accordance with the protocols approved by the Institutional Animal Care and Use Committee at the University of Georgia. For the two-dose experiment, six-to-eight-week-old female BALB/c mice (Harlan Laboratories, Indianapolis, IN) were anesthetized with 200 µL of 2,2,2-tribromoethanol in tert-amyl alcohol (Avertin; 180 to 250 µL/kg of body weight) injected intraperitoneally. The mice (n = 10, per group) were immunized by intranasal administration of  $1 \times 10^5$  AP of AVLPL-F or AVLPL-M2-1, or  $1 \times 10^5$  PFU of RSV A2 in a 100 µL volume. Negative control mice received 100 µL of PBS intranasally. Mice were boosted with the same dose 21 days post-immunization with the same dose as the primary immunization.

For the single-dose experiment six-to-eight-week-old female BALB/c mice (n = 13, per group) were anesthetized as previously described, and then immunized by intranasal administration of  $1 \times 10^5$  AP of AVLPL-EGFP,  $1 \times 10^5$  AP,  $4 \times 10^5$  AP, or  $1.3 \times 10^6$  AP of AVLPL-F, and  $1 \times 10^5$  AP or  $4 \times 10^5$  AP of AVLPL-M2-1 in a 100 µL volume. Negative control mice received 100 µL of PBS intranasally.

Blood was collected via cardiac bleed (n = 5, per group) for serological analysis at 6-days post-boost for the two-dose experiment, and 27 days post-immunization for the single-dose experiment, and spleens were harvested for enzyme-linked immunospot assay. Mice were challenged intranasally with  $5 \times 10^5$  PFU of RSV A2 in a 100 µL volume at 11 days post-boost for the two-dose experiment, and 32 days post-immunization for the single-dose experiment. Four days later, lungs from 5 mice in each group were collected to measure lung viral titers. The lungs from the remaining 3 mice in each group from the single-dose experiment were perfused with 10% formalin solution and sent for histology.

For the mouse experiment comparing cell-mediated immune responses six-to-eight-week-old female BALB/c mice (n = 6, per group) were anesthetized as described, and then immunized by intranasal administration of  $2 \times 10^4$  PFU of RSV A2 or PIV5-RSV-F (SH/HN), or  $2 \times 10^4$  AP of AVLPL-EGFP or AVLPL-F in 100 µL volume. Negative control mice received 100 µL of PBS intranasally. All groups, except the AVLPL-F group, were boosted 21 days post-immunization with the same dose as the primary immunization. At 6 days post-boost (27 days post-primary immunization), spleens were harvested for enzyme-linked immunospot assay.

## 2.6. ELISAs to measure total RSV antigen-specific IgG, IgG1 and IgG2a

RSV F-specific serum antibody titers were measured by ELISA as described before [22]. Immulon® 2HB 96-well microtiter plates were coated with 100  $\mu$ L per well of 1  $\mu$ g/ml of purified RSV-F protein in PBS. Two-fold serial dilutions of the serum samples were made in blocking buffer and 100  $\mu$ L were added to the plates. Secondary antibody was diluted 1:2500 [horseradish peroxidase-labeled goat anti-mouse IgG or horseradish peroxidase-labeled goat anti-mouse IgG1 or IgG2a (SouthernBiotech, Birmingham, AL)] in blocking buffer, and 100  $\mu$ L of the diluted antibody was added to each well. The endpoint titer was defined as the highest serum dilution at which the OD was greater than two standard deviations above the mean OD of the naïve serum.

## 2.7. Neutralizing antibody assay

Neutralizing antibody titers were measured by plaque reduction assay. Two-fold serial dilutions of heat-inactivated serum starting at a 1:8 dilution were prepared in Opti-MEM supplemented with 2% FBS, 1% P/S, and 5% guinea pig complement (Sigma-Aldrich, St. Louis, MO). The diluted serum was incubated with 150 PFU of RSV A2 for one hour at 37 °C, 5% CO<sub>2</sub>. After incubation, 50  $\mu$ L of the serum and virus mixture were added to confluent monolayers of Vero cells in 12-well plates, and the cells were incubated for one hour at 37 °C, 5% CO<sub>2</sub>, with rocking every 15 min. After adsorption, the cells were overlaid with 2 mL of Opti-MEM with 2% FBS, 1% P/S, and 0.8% methylcellulose. After incubating for 7 days at 37 °C, 5% CO<sub>2</sub>, the plaques were visualized by immunostaining. The neutralizing antibody titers were defined as the highest serum dilution at which 50% neutralization of the input virus control was achieved.

## 2.8. Enzyme-Linked ImmunoSpot assay (ELISPOT)

BD™ ELISPOT Mouse IFN- $\gamma$  Set (BD Biosciences, San Jose, CA) was used for analyzing cellular immune responses. BD™ ELISPOT plates were coated with purified anti-mouse IFN- $\gamma$  antibody 24 h prior to performing assay. Mouse spleens (n = 5, per group) were collected at 6 days post-boost for the two-dose experiment and 27 days post-immunization for the single-dose experiment and put into 15 mL conical tubes containing 5 mL of HBSS. Splenocytes were prepared by pressing spleens through a 70  $\mu$ m cell strainer, incubating them with ACK lysis buffer, washing them with HBSS, and re-suspending in complete tumor medium (CTM) to a concentration of  $5 \times 10^6$  cells/mL. The capture antibody solution was removed from the plates and then the plates were washed 5–6 times with PBS. The plates were then blocked with CTM for 90 min. The blocking solution was discarded, and 0.1  $\mu$ g of RSV F peptide (85–93), RSV M2-1 peptide (82–90), GFP peptide (200–208), or PMA/Ionomycin in 50  $\mu$ L of CTM were added to the wells. 50  $\mu$ L of splenocytes were added to plates ( $2.5 \times 10^5$  cells/well) and incubated at 37 °C, 5% CO<sub>2</sub>, for 48 h. The spots were immunostained according to the BD™ ELISPOT Set instruction manual and counted using an ImmunoSpot® analyzer (Cell Technology Limited, CTL). Results were presented as mean number of IFN- $\gamma$  secreting cells per  $10^6$  splenocytes.

## 2.9. Titration of RSV from mouse lungs

Mouse lungs were collected in gentleMACS M tubes (Miltenyi Biotec Inc., Auburn, CA) containing 3 mL of Opti-MEM with 1% BSA and kept on ice. The lungs were homogenized using the Protein\_01 program of a gentleMACS Dissociator (Miltenyi Biotec Inc.) at 4 °C and then centrifuged for 10 min at  $3000 \times g$ . RSV titers in the supernatant were measured by plaque assay [24].

## 2.10. Histology

For the single-dose experiment, mouse lungs (n = 3, per group) were harvested four days after challenge and then perfused with 1 mL of 10% formalin. The lungs were immersed in 10% formalin for at least 24 h. The fixed lungs were transferred to 70% ethanol, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. A pathologist scored the lung lesions in the sections for alveolitis, perivascular cuffing, vasculitis, interstitial pneumonia, and pleuritis. The scores ranged from 0 to 4, with 0 indicating no lesions and 4 indicating severe lesions.

## 2.11. Statistics

Graphpad Prism software for Windows (Graphpad Software, La Jolla, CA) was used for the statistical analysis. Total serum IgG antibody titers and neutralizing antibody titers were analyzed using the one-way analysis of variance (ANOVA) test and Bonferroni's multiple comparisons test. IgG1 or IgG2a antibody titer ratios were analyzed using the unpaired, two-tailed *t*-test. Levels of IFN- $\gamma$  secreting cells and lung viral loads were analyzed using one-way ANOVA followed by Dunnett's multiple comparisons test. Kruskal-Wallis test was used to analyze the histology data. For the two-dose experiments, the asterisks mark the significance between the PBS and vaccine groups. For the single-dose experiment and histopathology, the asterisks mark the significance between the AVLP-EGFP group and the vaccine groups.

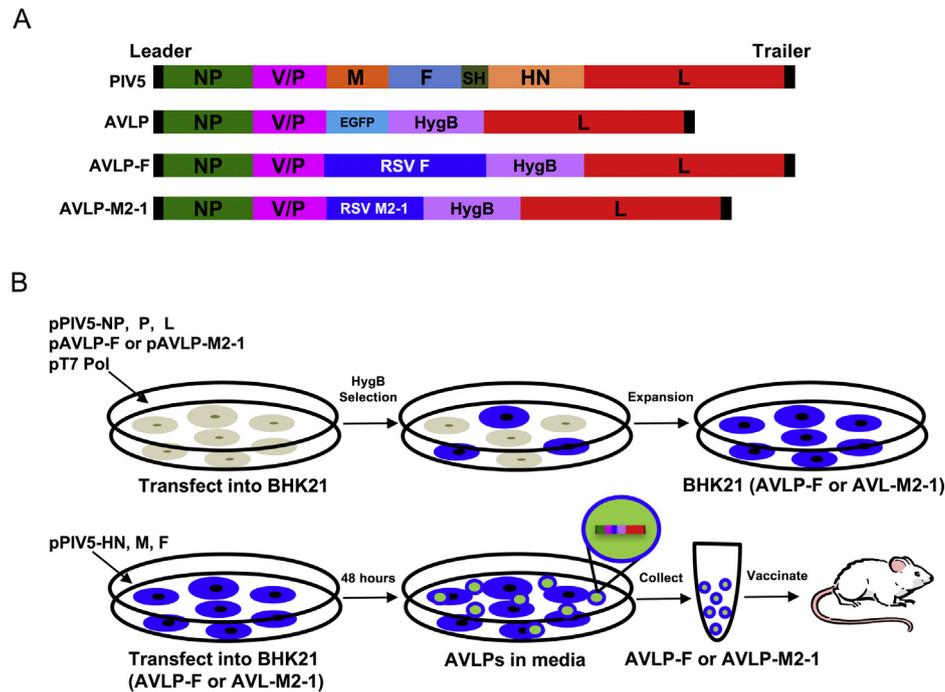
## 3. Results

### 3.1. Construction and characterization of PIV5-AVLP-based RSV vaccines

RSV F and RSV M2-1 genes from RSV A2 were cloned into a plasmid containing the PIV5-AVLP backbone, which contains the PIV5 NP, PIV5 V/P, and PIV5 L genes, in addition to a Hygromycin B (HygB) gene (Fig. 1A). Stable BHK21 cell lines containing the AVLP-F and AVLP-M2-1 genomes were created by transfecting each individual plasmid along with plasmids encoding for PIV5 NP, P, L, genes and the 437-T7 polymerase as described before (Fig. 1B) [21]. To generate AVLP-F and AVLP-M2-1 particles, AVLP-F and AVLP-M2-1 stable cell lines were transfected with plasmids encoding PIV5 HN, PIV5 M, and PIV5 F as described before (Fig. 1B) [21]. The AVLPs collected in the media were then used to immunize mice (Fig. 1B). RSV M2-1 incorporation into AVLPs was confirmed by western blotting using an RSV M2-1-specific monoclonal antibody (Fig. 2A). We were not able to detect RSV F in AVLP-F particles. Even though RSV F incorporation into the AVLPs was not detected, RSV F expression was confirmed in AVLP-F-infected cells by immunofluorescence assay (Fig. 2B) using an RSV F-specific monoclonal antibody. This demonstrated that even though RSV F was not detected in the AVLPs, cells infected with these particles still produce *de novo* antigen leading to an induction of an immune response. RSV M2-1 expression in AVLP-M2-1-infected cells was confirmed by the same methods using RSV M2-1 specific antibody (Fig. 2B). AVLP-F and AVLP-M2-1 particles were visualized by transmission electron microscopy (Fig. 2C). We observed that the AVLP-F and AVLP-M2-1 particles were similar in shape and size to wild-type PIV5 particles.

### 3.2. Immunization with two doses of AVLP-F vaccine induces RSV antigen-specific antibodies and generates RSV-neutralizing antibodies

To determine immunogenicity of AVLP-F and AVLP-M2-1, mice were immunized intranasally with either vaccine at a dose of



**Fig. 1.** Construction and production of AVLP-F and AVLP-M2-1 vaccines. (A) Schematic of AVLP-F and AVLP-M2-1 vaccine constructs. NP, nucleoprotein; V, V protein; P, phosphoprotein; L, RNA-dependent RNA polymerase; EGFP, enhanced green fluorescent protein; HygB, hygromycin B phosphotransferase; RSV F, respiratory syncytial virus fusion protein; RSV M2-1, respiratory syncytial virus M2-1 protein. (B) Schematic of AVLP-F and AVLP-M2-1 vaccine production. BHK21 cells were transfected with plasmids encoding for PIV5 NP, P, L, and the 437-T7 polymerase along with the AVLP-F or AVLP-M2-1 plasmids. Hygromycin B was added to the transfected cells for selection. BHK21 (AVLP-F and AVLP-M2-1) cell clones were then allow to expand under hygromycin B selection. AVLP-F and AVLP-M2-1 BHK21 stable cells lines were then transfected with plasmids encoding PIV5 HN, M, and F. Media containing AVLP-F and AVLP-M2-1 particles was collected 48 h after transfection. These particles were then used to vaccinate BALB/c mice. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

$1 \times 10^5$  AP, and then boosted with the same dose by the same route of administration at 21 days post-primary vaccination. Total serum IgG antibody titers to RSV were measured 6 days post-boost. Serum from mice immunized with the AVLP-F vaccine had increased F-specific total IgG antibody titers compared to the mice immunized with PBS, although to a lesser degree than RSV A2-immunized mice (Fig. 3A). We also measured F-specific IgG2a/IgG1 antibody ratios in immunized mice. Immunization with this vaccine produced RSV antigen-specific IgG2a/IgG1 antibody ratios that were not significantly different from ratios observed in mice immunized with RSV A2 (Fig. 3B).

Neutralizing antibody titers were measured using a complement-enhanced neutralization assay. At 6 days post-boost, mice immunized with the AVLP-F vaccine had significantly higher levels of neutralizing antibodies compared to the PBS-immunized group, although at lower levels than RSV A2-immunized mice (Fig. 3C). Sera from mice immunized with the AVLP-M2-1 vaccine did not have any neutralizing activity (Fig. 3C).

### 3.3. Immunization with AVLP-F and AVLP-M2-1 induces RSV antigen-specific cell-mediated responses

To determine if the vaccine candidates elicited cell-mediated immune responses, spleens were harvested from the mice at 6 days post-boost and enzyme-linked immunospot (ELISPOT) assays were performed. Splenocytes isolated from mice immunized with the different vaccine candidates were stimulated with RSV F and RSV M2-1 peptides, and the number of IFN- $\gamma$  secreting cells were measured. Splenocytes from AVLP-F-immunized mice showed a significant increase in IFN- $\gamma$  secreting cells when stimulated with the RSV F peptide compared to mice immunized with RSV A2 (Fig. 4). Spleens from mice immunized with the AVLP-M2-1 vaccine showed an increase in IFN- $\gamma$  secreting cells when

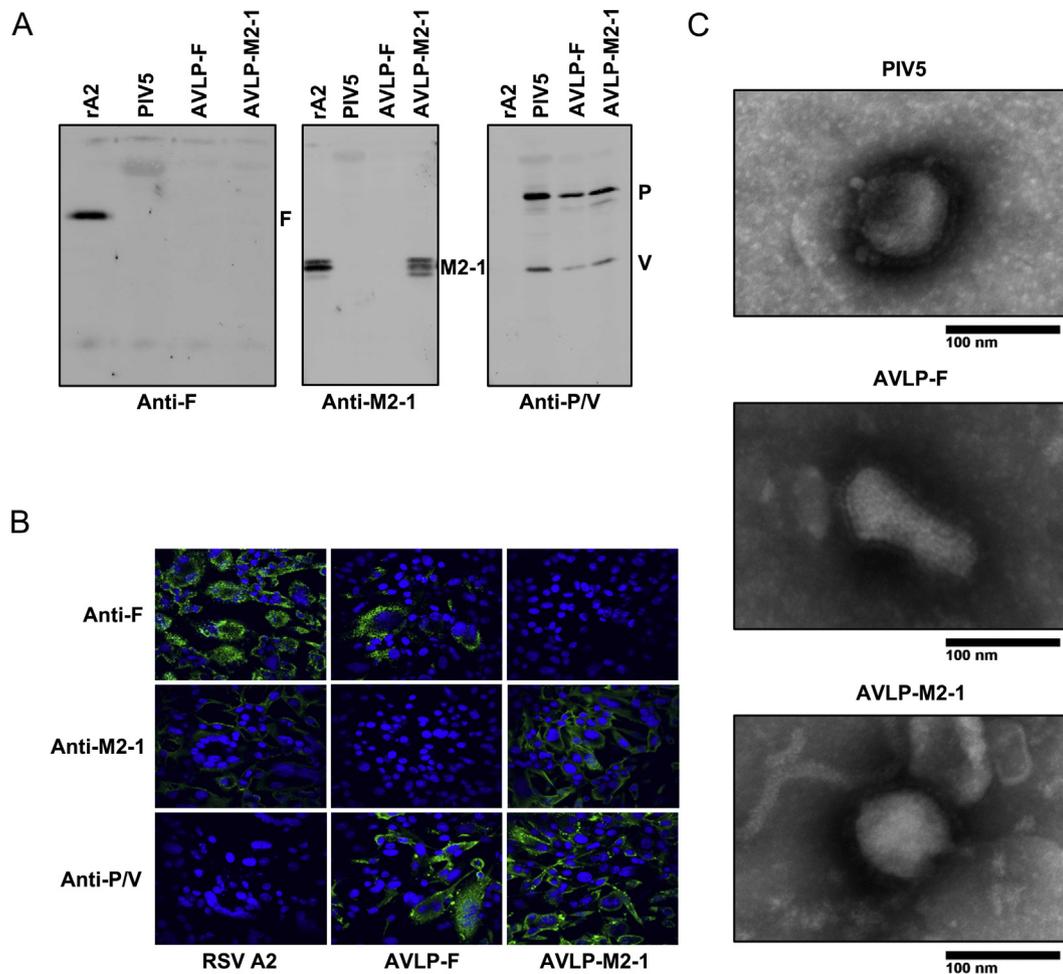
stimulated with the RSV M2-1 peptide comparable to mice immunized with RSV A2 (Fig. 4). These results indicated that both vaccine candidates, AVLP-F and AVLP-M2-1, are capable of eliciting cell-mediated responses. Interestingly, AVLP-F generated more robust F-specific cellular immune responses than RSV infection.

### 3.4. PIV5-AVLP-based RSV vaccines generate protective immunity against RSV A2 challenge in the mouse model

The immunized mice were challenged with  $5 \times 10^5$  PFU of RSV A2 11 days post-boost. Lungs were collected 4 days post-challenge to measure RSV A2 titers. Mice immunized with the AVLP-F vaccine had no detectable virus in the lungs, similar to the RSV A2-immunized group. Two of the AVLP-M2-1-immunized mice had an average lung viral titer of 81 PFU/lung, while the other two mice in the group had no detectable virus. Overall, the lung viral titers from both vaccine groups were significantly lower than the lung viral titers from the PBS control group, which had an average titer of  $2.4 \times 10^4$  PFU/lung (Fig. 5). These results indicate that vaccination with either the AVLP-F or AVLP-M2-1 vaccine candidate provides protection against RSV A2 challenge.

### 3.5. Immunization with a single dose of AVLP-F induces RSV antigen-specific antibodies and generates RSV-neutralizing antibodies

Since immunizing mice with two doses of either vaccine candidate protected mice against RSV A2 challenge, we wanted to determine if a single dose of each vaccine would confer protective immunity against RSV infection. A low-dose ( $1 \times 10^5$  AP), a medium-dose ( $4 \times 10^5$  AP), and a high-dose ( $1.3 \times 10^6$  AP) of AVLP-F, as well as a low-dose ( $1 \times 10^5$  AP) and a high-dose ( $4 \times 10^5$  AP) of AVLP-M2-1 were used to immunize the animals as before. Total serum IgG antibody titers to RSV F were measured



**Fig. 2.** Characterization of AVL-P-F and AVL-P-M2-1 vaccines. (A) Detection of RSV F, RSV M2-1, and PIV5 P/V in AVL-P-F and AVL-P-M2-1 particles by western blotting. AVL-P-F, AVL-P-M2-1, RSV A2, and PIV5 were stained with anti-RSV F, anti-RSV M2-1, and anti PIV5-P/V antibodies. RSV A2 and PIV5 were used as controls. (B) Detection of RSV F, RSV M2-1, and PIV5-P/V expression in AVL-P-F and AVL-P-M2-1-infected BHK cells by immunofluorescence assay. BHK cells were infected with AVL-P-F, AVL-P-M2-1, or RSV A2 as a control. After 48 h, the cells were fixed with 60%/40% acetone/methanol and stained with the indicated antibodies. (C) EM analysis of particles. Concentrated AVL-P-F and AVL-P-M2-1 particles were examined using an electron microscope to determine the morphology of the particles. Representative images of each vaccine candidate were taken at 30,000 $\times$  magnification.

27 days post-immunization. Similar to what was observed in mice immunized with the two-dose regimen, mice immunized with a single-dose regimen showed increased F-specific total IgG antibody titers compared with mice immunized with AVL-P-EGFP (Fig. 6A). Mice immunized with the high-dose of AVL-P-F had the highest IgG titers against RSV F among the AVL-P-F-immunized groups, compared with AVL-P-EGFP-immunized mice.

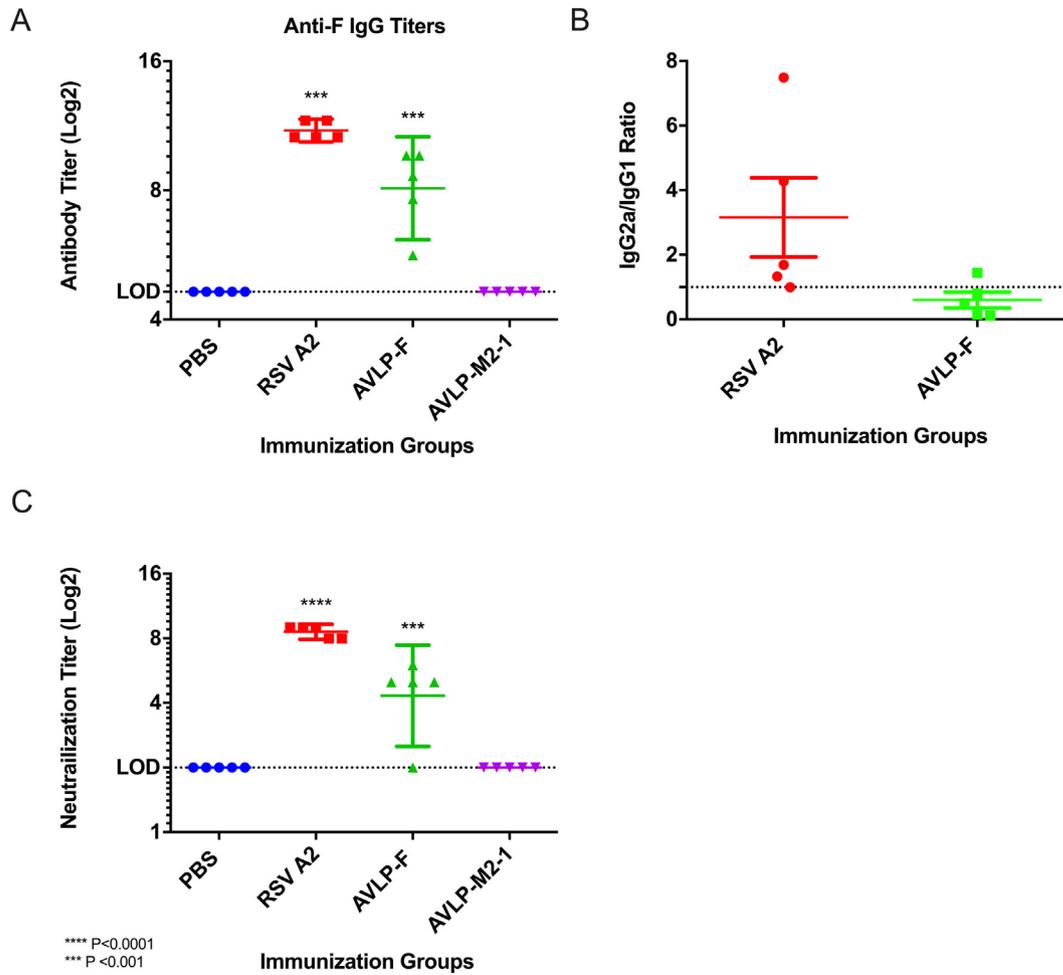
At 27 days post-immunization serum neutralizing antibody titers of mice immunized with the AVL-P-F vaccine were significantly increased compared with the AVL-P-EGFP-infected mice (Fig. 6B). Mice immunized with the medium- and high-dose of AVL-P-F had higher neutralizing antibody titers than the mice from the low-dose group, indicating that the neutralizing antibody titers were AVL-P-F-dose dependent. Sera from mice immunized with the AVL-P-M2-1 vaccine (low-dose or high-dose) did not have any neutralizing activity (Fig. 6B).

### 3.6. Immunization with a single dose of AVL-P-F and AVL-P-M2-1 induces RSV antigen-specific cell-mediated responses

The spleens from immunized mice were harvested at 27 days post-immunization. The splenocytes were stimulated with RSV F

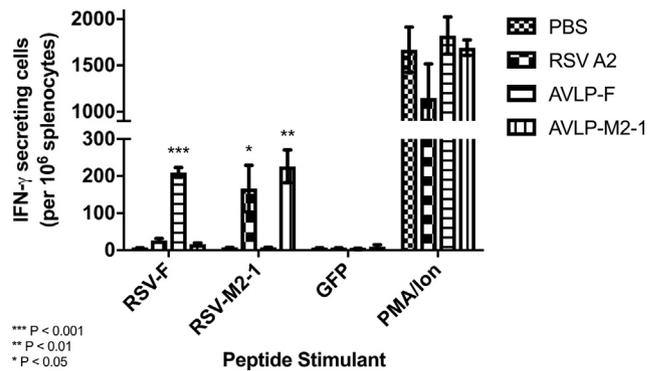
and RSV M2-1 peptides, and the number of IFN- $\gamma$  secreting cells was measured. GFP and PMA/Ionomycin peptide stimulants were used as negative and positive controls, respectively. Splenocytes from mice immunized with a single-dose of AVL-P-F showed a significant increase in IFN- $\gamma$  secreting cells when stimulated with the RSV F peptide compared with mice immunized with AVL-P-EGFP, with the medium- and high-dose groups showing the greatest increase (Fig. 7A). Similarly, splenocytes isolated from mice immunized with a single-dose of AVL-P-M2-1 vaccine showed a significant increase in the number of IFN- $\gamma$  secreting cells when stimulated with the RSV M2-1 peptide compared with mice immunized with AVL-P-EGFP. In this case, there was no significant difference in the number of IFN- $\gamma$  secreting cells between the low-dose and high-dose groups (Fig. 7A). Splenocytes from AVL-P-EGFP-immunized mice showed a significant increase of IFN- $\gamma$  secreting cells when stimulated with GFP peptide. These results indicated that single immunizations with both vaccine candidates, AVL-P-F and AVL-P-M2-1, elicit antigen-specific cell-mediated responses. But unlike the antibody response, these results were not dose-dependent.

In order to compare cellular immune responses induced by the AVL-P vaccine platform with those induced by natural RSV infection and the live PIV5 platform [22], mice were immunized intranasally

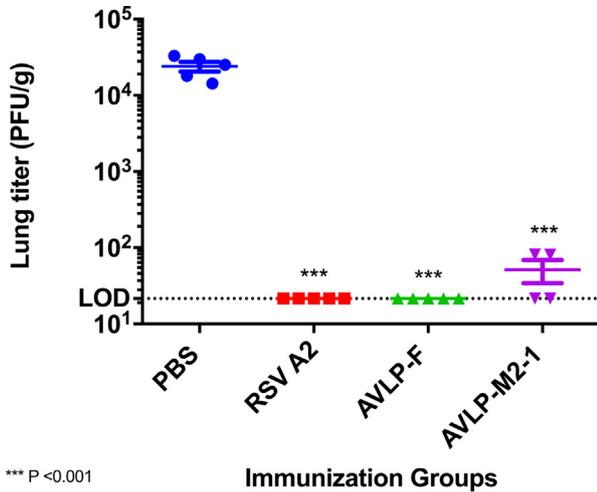


**Fig. 3.** Immunization with two doses of the AVL-P-F vaccine induces RSV antigen-specific IgG, IgG1, and IgG2a responses and RSV-neutralizing antibodies. Six-to-eight week old BALB/c mice were immunized with  $1 \times 10^5$  AP of AVL-P-F or AVL-P-M2-1,  $1 \times 10^5$  PFU of RSV A2, or PBS in 100- $\mu$ L volumes. Mice were boosted at 21-days-post-immunization and serum samples were collected at 6 days post-boost. (A) Anti-RSV F IgG antibody responses. Total RSV F-specific IgG antibody titers were measured by ELISA with serum collected on day 6 days post-boost. (B) Anti-RSV F IgG1 and IgG2a antibody responses. RSV F-specific IgG1 and IgG2a antibodies were measured by ELISA with serum from day 6 days post-boost. Values from the titration curves were used to calculate RSV F-specific IgG2a/IgG1 ratios from sera collected from RSV A2 and AVL-P-F-immunized mice. Solid horizontal lines represent the mean value in each group and the dotted horizontal line represents a ratio of 1. (C) RSV neutralizing antibody responses. RSV neutralizing antibodies were measured by plaque reduction assay. Two-fold serial dilutions of heat-inactivated serum were incubated with 150 PFU of RSV A2. The mixture was added to Vero cells, and plaques were enumerated 7 days later. The neutralizing antibody titer was defined as the reciprocal of the highest dilution at which there was a 50% reduction in input virus. Each group consisted of 5 mice. Horizontal lines represent the geometric mean value in each group. LOD, limit of detection. Error bars in (A) and (C) represent 95% CI, and error bars in (B) represent standard error of the mean. \*\*\* (P < 0.001), \*\*\*\* (P < 0.0001), significance between PBS and vaccine groups.

with RSV A2 ( $2 \times 10^4$  PFU), PIV5-RSV-F (SH/HN,  $2 \times 10^4$  PFU), AVL-P-EGFP ( $2 \times 10^4$  AP), and AVL-P-F ( $2 \times 10^4$  AP). All but one group, the AVL-P-F group, were then boosted with the same dose by the same route of administration 21 days post-primary vaccination. The spleens from immunized mice were harvested at 6 days post-boost (or 27 days post-immunization for the AVL-P-F group). The splenocytes were stimulated with RSV F and RSV M2-1 peptides, and the number of IFN- $\gamma$  secreting cells was measured. Splenocytes from mice immunized with a single-dose of AVL-P-F showed a significant increase in IFN- $\gamma$  secreting cells when stimulated with the RSV F peptide compared with mice immunized with two-doses of AVL-P-EGFP (Fig. 7B). This difference between AVL-P-EGFP and AVL-P-F is far greater than the difference between AVL-P-EGFP and two-doses of RSV A2 or PIV5-RSV-F (SH/HN), with the RSV A2-immunized group not showing any significance at all (Fig. 7B). These results demonstrate that a single-dose of AVL-P-F is capable of inducing a significantly higher cellular-immune response compared to two-doses of RSV A2 or PIV5-RSV-F (SH/HN). More importantly, these results indicate that AVL-P vaccine platform is a promising vector for generating cell-mediated immune responses.



**Fig. 4.** Immunization with the AVL-P-F and AVL-P-M2-1 vaccines induce RSV antigen-specific cell-mediated responses. Six-to-eight week old BALB/c mice were immunized with  $1 \times 10^5$  AP of AVL-P-F or AVL-P-M2-1,  $1 \times 10^5$  PFU of RSV A2, or PBS in 100- $\mu$ L volumes. Mice were boosted 21 days-post-immunization and spleens were collected 6 days post-boost. Splenocytes were harvested and stimulated with RSV F, RSV M2-1, PMA/Ionomycin, or GFP peptides. Results were presented as number of IFN- $\gamma$ -secreting cells per  $10^6$  splenocytes. Each group consisted of 3 to 5 mice. Error bars are standard error of the mean. (P < 0.05), \*\* (P < 0.01), \*\*\* (P < 0.001), significance between PBS and vaccine groups.



**Fig. 5.** PIV5-AVLP-based RSV vaccines are protective against RSV A2 challenge Six-to-eight week old BALB/c mice were immunized with  $1 \times 10^5$  AP of AVLP-F or AVLP-M2-1,  $1 \times 10^5$  PFU of RSV A2, or PBS in 100- $\mu$ L volumes. Mice were boosted 21 days post-immunization and challenged with  $5 \times 10^5$  PFU of RSV A2 11 days post-boost. Four days later, lungs were collected, and RSV titers were measured by plaque assay in Vero cells. Groups consisted of 4 to 5 mice. Horizontal lines represent the mean value in each group. LOD, limit of detection. Error bars are standard error of the mean. \*\*\* ( $P < 0.001$ ), significance between PBS and vaccine groups.

**3.7. PIV5-AVLP-based RSV vaccines generate protective immunity against RSV A2 challenge after a single dose in the mouse model**

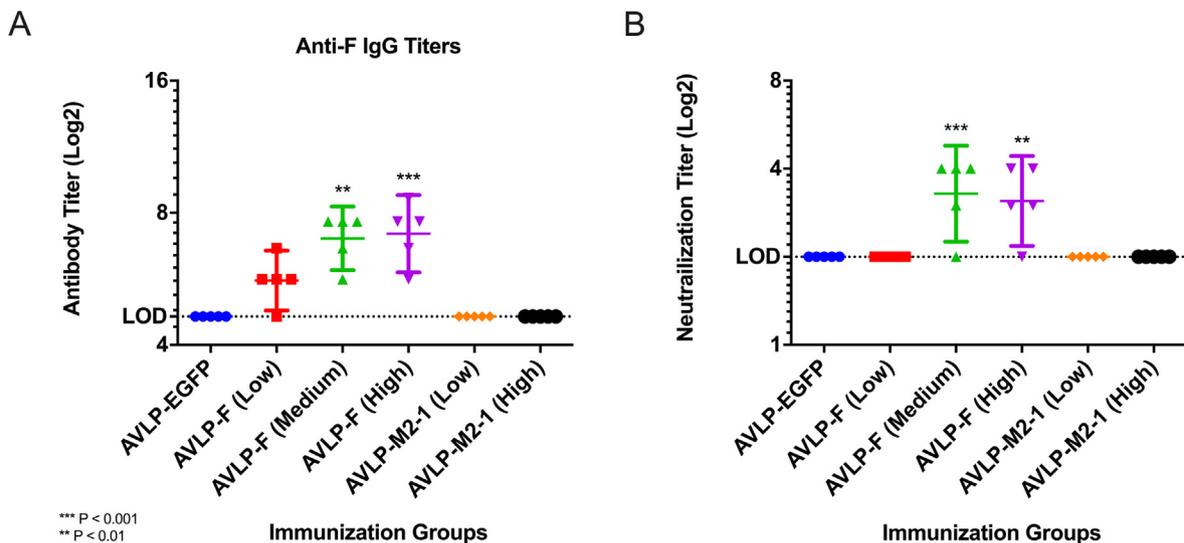
To determine the protective efficacy of the vaccine candidates after a single dose, the mice were challenged with  $5 \times 10^5$  PFU of RSV A2 and lungs were collected 4 days later to measure viral titers. Mice vaccinated with either the AVLP-F or AVLP-M2-1 showed significant reductions in lung viral titers compared to the AVLP-EGFP control group. Mice immunized with the low- and medium-dose of AVLP-F had lung viral titer averages of 121 PFU/lung and 131 PFU/lung, respectively. Mice immunized with the

low- and high-dose of AVLP-M2-1 vaccine had lung viral titer averages of 177 PFU/lung and 215 PFU/lung, respectively. More importantly, mice immunized with the high dose of AVLP-F vaccine had no detectable virus in the lungs. Overall, the lung viral titers from all vaccine groups were significantly lower than the lung viral titers from the AVLP-EGFP control group, which had an average titer of  $2.3 \times 10^4$  PFU/lung (Fig. 8). These results indicate that a single-dose immunization with either of the vaccine candidates is sufficient to induce potent immunity against RSV A2 challenge.

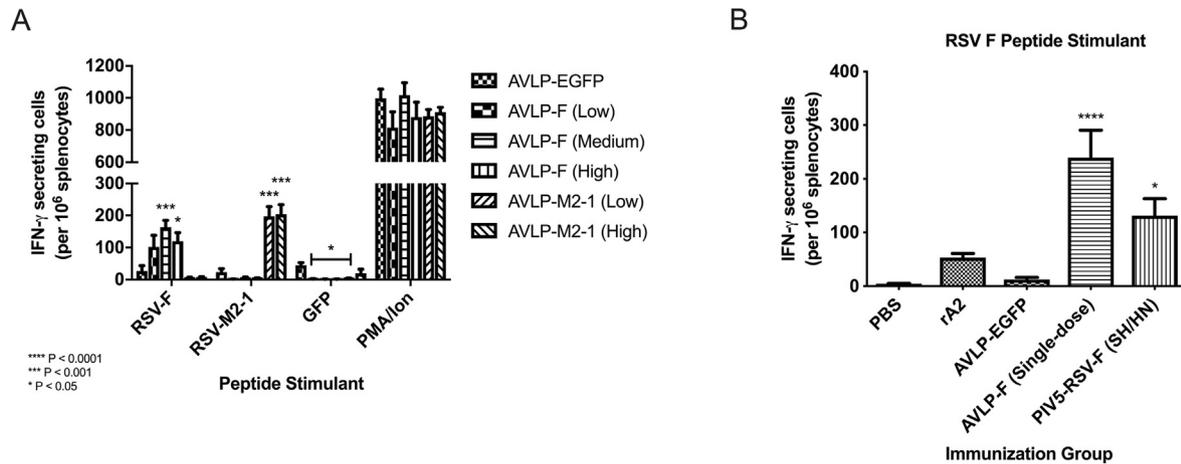
Lung histology from three mice from each vaccine group was examined after RSV A2 challenge to determine if immunization with the PIV5-AVLP-based vaccine candidates enhanced RSV-induced lung pathology. Lung tissue from the AVLP-F and AVLP-M2-1 vaccination groups had similar levels of inflammation compared to the AVLP-EGFP control group. The inflammation in the mice vaccinated with AVLP-EGFP or the AVLP-F and AVLP-M2-1 vaccine candidates was predominantly characterized by perivascular cuffing (Fig. 9A). The layer of leukocytes cuffing the pulmonary blood vessels mainly consisted of lymphocytes and macrophages, with neutrophils and eosinophils surrounding the vessels at a lower frequency. Apart from scoring the tissue sections for perivascular cuffing (Fig. 9B), tissue sections were scored for alveolitis, vasculitis, interstitial pneumonia, and pleuritis (Fig. 9C–F). No enhanced pathology was observed in the vaccine groups compared to the control group, indicating that the PIV5-AVLP-based system did not cause enhanced disease.

**4. Discussion**

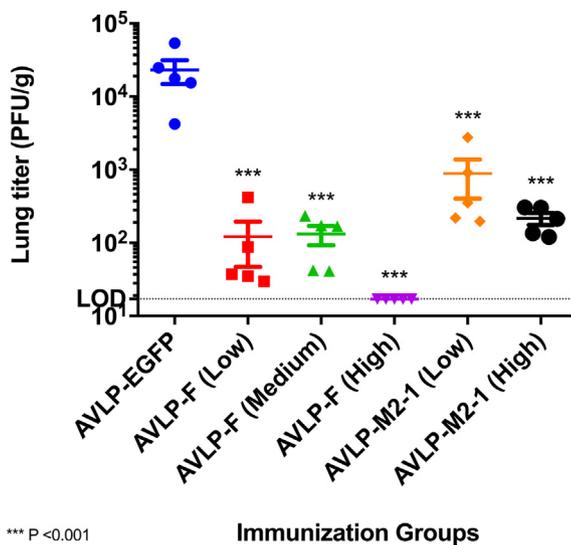
RSV is a serious health threat to infants as well as the elderly and immunocompromised individuals worldwide, yet there is still no licensed vaccine. In this study, we aimed to produce a safe and effective RSV vaccine by using the PIV5-AVLP system. We demonstrated that even though a PIV5-AVLP-based vaccine expressing either RSV F or RSV M2-1 proteins did not generate robust antibody responses, they did generate robust cellular immune responses, and they were effective in protecting against RSV



**Fig. 6.** Immunization with a single dose of the AVLP-F vaccine induces RSV antigen-specific IgG responses and RSV-neutralizing antibodies. Six-to-eight week old BALB/c mice were immunized with  $4 \times 10^5$  AP of AVLP-EGFP,  $1 \times 10^5$  AP,  $4 \times 10^5$  AP, or  $1.3 \times 10^6$  AP of AVLP-F,  $1 \times 10^5$  AP or  $4 \times 10^5$  AP of AVLP-M2-1, or PBS in 100- $\mu$ L volumes. Serum samples were collected 27 days post-immunization. (A) Anti-RSV F IgG antibody responses. Total anti-RSV F IgG antibody titers were measured by ELISA using serum collected on day 27 post-immunization. (B) RSV neutralizing antibody responses. RSV neutralizing antibodies were measured by plaque reduction assay. Two-fold serial dilutions of heat-inactivated serum were incubated with 150 PFU of RSV A2, and the mixture was used to infect Vero cells. Plaques were enumerated 7 days later. The neutralizing antibody titer was defined as the reciprocal of the highest dilution at which there was a 50% reduction in input virus. Each group consisted of 5 mice. Horizontal lines represent the geometric mean value in each group. LOD, limit of detection. Error bars in (A) and (B) represent 95% CL. \*\* ( $P < 0.01$ ), \*\*\* ( $P < 0.001$ ), significance between AVLP-EGFP and vaccine groups.



**Fig. 7.** Immunization with a single-dose of the AVLP-F and AVLP-M2-1 vaccines induce RSV antigen-specific cell-mediated responses. (A) Six-to-eight week old BALB/c mice were immunized with  $4 \times 10^5$  AP of AVLP-EGFP,  $1 \times 10^5$  AP,  $4 \times 10^5$  AP, or  $1.3 \times 10^6$  AP of AVLP-F,  $1 \times 10^5$  AP or  $4 \times 10^5$  AP of AVLP-M2-1, or PBS in 100- $\mu$ L volumes. Splenocytes were collected at 27 days post-immunization. Splenocytes were harvested and stimulated with RSV F, RSV M2-1, or GFP peptides, or PMA/Ionomycin. Each group consisted of 3 to 5 mice. (B) Six-to-eight week old BALB/c mice were immunized with  $2 \times 10^4$  PFU of RSV A2 or PIV5-RSV-F (SH/HN),  $2 \times 10^4$  AP of AVLP-EGFP or AVLP-F, or PBS in 100  $\mu$ L volumes. All groups, except the AVLP-F group, were boosted 21 days post-immunization with the same dose as the primary immunization. Splenocytes were collected at 6 days post-boost (27 days post-primary immunization). Splenocytes were harvested and stimulated with RSV F. Each group consisted of 6 mice. Results were presented as the number of IFN- $\gamma$  secreting cells per  $10^6$  splenocytes. Error bars are standard error of the mean. \* ( $P < 0.05$ ), \*\* ( $P < 0.001$ ), \*\*\* ( $P < 0.0001$ ) significance between AVLP-EGFP and vaccine groups.



**Fig. 8.** Single-dose immunizations are protective against RSV A2 challenge Six-to-eight week old BALB/c mice were immunized with  $4 \times 10^5$  AP of AVLP-EGFP,  $1 \times 10^5$  AP,  $4 \times 10^5$  AP, or  $1.3 \times 10^6$  AP of AVLP-F,  $1 \times 10^5$  AP or  $4 \times 10^5$  AP of AVLP-M2-1, or PBS in 100- $\mu$ L volumes. Mice were challenged with  $5 \times 10^5$  PFU of RSV A2 at 32 days post-immunization. Four days later, lungs were collected, and RSV titers were measured by plaque assay in Vero cells. Each group consisted of 5 mice. Horizontal lines represent the mean value in each group. LOD, limit of detection. Error bars are standard error of the mean. \*\*\* ( $P < 0.001$ ), significance between AVLP-EGFP and vaccine groups.

infection. These results are consistent with previous reports demonstrating that cell-mediated immunity is important for protection and for clearing an infection, and can be explored for RSV vaccine development. Interestingly, mice vaccinated with the AVLP-F vaccine had a higher number of IFN- $\gamma$  secreting cells compared to natural RSV A2 infection and mice vaccinated with PIV5-RSV-F (SH/HN). The result that an AVLP-based vaccine generates more robust cellular immune responses than RSV A2 infection or PIV5-RSV-F (SH/HN) vaccination indicate that AVLP is an excellent

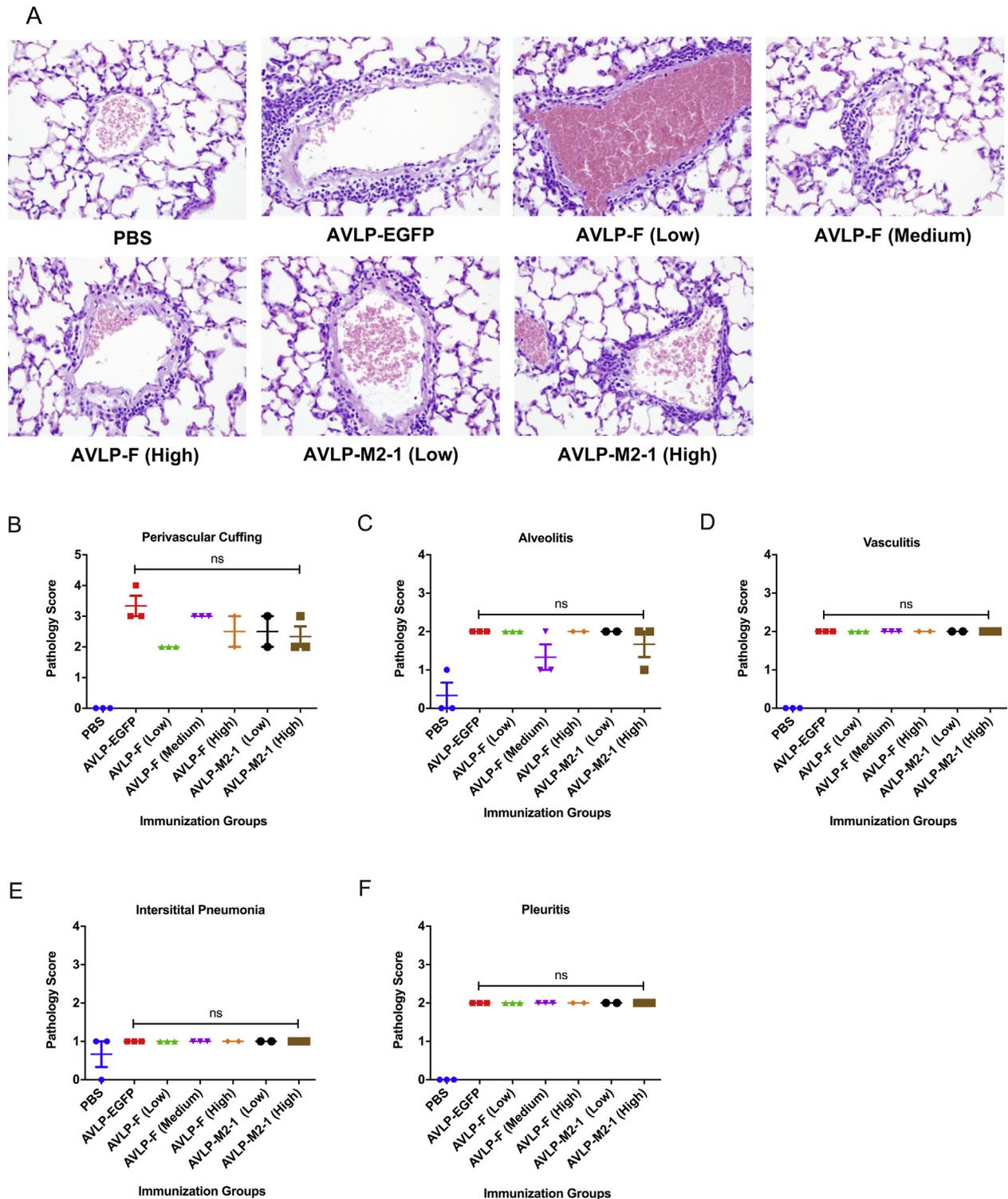
system to explore for vaccine development when cellular immune responses are critical for protection such as a cancer vaccine.

Here we showed that a single high-dose of AVLP-F was able to provide sterilizing immunity against RSV infection. When we compared the single low-dose AVLP-F data to the two-dose data we see a boosting effect in protection, even though we do not see a significant difference in the immune responses. Mice vaccinated with a single low-dose of AVLP-F showed over a 2-log reduction in lung viral titers compared to the control group. Mice that received two low-doses of AVLP-F achieved complete protection against RSV infection, similar to the RSV A2-vaccinated group. These data demonstrate that even though a single low-dose of AVLP-F significantly reduces lung viral titers, boosting with the same low-dose will help achieved sterilizing immunity in RSV infected mice. The advantage of single dose is that it can be administrated once without requiring a second visit. However, downside of single dose is that it requires a much higher dose. Prime-boost requires two visits to get immunized. The advantage is that a much lower dose can be used in each inoculation.

Since RSV vaccination has been associated with the development of enhanced disease upon natural infection with RSV, histopathology was performed to determine if the vaccine candidates produced enhanced lung pathology. Immunization with either AVLP-F or AVLP-M2-1 did not cause enhanced lung pathology in mice after RSV challenge, with pathology scores in all immunization groups being similar to the control group.

Multiple immune factors are associated with RSV disease. These include excessive inflammatory response, robust CD8<sup>+</sup> T cell responses, and Th2-biased CD4<sup>+</sup> T cell responses [25]. The low levels of histopathology seen in this study were likely a result of the host immune response induced by the vaccines, not necessarily the lung viral loads. As mentioned above, pathology scores had an approximate average of 2, if not lower, for most parameters, suggesting that the AVLP-based vaccines did not induce enhanced disease upon RSV challenge. However, mouse is not an ideal model for studies of RSV enhanced diseases. Further studies of AVLP safety using cotton rats will be needed.

Since RSV F is the major protective antigen, most vaccine candidates for RSV are developed using this protein as the antigen. Other



**Fig. 9.** High-magnification images post-challenge lung lesions in mice immunized with single-doses of PBS, AVLP-EGFP, AVLP-F, or AVLP-M2-1 vaccine candidates. Six-to-eight week old BALB/c mice were immunized with  $4 \times 10^5$  AP of AVLP-EGFP,  $1 \times 10^5$  AP,  $4 \times 10^5$  AP, or  $1.3 \times 10^6$  AP of AVLP-F,  $1 \times 10^5$  AP or  $4 \times 10^5$  AP of AVLP-M2-1, or PBS in 100- $\mu$ L volumes. Mice were challenged with  $5 \times 10^5$  PFU of RSV A2 at 32 days post-immunization. Four days later, lungs were collected for histology. Lungs were fixed in 10% formalin, processed, and sections were stained with hematoxylin and eosin. (A) High-magnification images of lungs showing that inflammation was predominantly characterized by perivascular cuffing. (B–F) Lung sections were scored on a scale of 0 to 4 for (B) perivascular cuffing, (C) alveolitis, (D) vasculitis, (E) interstitial pneumonia, and (F) pleuritis. Groups consisted of 2 to 3 mice each. Horizontal lines represent the mean value in each group. Error bars are standard error of the mean. ns, significance between AVLP-EGFP and vaccine groups.

groups have also used the RSV M2-1 protein as a vaccine antigen because of its ability to induce cell-mediated immune responses [26,27]. One group created two different viral-vectored vaccines for RSV using chimpanzee Adenovirus (PanAd3-RSV) and Modified

Vaccinia Ankara RSV (MVA-RSV) as vectors. Both of these vaccines encoded the F, N, and M2-1 proteins to induce both humoral and cellular immune responses. Like the AVLP-M2-1 vaccine candidate described here, both the PanAd3-RSV and MVA-RSV vaccine

candidates induced antigen-specific T-cell responses, and a single-dose immunization with PanAd3-RSV protected mice against RSV challenge [26]. Unlike Pierantoni et al., who used RSV F, N and M2-1 as antigens for their vaccine, the PIV5-AVLP system protected mice against RSV challenge using only RSV M2-1 as an antigen. While this demonstrates that PIV5-AVLP-based vaccine is quite robust, we can further improve efficacy of candidate vaccines by expressing more than one antigen. We can also improve the PIV5-AVLP vaccine candidates by combining the AVLP-F and AVLP-M2-1 vaccines and administering them as a multi-valent vaccine, or by making an AVLP expressing both RSV F and M2-1. The addition of RSV N as an antigen for vaccines has been demonstrated to be protective against RSV challenge [26,28,29]. We can improve our PIV5-AVLP vaccines by incorporating an RSV N vaccine in the multi-valent approach, or combine RSV F, M2-1, and N in a single AVLP.

It is known that the assembly and budding machinery for PIV5 (M, F, HN, and NP) are necessary for VLP production [15,16]. Transfection of plasmids encoding these proteins into cells can lead to the production of VLPs to similar levels as a PIV5 infection [15], indicating the possibility of optimizing our PIV5-AVLP production system to achieve high titers every time AVLPs are produced. It is interesting that RSV F was not detected in the AVLP-F particles. Previously, HA of influenza A virus was readily detected in AVLP expressing HA (AVLP-HA) particles [21]. We speculate that small amount of RSV F was incorporated into AVLP-F particles and this low level of F incorporation was not detectable in our current system and using our existing techniques. We can improve our PIV5-AVLP vaccine candidates by increasing the rate of incorporation of the antigen into the AVLPs. This can be done by substituting the cytoplasmic domain of RSV F with the cytoplasmic domain of PIV5 F, since it has been shown to play a critical role in virus assembly and budding, along with the cytoplasmic tail of PIV5 HN [30,31].

Comparing to live PIV5-based vector system, PIV5 AVLP-based system does not produce replication competent progeny, reducing anxiety on safety of live viral vector. Furthermore, it is possible that PIV5-based AVLPs can be pseudotyped by different viral glycoproteins, affording this system the versatility a system like the adenovirus-based platform is incapable of.

In summary, both PIV5-AVLP-based RSV vaccines described here show great potential as effective RSV vaccines. They both generated robust cellular immune responses and prevented RSV infection. Because of the lower dose required for protection, their production will be less of an issue for mass immunization. This work also validates the use of the PIV5-AVLP system as a new and exciting platform for vaccine development.

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## Conflict of interest

B.H. is the inventor of a patent application filed by the University of Georgia Research Foundation on AVLP.

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