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Venezuelan Equine Encephalitis Virus Replicon Particles Encoding Respiratory Syncytial Virus Surface Glycoproteins Induce Protective Mucosal Responses in Mice and Cotton Rats▽

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Respiratory syncytial virus (RSV) is an important viral pathogen that causes severe lower respiratory tract infection in infants, the elderly, and immunocompromised individuals. There are no licensed RSV vaccines to date. To prevent RSV infection, immune responses in both the upper and lower respiratory tracts are required. Previously, immunization with Venezuelan equine encephalitis virus replicon particles (VRPs) demonstrated effectiveness in inducing mucosal protection against various pathogens. In this study, we developed VRPs encoding RSV fusion (F) or attachment (G) glycoproteins and evaluated the immunogenicity and efficacy of these vaccine candidates in mice and cotton rats. VRPs, when administered intranasally, induced surface glycoprotein-specific virus neutralizing antibodies in serum and immunoglobulin A (IgA) antibodies in secretions at the respiratory mucosa. In addition, fusion protein-encoding VRPs induced gamma interferon (IFN-γ)-secreting T cells in the lungs and spleen, as measured by reaction with an *H-2K*^{d}-restricted CD8$^+$ T-cell epitope. In animals vaccinated with F protein VRPs, challenge virus replication was reduced below the level of detection in both the upper and lower respiratory tracts following intranasal RSV challenge, while in those vaccinated with G protein VRPs, challenge virus was detected in the upper but not the lower respiratory tract. Close examination of histopathology of the lungs of vaccinated animals following RSV challenge revealed no enhanced inflammation. Immunization with VRPs induced balanced Th1/Th2 immune responses, as measured by the cytokine profile in the lungs and antibody isotype of the humoral immune response. These results represent an important first step toward the use of VRPs encoding RSV proteins as a prophylactic vaccine for RSV.

Respiratory syncytial virus (RSV) is a major human pathogen that causes serious lower respiratory tract illness in infants and the elderly. Significant morbidity and mortality for RSV are especially common in certain high-risk pediatric populations such as premature infants and infants with congenital heart or lung disorders. RSV bronchiolitis in infants is associated with recurrent wheezing and asthma later in childhood (53, 76). There are currently no FDA-approved vaccines for prevention of RSV disease by active immunization. Immunoprophylaxis by passive transfer of a humanized murine RSV fusion (F) protein-specific antibody is licensed for much of the high-risk infant population but is not cost-effective in otherwise healthy infants, who represent the majority of those hospitalized with RSV. There is also a high rate of RSV reinfection during childhood, which suggests that a protective immune response to a vaccine may need to differ either quantitatively or qualitatively from that induced by natural infection.

Previous attempts to develop RSV vaccines have faced significant obstacles. An experimental formalin-inactivated RSV vaccine in the 1960s induced exacerbated disease and death in some vaccinated children during subsequent natural infection. It was shown subsequently that the formalin-inactivated RSV vaccine induced serum antibodies with poor neutralizing activity in infants (50) and an atypical Th2-biased T-cell response associated with enhanced histopathology following experimental immunization in small animals (58, 68). Treatment of RSV antigens with formaldehyde modifies the protein with carbonyl groups, which preferentially induces Th2-type responses and leads to enhanced disease (47). Other attempts to generate RSV vaccines include using live-attenuated cold-adapted, temperature-sensitive mutant strains of RSV (10, 12–17, 22, 32, 39, 41, 42), protein subunit vaccines coupled with adjuvant (30, 56, 70, 73), and RSV proteins expressed from recombinant viral vectors, including vaccinia virus (52, 75), adenovirus (31), vesicular stomatitis virus (37), Semliki Forest virus (8), bovine/human parainfluenza virus type 3 (26), Sendai virus (64), and Newcastle disease virus (45). Although some of these vaccines showed promising preclinical data, no vaccine has been licensed for human use due to safety concerns and lack of efficacy data. RSV vaccines under development have not been

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tested in efficacy trials. In addition, many of these vaccines face significant hurdles when they are introduced into very young infants, who are one of the principal target populations for RSV vaccines. Infants have circulating maternal antibodies against RSV and against most of the candidate viral vectors, which likely would cause a blunting of the efficacies of these vaccines in vivo.

The two surface glycoproteins of RSV, F protein and attachment (G) protein, are the major antigenic targets for neutralizing antibodies. Serum neutralizing antibodies in high titer are sufficient to protect the lower respiratory tract (9). F and G proteins, therefore, have been used separately or in combination in many experimental RSV vaccines. Immunization with purified F protein alone or F protein expressed from a recombinant viral vector such as vaccinia virus induces RSV-specific neutralizing antibodies, $CD8^+$ cytotoxic T lymphocytes, and protection against subsequent RSV challenge in mice or cotton rats (52). Vaccination with G protein alone, however, often induces only partial protection against RSV challenge. In mice, the immune response against G is associated with eosinophilia and the induction of Th2-type $CD4^+$ lymphocytes in some experiments (27, 35, 65).

A key determinant for optimal vaccination against respiratory viruses, such as RSV, is the ability of the vaccine to induce mucosal immunity. This goal can be achieved by using a mucosal route for vaccination or possibly by use of a vaccine construct that preferentially induces mucosal responses. Protection in the upper respiratory tract has been demonstrated in several animal models (22, 51) and in humans (42) following immunization by the intranasal (i.n.) route and has been linked to the induction of virus-specific mucosal immunoglobulin A (IgA) antibodies.

Venezuelan equine encephalitis (VEE) virus is an RNA virus of the *Togaviridae* family. Virus replicon particles (VRPs) are defective nonpropagating VEE particles developed by Pushko et al. in 1997 (60). VRPs have been used successfully and safely in immunization and challenge studies for a wide range of viral and bacterial pathogens in animal model systems (2, 4, 24, 28, 29, 36, 43, 59, 60, 63, 69, 71). More importantly, these particles induce mucosal immune responses after nonmucosal inoculation in animals (18, 28) and confer protection to the primary mucosal target tissue (25; E. M. Richmond, K. W. Brown, N. L. Davis, and R. E. Johnston, unpublished results). VEE virus is also known to be infectious by aerosol and intranasal (i.n.) routes, which would allow the VRPs to access target cells to induce an immune response (6, 7, 33).

VRPs contain a modified positive-sense RNA viral genome designed to express the VEE nonstructural replicase proteins, but no VEE structural proteins, as the structural protein genes have been replaced by the gene encoding the heterologous antigen. These particles are produced in a cellular packaging system in which structural proteins are supplied in *trans* and only the modified viral genome is packaged into an intact VRP. The resulting replicons express high levels of antigens in infected cells and induce humoral and cellular immune responses in vivo (60). Moreover, these replicons are potential vaccine vectors for use in very young infants, since they display VEE viral coat proteins and thus are not neutralized by maternal RSV antibodies. Other advantages of using VRPs over other viral vaccines include the lack of preexisting immunity to VEE in the target populations and their systemic and mucosal adjuvant activities (67).

Here, we tested whether VEE replicon vaccine candidates could induce effective mucosal protection against RSV following i.n. immunization in BALB/c mice or cotton rats. These two animal models had previously been shown to be semipermissive to RSV infection. BALB/c mice were used to delineate the underlying mechanism of vaccine-enhanced RSV disease, and cotton rats were used in preclinical testing for their ability to allow RSV replication to high titers. Combination of the results from these animal models allowed us to compare directly the immune responses induced by the vaccine to those induced by natural infection, both quantitatively and qualitatively, and to look at the ability of those responses to inhibit viral replication in both the upper and lower respiratory tracts. In this study, we found that VRPs encoding the RSV F protein induced both systemic and mucosal antibody responses. These VRPs also induced antigen-specific T cells in both the lungs and spleens of immunized animals. The T-cell response was Th1/Th2 balanced, and aggravated histopathology was not observed. In addition, following i.n. challenge of these animals with wild-type RSV, virus replication was below the level of detection. In contrast, animals vaccinated with VRPs encoding the RSV attachment protein G showed challenge virus replication in the upper but not the lower respiratory tract. These findings provide proof-of-principle that VEE VRPs expressing the RSV F protein can be used to prevent RSV infection.

MATERIALS AND METHODS

Animals and cell lines. Specific-pathogen-free 5- to 6-week-old BALB/c mice and cotton rats were purchased from Harlan (Indianapolis, IN). Animals were housed in microisolator cages throughout the study. All experimental procedures performed were approved by the Institutional Use and Care of Animals Committee at Vanderbilt University Medical Center.

HEp-2 cells were obtained from ATCC (CCL-23) and maintained in Opti-MEM medium (Invitrogen, CA) supplemented with 2% fetal bovine serum, 4 mM L-glutamine, 5 μg/ml amphotericin B, and 50 μg/ml gentamicin sulfate at 37°C with 5% CO_2.

VEE constructs and generation of VRPs containing RSV F or G genes. The method for construction and packaging of VRPs was described previously (18). Heterologous genes were inserted into a VEE-based replicon, pVR21, which was derived from mutagenesis of a cDNA clone of the Trinidad donkey strain of VEE. The RSV F and G sequences used were based on the previously published low-passage RSV strain A2/HEK-7 sequence (3, 10) or human metapneumovirus (MPV) F genes from the MPV A2 strain (GenBank accession no. EF051124). Optimized sequences were cloned into pVR21 downstream of the subgenomic 26S promoter via a two-step PCR and ligation process. First, a region of pVR21 DNA was PCR amplified with primers to generate amplicons that included a unique 5′ SwaI restriction site and the 26S mRNA leader at the 3′ end of the amplicon. Second, the RSV F, G, or MPV F gene was PCR amplified to obtain amplicons that contained the 26S mRNA leader at the 5′ end, the heterologous gene, and a PacI restriction site at the 3′ end. The two amplicons then were used as a template for a third PCR using a forward primer hybridizing to the pVR21 amplicon and a reverse primer hybridizing to the RSV F, G, or MPV F amplicon. This PCR generated an overlapping fragment that spanned the 26S promoter leader sequence and the RSV F, G, or MPV F sequence and that contained the unique 5′ SwaI and 3′ PacI restriction sites that could be directionally ligated back into a digested pVR21 plasmid.

For generation of VRPs, capped RNA transcripts of pVR21 containing the RSV F or G or MPV F gene were generated in vitro with the mMESSAGE mMACHINE T7 kit (Ambion, Austin, TX). Similarly, helper transcripts that contained the VEE capsid and glycoprotein genes were generated in vitro. Baby hamster kidney (BHK) cells then were cotransfected by electroporation with 30 μg of pVR21 and helper RNAs, and culture supernatants were harvested at 30 h after transfection. VRPs were partially purified and concentrated by pelleting

through 20% (wt/vol) sucrose in phosphate-buffered saline (PBS) and then resuspended in endotoxin-free PBS.

VRP titration. Serial dilutions of VRPs encoding RSV F (designated VRP-RSV.F) or RSV G (designated VRP-RSV.G) were used to infect BHK cells in eight-chamber slides (Nunc) for 20 h at 37°C. Infected BHK cells were fixed and immunostained for VEE nonstructural proteins. Infectious units then were calculated from the number of stained cells per dilution and converted to infectious units (IU) per milliliter. A typical yield of VRPs was 1×10^9 IU/ml.

Western blot. BHK cells were infected at a multiplicity of infection (MOI) of 5 with VRP-RSV.F, VRP-RSV.G, or VRP-MPV.F for 24 h at 37°C. Infected BHK cells were washed twice with ice-cold PBS and scraped into microcentrifuge tubes. The cells were pelleted for 10 s at 6,000 rpm and lysed in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.5% [vol/vol] protease inhibitor cocktail, pH 8.0) (Sigma, St. Louis, MO) for 10 min on ice. The resulting cell lysates then were cleared from debris by centrifugation at 13,000 rpm for 5 min.

Proteins were separated by electrophoresis using a NuPAGE 4 to 12% Bis-Tris gel (Novex) and transferred onto an Invitrolon polyvinylidene difluoride membrane (Invitrogen). The membrane was blocked with Tris-buffered saline with 0.05% Tween 20 (TBST)–5% nonfat dry milk at 4°C overnight. The blot then was washed and stained for the presence of RSV F or RSV G proteins with mouse monoclonal antibodies (1:1,000 dilution of RSV F [clone mab19] or RSV G [clone SL1860] antibodies in TBST–1% nonfat dry milk) for an hour at room temperature. After the primary antibody incubation, secondary goat anti-mouse horseradish peroxidase (HRP)-conjugated antibodies (1:5,000 dilution in TBST–1% nonfat dry milk) were added. The blot was washed again with TBST after a 1-h incubation and developed using SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL).

Immunofluorescence staining. BHK cells were infected at an MOI of 5 with VRP-RSV.F or VRP-RSV.G in eight-chamber slides (Nunc) for 24 h at 37°C. Infected BHK cells were fixed in 80% methanol for an hour at 4°C. The cells then were blocked with PBS–3% bovine serum albumin (BSA) for 2 h at room temperature. Primary antibodies against RSV F or RSV G (1:1,000 dilution in PBS–1% BSA) were added and allowed to incubate for an hour at room temperature. Cells were washed twice with TBST after the primary antibodies incubation. Secondary goat anti-mouse AlexaFluor C568-conjugated antibodies were added (1:1,000 dilution in TBST–1% BSA) to the cells for an additional hour. The slide then was washed with TBST and mounted with Prolong antifade medium (Invitrogen). The slide was visualized under a LSM510 inverted laser-scanning confocal microscope (Carl Zeiss Microimaging, Thornwood, NY).

Vaccination and challenge of mice or cotton rats. BALB/c mice were anesthetized with isoflurane by inhalation and vaccinated i.n. with various titers of VRP-RSV.F or VRP-RSV.G in a 100-μl inoculum. Some groups of BALB/c mice were injected with 10^6 IU of VRP-RSV.F intraperitoneally (i.p.) or intramuscularly (i.m.). Control groups were inoculated with PBS, 5×10^5 PFU of RSV wild-type strain A2, or 10^6 IU of VRP-MPV.F via the same route. Mice that were vaccinated with VRPs were boosted with the same dose 2 and 4 weeks later. The mice were observed for clinical signs daily and bled at 14-day intervals to follow immune responses.

Twenty-eight days after the third immunization, mice from all groups were challenged with 5×10^5 PFU of RSV wild-type strain A2 i.n. To monitor virus replication in the upper and lower respiratory tracts, nasal turbinates and lungs were harvested on day 4 postchallenge and subsequently assayed for virus titer for each animal. The mean values then were calculated for each experimental group. Similarly, cotton rats were vaccinated on day 0 and day 14 with 10^6 IU of VRP-RSV.F or VRP-RSV.G i.n. in groups of four. Control groups were vaccinated with PBS, 5×10^5 PFU of RSV A2, or 10^6 IU of VRP-MPV.F. They then were bled on day 35 to monitor immune responses and were challenged with 5×10^5 PFU of RSV A2 on day 42 and sacrificed on day 46. Lung and nasal turbinates were harvested separately and homogenized to determine viral titers for each individual animal. The mean values then were calculated for the group.

BAL fluid and nasal wash collection. A subset of animals (BALB/c mice or cotton rats) was sacrificed on day 56 (28 days postvaccination) to collect bronchoalveolar lavage (BAL) fluids and nasal washes. BAL fluids were collected by ligation of the trachea with suture and insertion of a 23-gauge blunt needle into the distal trachea, followed by three in-and-out flushes of the airway with 1 ml of sterile PBS. Nasal washes were obtained by flushing 3 ml PBS through the upper trachea and out the nasal orifice into a sterile receptacle. Both BAL and nasal washes were concentrated 10-fold using 10-kDa molecular mass-cutoff Centricon concentrators (Millipore, Bedford, MA).

Collection of splenocytes and lung lymphocytes. Spleens were harvested from vaccinated and control mice 14 days after the second VRP boosting. Spleens were placed in RPMI medium supplemented with 10% fetal bovine serum, 10 mM HEPES buffer, 2 mM L-glutamine, 0.5 mg/ml gentamicin, and 50 mM 2-mercaptoethanol (designated "complete RPMI"). The spleens were minced and ground gently through cell strainers (Becton-Dickinson, San Jose, CA) to obtain single-cell suspensions. The cells then were lysed with red blood cell lysing buffer (Sigma-Aldrich, St Louis, MO) and washed with complete RPMI before use. Lungs were excised and washed in PBS once. The lungs were placed in complete RPMI, minced, ground, and passed through cell strainers. The resulting suspensions were underlaid with Ficoll gradient and centrifuged at 1,000 rpm for 10 min. Buffy coats then were removed, and lymphocytes were counted.

RSV F protein-specific ELISA. For the RSV F protein-specific enzyme-linked immunosorbent assay (ELISA), sera collected at day 14, 28, or 42 were tested for the presence of F protein-specific antibodies. Concentrated nasal washes and BAL fluids also were tested. Briefly, 150 ng of purified recombinant soluble RSV F protein expressed from mammalian cells was adsorbed onto Immulon 2B plates overnight in carbonate buffer (pH 9.8) at 4°C. The plate then was blocked with 1% BSA in PBS for 2 h at room temperature. After thorough washing with TBST–1% BSA, a 1:1,000 dilution of serum, concentrated nasal wash, or concentrated BAL fluid samples were added to the plate and allowed to incubate for an hour at room temperature. The plates were washed again, and HRP-conjugated anti-mouse IgA (1:500 dilution), IgG (1:5,000 dilution), IgG1 (1:500 dilution), or IgG2a (1:500 dilution) antibodies were added (Southern Biotech, Birmingham, AL) and allowed to incubate for another hour. Finally, the plate was washed and 100 μl of One-Step Turbo tetramethylbenzidine peroxidase substrate (Pierce, Rockford, IL) was added per well to quantify the relative amounts of F-specific IgA, IgG, IgG1, or IgG2a in the samples. The reactions then were stopped by adding 50 μl of 1 M HCl, and the absorbances of the samples were read at 450 nm. The amounts of IgG1 and IgG2a were calculated by interpolating experimental optical density at 450 nm (OD_{450}) readings onto curves determined by using purified IgG1 or IgG2a standard preparations of known concentration, and the ratios were determined by dividing the mass of IgG1 by that of IgG2a.

Neutralizing antibody assay. Serum samples were tested for the presence of RSV neutralizing antibodies. Briefly, a viral suspension that was standardized to yield 50 plaques per well in HEp-2 cell monolayer cultures was used. An aliquot of the RSV suspension was incubated with serial dilutions of the serum samples. After an hour, the suspension was absorbed onto HEp-2 cells and then overlaid an hour later with a semisolid methylcellulose overlay. After 5 days, the cell culture monolayers were fixed and stained by immunoperoxidase using anti-F monoclonal antibodies to identify plaques. Plaques were counted, and plaque reduction was calculated by regression analysis to provide a 60% plaque reduction titer.

Viral plaque titer assay. Serial dilutions of nasal turbinates or lung homogenates were inoculated onto HEp-2 cell monolayer cultures, and plaque assays were performed as described above.

ELISPOT assay. Gamma interferon (IFN-γ)-secreting T cells were quantified in an enzyme-linked immunospot (ELISPOT) assay. Briefly, 1 μg of anti-mouse IFN-γ capture antibody per well was adsorbed onto methanol-activated Millipore ELLIP 10SSP multiscreen plates overnight at 4°C. The plates then were washed three times with PBS and blocked with complete RPMI for 2 h at room temperature. Peptides that correspond to a known major histocompatibility complex (MHC)-restricted RSV F protein epitope (KYKNAVTEL), RSV G protein epitope (WAICKRIPNKKPGKK), or unrelated influenza virus nucleocapsid protein epitope (TYQRTRALV) were added into each well in a 50-μl volume. Freshly isolated splenocytes or lung lymphocytes then were added at a concentration of 2×10^5 cells per well in 50 μl complete RPMI in duplicate. The plates were incubated for 20 h at 37°C in 5% CO_2 before harvest. On the day of harvest, the plates were washed three times with PBS-Tween and 0.2 μg of biotinylated anti-IFN-γ antibodies in PBS was added to each well, followed by a 3-h incubation at room temperature. Plates were washed again before the addition of 100 μl of avidin-peroxidase complex (Vector Laboratories, Burlingame, CA). Plates were washed after an hour at room temperature, and 100 μl of AEC substrate (Sigma, St. Louis, MO) was added to the plate. The substrate was allowed to incubate for 4 min at room temperature before the plates were rinsed in cold tap water. The plates then were air dried overnight before spots were counted by an automatic reader (Cellular Technology, Cleveland, OH) and expressed as the number of IFN-γ-expressing cells per 10^6 cells.

Histology. Four days after RSV challenge, mice were euthanized by CO_2 inhalation and lungs were harvested. To preserve structural integrity of the lungs, 1 ml of 10% neutral buffered formalin was instilled into the lungs via tracheotomy, followed by ligation of the trachea with suture. The whole lung then was immersed in 10% neutral buffered formalin overnight. After fixation, the lungs were dehydrated by immersion in 70% ethanol for another day. The lungs then were embedded in paraffin, sectioned, and stained with hematoxylin/eosin or periodic-acid Schiff reaction mixture for detection of mucin. The severity of inflammation was evaluated separately for the alveolar and peribronchial tissue

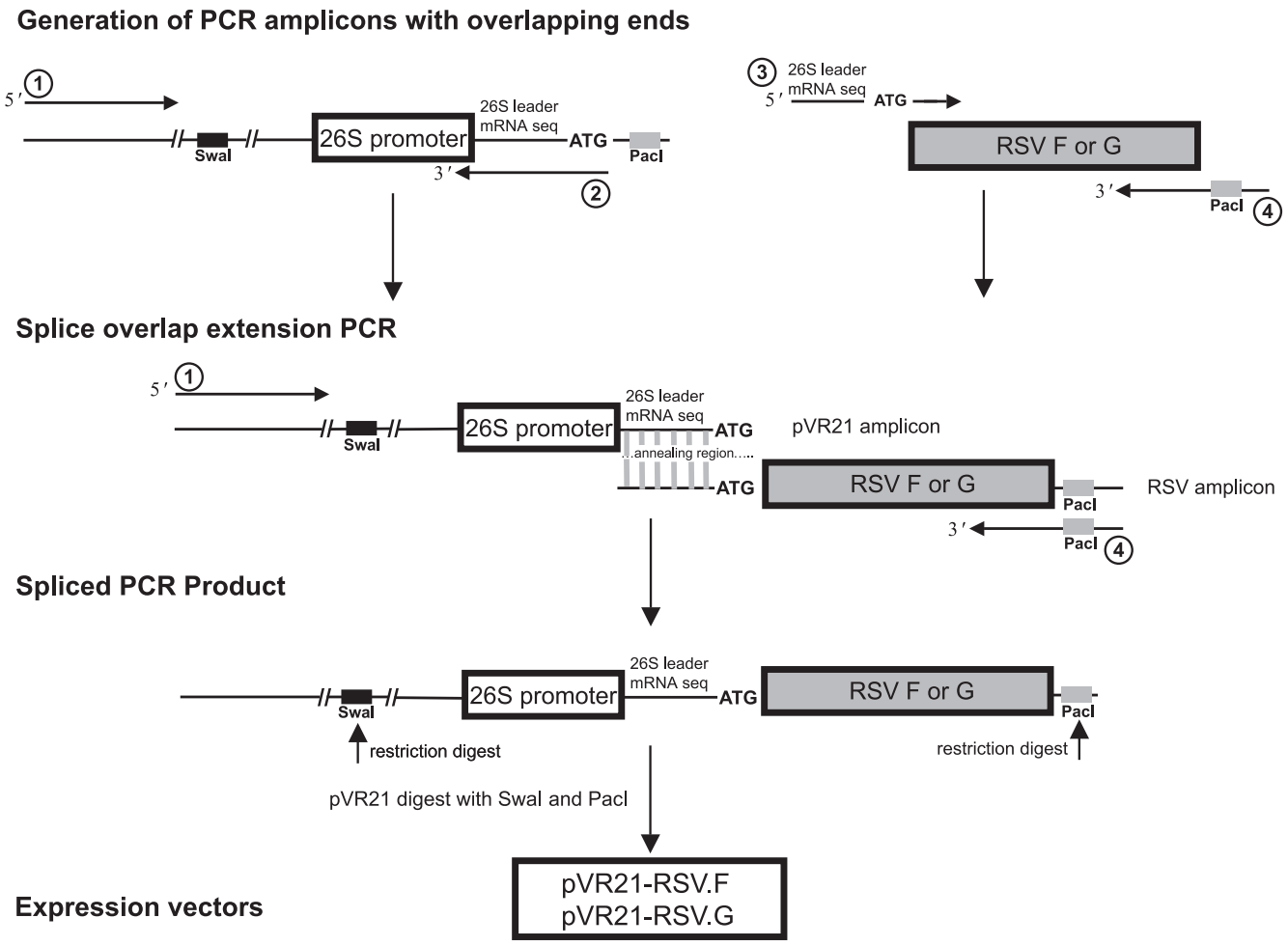

FIG. 1. Construction of VEE expression vector. RSV.F and RSV.G open reading frames were cloned into the VEE vector pVR21 via several steps. First, the VEE subgenomic 26S promoter was PCR amplified from pVR21 to generate amplicons that included the 26S mRNA leader sequence on the 3′ end. Second, RSV F or G amplicons were generated with a 26S leader mRNA sequence on the 5′ end. The two amplicons then were amplified to generate overlapping PCR products that contained RSV F or G genes under the control of the VEE subgenomic 26S promoter. Finally, the spliced PCR products were each cloned back into pVR21 using unique restriction enzyme sites, SwaI and PacI, to produce pVR21-RSV.F or pVR21-RSV.G. Numbers in circles denote primers used in each PCR.

and perivascular spaces in a group-blind fashion. The degree of inflammation in the alveolar tissue was graded as follows: 0, normal; 1, increased thickness of the interalveolar septa (IAS) by edema and cell infiltration; 2, increased thickness of IAS with presence of luminal cell infiltration; 3, abundant luminal cell infiltration; and 4, inflammatory patches formed. The degrees of inflammation in the peribronchial and perivascular spaces were graded as follows: 0, no infiltrate; 1, slight cell infiltration noted; 2, moderate cell infiltration noted; and 3, abundant cell infiltration noted. In each tissue section, 10 alveolar tissue fields, 10 airways, and 10 blood vessels were analyzed at a ×200 magnification. Mean scores were calculated for each mouse.

Cytokine gene expression and RSV genome detection in the lungs after RSV challenge. Lungs from unvaccinated or vaccinated mice were harvested 4 days after RSV challenge and placed into RNeasy RNA tissue lysis buffer (QIAGEN). The tissues were homogenized, and RNAs were extracted according to the manufacturer's protocol. Primers and probes were purchased from Applied Biosystems (Foster City, CA) to measure mRNA for Th1 or Th2 cytokines based on GenBank sequences for murine glyceraldehye-3-phosphate dehydrogenase (GAPDH), IFN-γ, and interleukin-2 (IL-2), IL-4, IL-5, IL-10, and IL-12. RSV primers and probes were used to detect the RSV F gene as previously described (46). Probes were labeled at the 5′ end with 6-carboxyfluorescein (FAM) and at the 3′ end with the nonfluorescent quencher Blackhole Quencher 1 (BHQ1; Operon Biotechnologies, Huntsville, AL). Reverse-transcribed real-time PCR was performed using a Quantitect probe RT-PCR kit (QIAGEN, Valencia, CA) and a Smart Cycler II (Cepheid, Sunnyvale, CA) using 5 μl of extracted mRNA. The parameters used were 1 cycle of 50°C for 30 min, 1 cycle of 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. Reactions were performed in triplicate, with no template as a negative control. Relative amounts of cytokine gene mRNAs and RSV F RNA were determined by normalizing to the level of GAPDH mRNA, and uninfected mice were used as baseline controls. Differences in cytokine mRNA levels were computed by the $\Delta\Delta C_T$ method comparing infected to uninfected mice. Similarly, differences (fold) in RSV F genes were computed using the $2^{-\Delta\Delta CT}$ method comparing vaccinated to mock-vaccinated animals.

Statistics. GraphPad Prism software was used to analyze the data (GraphPad Software Inc., San Diego, CA). All data were expressed as the mean and standard error of the mean. Data also were analyzed by Mann-Whitney rank sum test to compare the data distribution between any two experimental groups.

RESULTS

Cloning and expression of RSV antigens using VEE VRPs. RSV.F and RSV.G genes were cloned into the pVR21 VEE replicon vector under the control of a subgenomic 26S promoter (Fig. 1). VRPs then were produced in BHK cells by

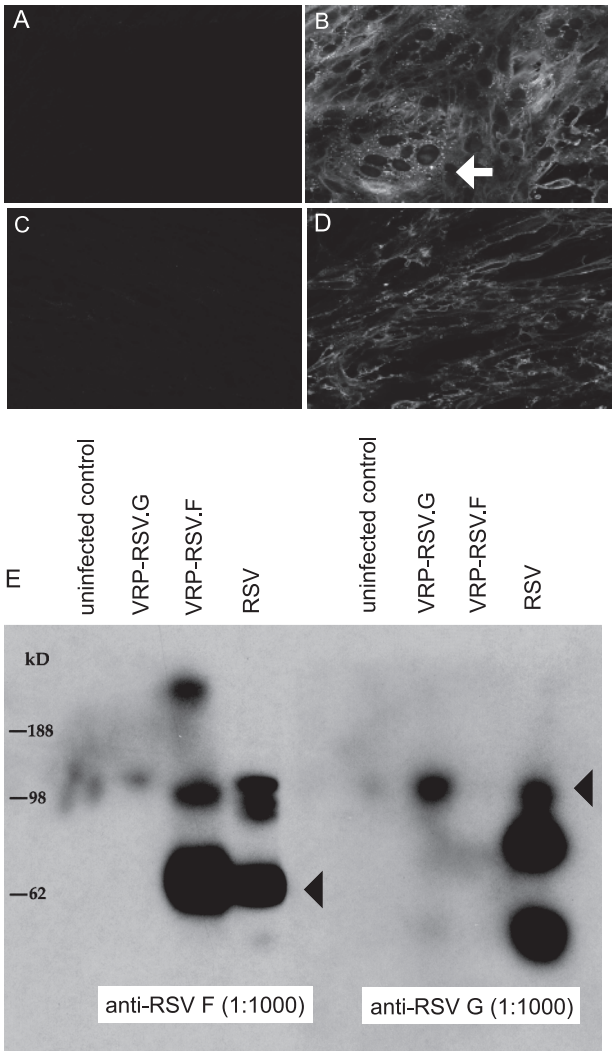

FIG. 2. Infection of BHK-21 cells with VEE replicon particles encoding RSV.F (VRP-RSV.F) or RSV.G (VRP-RSV.G) leads to robust protein expression. BHK cells were infected at an MOI of 5 with VRP-RSV.F or VRP-RSV.G. After 24 h, immunostaining was performed on uninfected (A) or VRP-RSV.F-infected (B) BHK-21 cells with RSV F-specific mouse monoclonal antibodies. Secondary AlexaFluor C568-conjugated goat anti-mouse antibodies were used for fluorescence labeling. A white arrow indicates fusion of multiple cells. Similar staining was performed with uninfected (C) or VRP-RSV.G-infected (D) BHK cells with RSV G-specific mouse monoclonal antibodies. (E) In addition, Western blotting was used to detect the presence of RSV F or G proteins in VRP-infected BHK-21 cell lysates. Western blots were probed with mouse monoclonal antibodies against RSV F or G proteins. Black arrowheads indicate the predicted apparent molecular masses of the proteins. Uninfected or RSV-infected cell lysates were used as negative or positive controls, respectively.

cotransfecting the replicon vector with plasmids encoding VEE capsid and structural proteins.

To ensure these replicons expressed the desired antigens, BHK cells were infected at an MOI of 5 with VRPs. Antigen expression then was measured by Western blotting and indirect immunofluorescence with RSV.F- or RSV.G-specific monoclonal antibodies. A robust amount of RSV F protein was expressed, as evident by the intense staining of BHK cells with anti-RSV F antibodies (Fig. 2B), compared to uninfected control cells (Fig. 2A). Examination by confocal microscopy revealed the formation of syncytia when RSV F proteins were expressed (Fig. 2B). RSV F expression also was confirmed by Western blotting of infected cell lysates, which showed a predicted band of RSV F at 60 kDa (Fig. 2E).

Similarly, cells infected with VRP encoding RSV.G expressed the predicted antigens when immunostained with anti-RSV G antibodies (Fig. 2D) and on Western blotting of cell lysates (Fig. 2E). Staining of cells infected with RSV.G VRP showed a membrane-bound pattern, which is consistent with previous reports of the distribution of G during RSV infection (54, 66). Large amounts of partially glycosylated and unglycosylated G were also observed in RSV lysates but were absent from BHK cells infected with VRP expressing RSV G.

Systemic IgG and mucosal IgA responses in VRP-vaccinated mice. To assess if VRPs could induce systemic humoral immune responses, we measured the amounts of RSV F-specific IgG antibodies in the serum of vaccinated mice by ELISA. Intranasal inoculation of VRPs induced significantly larger amounts of RSV F-specific IgG in the serum of vaccinated mice (1.4-fold higher) than in those infected once with RSV (Fig. 3A). Moreover, mucosal RSV F-specific IgA antibodies were detected in the nasal washes and BAL fluids, which reflect the presence of mucosal immunity in the upper and lower respiratory tracts of vaccinated animals, respectively (Fig. 3B and C).

Isotype profile of the serum IgG response. Formalin-inactivated RSV and subunit protein vaccines induce aberrant immune responses in naïve mice characterized by Th2-dominant cytokines and elevated IgG1/IgG2a ratios (72). The same Th2-dominant RSV response also has been noted in STAT1-deficient mice (21). We tested whether animals vaccinated with VRP-RSV.F exhibited a balanced response, as seen in those infected with wild-type RSV, or an aberrant response, as seen in RSV-infected STAT1-deficient mice. RSV-infected and VRP-RSV.F-vaccinated BALB/c mice exhibited a serum IgG profile characteristic of a balanced Th1/Th2 response, whereas STAT1-knockout mice showed the predicted atypical Th2-biased response. The ratio of IgG1 to IgG2a was fourfold lower for VRP-RSV.F-vaccinated and RSV-infected BALB/c mice than that for RSV-infected STAT1-deficient mice (Fig. 3D). A statistically significant difference between VRP-RSV.F-vaccinated and RSV-infected BALB/c mice was not detected.

Serum RSV neutralizing activity in VRP-vaccinated animals. The presence of neutralizing antibodies in the serum is an important parameter that has been correlated with protection of the lower respiratory tract against RSV infection (49, 57, 62). We therefore measured neutralizing activity of the sera from VRP-vaccinated mice and cotton rats using a 60% plaque reduction assay. The serum of mice vaccinated with PBS or VRP expressing MPV.F protein, a virus control expressing a heterologous transgene, did not possess any detectable neutralizing titer. Intranasal vaccination with VRP-RSV.F induced a 1.4- to 6.7-fold higher titer of serum neutralizing antibodies compared to mice infected with RSV. The increases in antibody titer were dose dependent and were significantly different in the 10^5 and 10^6 IU dose groups compared to the 10^4 IU dose group. VRP-RSV.G-vaccinated mice had a lower neutralizing titer than those vaccinated with VRP-RSV.F. At high dose, the

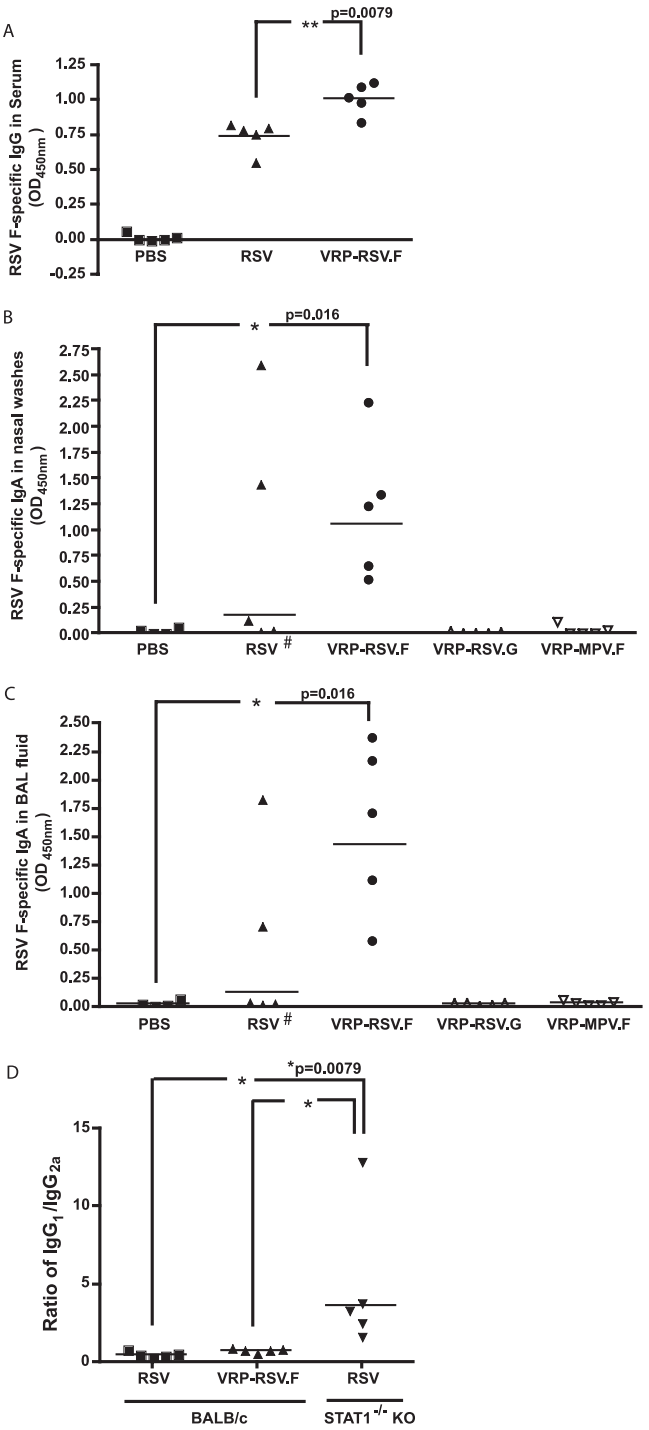

FIG. 3. VRP-RSV.F induces RSV-F-specific antibodies in the serum and mucosal secretions of VRP-vaccinated mice. BALB/c mice were vaccinated i.n. with 10^6 IU of VRP-RSV.F on days 0 and 14. (A) Sera from vaccinated mice were obtained 28 days post-second vaccination. RSV-F-specific ELISA was performed on diluted sera (1:1,000) with HRP-conjugated anti-mouse IgG antibodies. The amount of binding was determined from absorbance of HRP-substrate at $\lambda = 450$ nm (OD_{450nm}). Nasal washes (B) and BAL fluids (C) also were obtained from vaccinated mice 28 days post-second vaccination. The amounts of F-specific IgA antibodies were quantified similarly with HRP-conjugated anti-mouse IgA antibodies in an ELISA. #, two animals in the RSV-infected group did not make a detectable F-specific IgA response. (D) Sera from VRP-RSV.F-vaccinated mice were tested for the isotype of F-specific IgG1 and IgG2a antibodies. The ratios of IgG1 versus IgG2a were compared with sera from BALB/c or STAT1-deficient mice infected with 10^6 PFU of RSV A2. Each group in these experiments consisted of five animals. The horizontal lines in each panel indicate the geometric mean within each group.

neutralizing activity was comparable to that of the sera of RSV-infected mice, but the low dose of VRP-RSV.G did not induce any detectable responses (Fig. 4).

For cotton rats, i.n. vaccination with 10^6 IU of VRP-RSV.F induced a serum neutralizing activity of 1:210 compared to 1:170 from RSV-infected animals (see Table 2).

Kinetics of neutralizing activity after prime-boost immunization. We measured serum neutralizing antibody titers 2 weeks after each prime-boost vaccination. Animals were vaccinated with three doses of VRPs or infected with a single dose of RSV. As predicted, PBS-treated or VRP-MPV.F-vaccinated mice generated no detectable serum neutralizing titer (Fig. 5D). RSV-infected mice exhibited titers that peaked at day 28 postinfection and dropped gradually afterwards (Fig. 5A). VRP-RSV.F or VRP-RSV.G vaccination induced an increasing neutralizing titer after the first immunization, which peaked at 14 days after the first boost. Subsequent boosting did not enhance the level of neutralizing titer after the first boost, regardless of dosage (Fig. 5B and C). Therefore, a single prime boost was sufficient to generate effective neutralizing antibodies against RSV in vivo.

Cellular immunity in VRP-vaccinated mice. We performed an IFN-γ ELISPOT assay to detect RSV F- or G-specific T cells in the spleens or lungs of immunized animals. Lung lymphocytes and splenocytes were harvested separately 7 days after the second vaccination, stimulated in vitro with peptides representing known *H-2*^{d}-restricted RSV F (amino acids 85 to 93) or G (amino acids 183 to 197) CTL epitopes, and the numbers of IFN-γ-secreting cells were measured. The frequencies of RSV F-specific $CD4^+/CD8^+$ T cells were higher in the VRP-RSV.F-vaccinated group (ranging from 1,250 to 10,230 spots per 10^6 lung lymphocytes) than in the RSV-infected group (ranging from 1,285 to 3,180 spots per 10^6 lung lymphocytes) (Fig. 6A). Although two VRP-RSV.F-vaccinated animals showed significantly higher numbers of IFN-γ-secreting cells, than wild-type RSV-infected animals, the means for the two groups were not statistically different. The frequency of RSV F-specific $CD4^+/CD8^+$ T cells in the lungs was 10-fold higher than that in the spleen (Fig. 6B), and in this tissue, the mean number of IFN-γ-secreting cells in VRP-RSV.F-vaccinated mice showed an increase compared to the number in wild-type RSV-infected mice that was significant ($P = 0.0079$). The responses of splenocytes or lung lymphocytes to RSV G epitopes were low. The frequencies of RSV.G-specific $CD4^+/CD8^+$ T cells in RSV-infected mice averaged 418 or 20 spots per 10^6 lung lymphocytes or splenocytes, respectively (Fig. 6C and D). In the lungs, animals that were infected with RSV showed higher numbers of G protein-specific T cells than the VRP-RSV.G group. However, the mean values were not significantly different; we had low power to detect differences, based on the small sample size (Fig. 6C). Similarly, VRP-

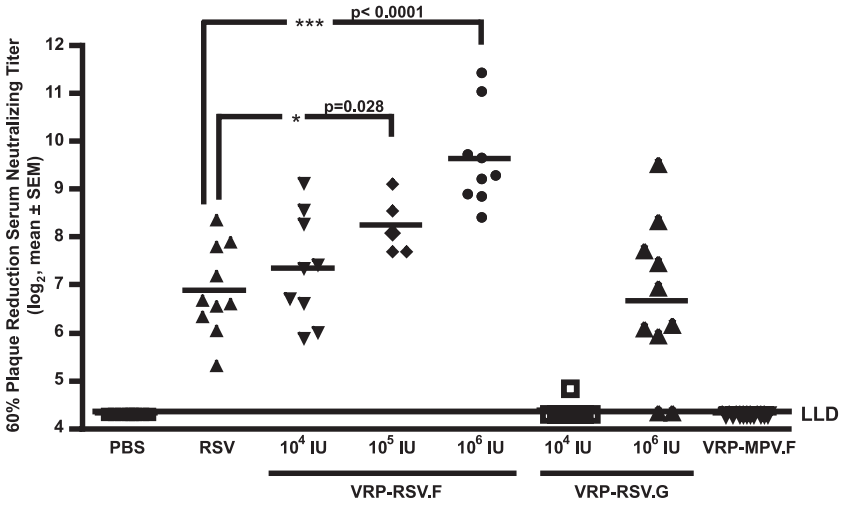

FIG. 4. VRP-RSV.F induced titers of RSV neutralizing antibodies in vaccinated mice equal to or higher than those of RSV infection or VRP-RSV.G vaccination. Naïve BALB/c mice were immunized i.n. with increasing doses of VRP-RSV.F (10^4, 10^5, or 10^6 IU) or VRP-RSV.G (10^4 or 10^6 IU) on days 0 and 14. Sera from vaccinated mice were tested for RSV neutralizing activity in a plaque reduction assay. Neutralizing activity is expressed as the geometric mean titer (GMT) of sera that neutralized 60% of plaques on RSV-infected HEp-2 cells and is denoted by a horizontal line drawn within a group. LLD, lower limit of detection.

RSV.G vaccination induced limited $CD4^+/CD8^+$ T-cell response in the spleen (Fig. 6D) (8).

Viral titer in lungs and nasal turbinates after challenge in vaccinated mice. To assess the protective efficacy of VRP vaccines in vivo, we measured the RSV titers in the lungs and nasal turbinates in mice and cotton rats following i.n. RSV challenge. Mice vaccinated with VRP-RSV.F showed no detectable challenge virus at any dosage tested (at least a 35-fold

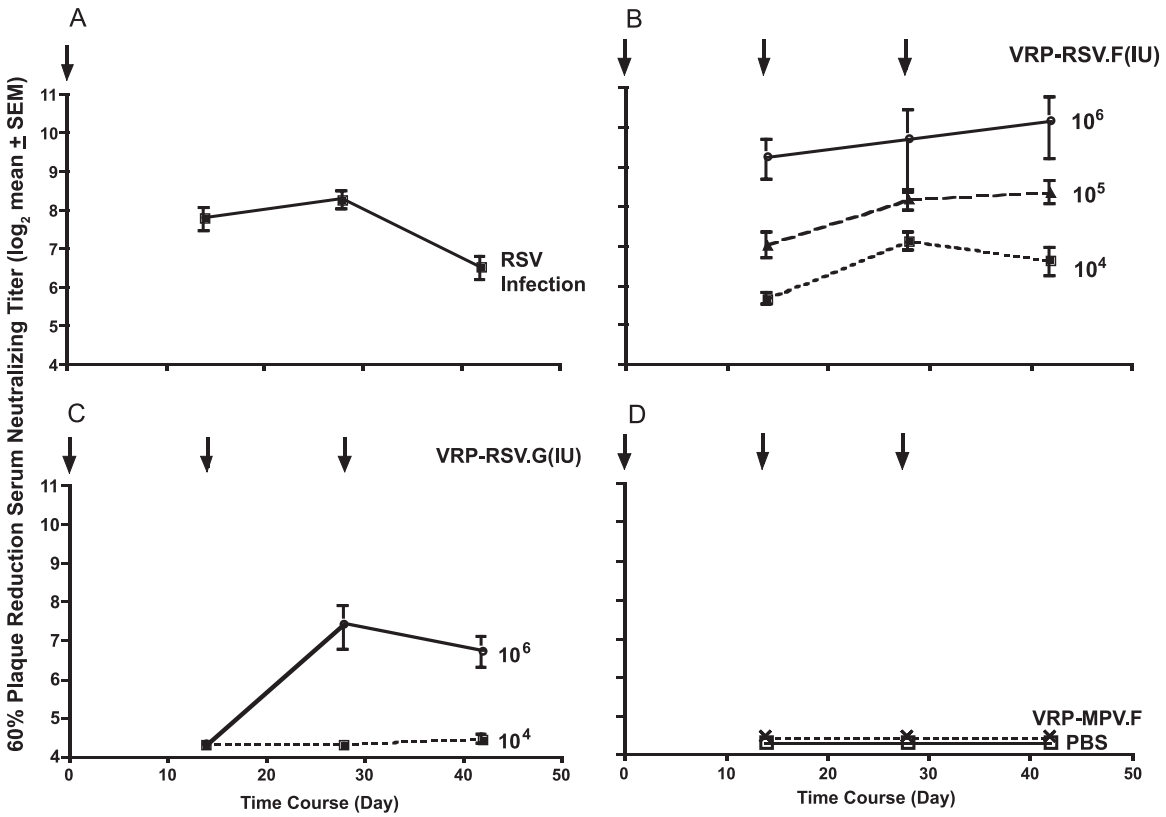

FIG. 5. Two immunizations were sufficient to generate a maximal serum neutralizing antibody response. BALB/c mice were vaccinated i.n. with VRP every 14 days for a total of three inoculations, as indicated by arrows. Sera were obtained every 2 weeks, and neutralizing activities against RSV were measured. Values represent the mean titer of five animals. Error bars indicate standard deviations from the mean.

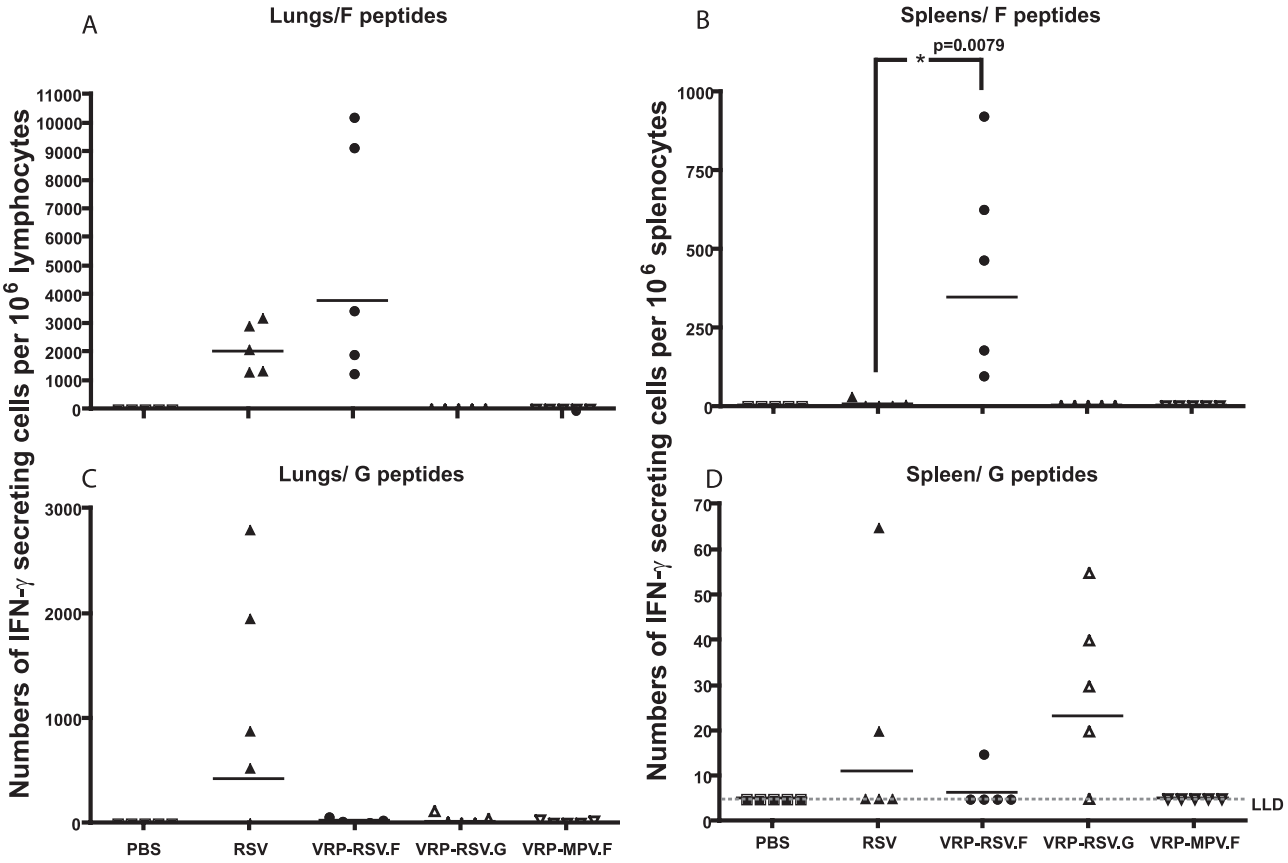

FIG. 6. RSV-F-specific lymphocytes and splenocytes were induced in the lungs and spleens of mice immunized i.n. with VRPs. BALB/c mice were vaccinated on days 0 and 14 with VRPs. Lymphocytes and splenocytes were harvested from the lungs (A and C) or spleens (B and D) 7 days after the second VRP vaccination or after the single RSV infection. A total of 1×10^5 cells were stimulated with RSV F (amino acids 85 to 93) peptide (A and B) or RSV G (amino acids 183 to 197) peptide (C and D) in vitro for 20 h, and the numbers of IFN-γ spot-forming cells were quantified by an ELISPOT assay. Spots were counted with an automated counting device and are expressed as numbers of spots per 10^6 cells. Each experimental group contained five animals. Each horizontal line denotes the geometric mean of the group. LLD, lower limit of detection.

TABLE 1. Reduction of titers of RSV in lungs and nasal turbinates of VRP-RSV.F-vaccinated BALB/c mice after challenge

Immunization[a]	Dose ($\log_{10}$ PFU/IU)[b]	RSV titer postchallenge ($\log_{10}$ PFU/g tissue) in[c]:		Fold reduction of RSV RNA in lungs[d]
		Lungs	Nasal turbinates	
PBS		3.25 ± 0.23	3.67 ± 0.23	1
RSV	6	≤1.7*	≤2.0*	23,042
VRP-RSV.F	4	≤1.7	≤2.0	ND
	5	≤1.7	≤2.0	ND
	6	≤1.7	≤2.0	12,077
VRP-RSV.G	4	≤1.7	3.00 ± 0.70	ND
	6	≤1.7	2.33 ± 0.85	204
VRP-MPV.F	6	3.03 ± 0.23	3.23 ± 0.25	3

[a] Animals in each VRP group received two doses of VRPs, while those in the RSV group were infected once with RSV.

[b] The titer of RSV (PFU) was determined by plaque formation in HEp-2 cells. The numbers of IU of VRP were determined by the number of infected BHK cells immunostained for VEE nonstructural proteins.

[c] Values are mean $\log_{10}$ PFU/g tissue ± standard error. Results are from groups of five animals. *, virus was not detected at the limit of detection: 1.7 in the lungs or 2.0 in the nasal turbinates.

[d] Differences (fold) were calculated based on the reduction of RSV RNA in the lungs 4 days after challenge compared to the amount of RSV RNA in the lungs of PBS-vaccinated animals. ND, not determined.

or 47-fold reduction in lungs or nasal turbinates, respectively). Previous infection with RSV also suppressed RSV growth below the limit of detection in the upper and lower respiratory tracts. In contrast, mice vaccinated with VRP-RSV.G showed no detectable challenge virus in the lungs but did have detectable virus in the nasal turbinates (Table 1). Real-time RT-PCR detection of RSV genome in the lungs of VRP-RSV.F-vaccinated mice after challenge revealed a 4-$\log_{10}$ reduction of viral RNA compared to PBS-vaccinated animals. Similarly, previous RSV infection also reduced copies of RSV genomes after challenge by 4.3 $\log_{10}$ compared to PBS controls. Immunization with VRP-RSV.G only reduced the RSV genome by 2.3 $\log_{10}$ (Table 1). In cotton rats, vaccination with VRP-RSV.F protected both the upper and lower respiratory tracts of these animals (at least 1,000-fold reduction in the lungs and 25-fold reduction in the nasal turbinates) (Table 2).

Immunogenicity of VRPs given by different routes of administration in mice. To assess the significance of immunization using different routes, we vaccinated BALB/c mice with the same dose (10^6 IU) of VRP-RSV.F i.n., i.p., and i.m. and compared immunogenicities based on serum neutralizing titers to RSV, number of RSV F-specific T cells generated, and ability to protect animals from RSV challenge. Similar serum

TABLE 2. RSV titers in the lungs and nasal turbinates were reduced in VRP-RSV.F vaccinated cotton rats after challenge

Immunization[a]	Dose (log_{10} PFU/IU)	Serum neutralizing antibody titer at challenge (log_2)[b]	RSV titer postchallenge (log_{10} PFU/g tissue) in[c]:	
			Lungs	Nasal turbinates
PBS		≤4.32	4.0 ± 0.4	3.4 ± 0.5
RSV	6	7.4 ± 1.0	≤ 1.0*	≤ 2.0*
VRP-RSV.F	6	7.7 ± 0.8	≤ 1.0	≤ 2.0

[a] Animals in each VRP group received two doses of VRPs, while those in the RSV group were infected once with RSV.
[b] Values are log_2 mean ± standard error.
[c] Values are mean log_{10} PFU/g tissue ± standard error. *, virus was not detected at the limit of detection: 1.0 in the lungs or 2.0 in the nasal turbinates.

neutralizing reciprocal titers were observed 14 days after a second immunization in animals vaccinated via different routes (8.8 log_2 for i.n., 8.4 log_2 for i.p., and 8.9 log_2 for i.m.). In addition, similar numbers of RSV F-specific T cells were detected in the spleens of mice vaccinated i.p. (367 cells/10^6 splenocytes), i.m. (330 cells/10^6 splenocytes), or i.n. (347 cells/10^6 splenocytes). In RSV challenge experiments, mice vaccinated with VRP-RSV.F by each of the three different routes had viral titers in the lungs and nasal turbinates that were below the level of detection.

Histopathology and cytokine gene expression profile in VRP-vaccinated mice after RSV challenge. Lungs from VRP-vaccinated and control mice were removed on day 4 after RSV challenge and tested for histopathology and for cytokine gene expression. Lung sections were scored in a group-blinded fashion. In naïve mice challenged with RSV, there were mild mononuclear infiltrates in the alveolar space compared to uninfected controls. There was a moderate increase in mononuclear infiltrates in the alveolar, peribronchial, and perivascular spaces of animals that were previously infected with RSV and in those that received VRP-RSV.F or VRP-RSV.G. The severities of inflammation were comparable between animals that were vaccinated with VRP-RSV.F and those previously infected with RSV. Animals vaccinated with VRP-RSV.G showed less inflammation. In contrast, mice vaccinated with formalin-inactivated RSV exhibited severe inflammation with alveolar inflammatory patches and abundant infiltration in the peribronchial and perivascular spaces. These animals also scored significantly higher for histopathology than their VRP-vaccinated counterparts (Table 3). Mucus was not detected in any of the sections (data not shown).

TABLE 3. Histopathology scores of lung tissues in vaccinated mice 4 days after wild-type RSV challenge

Immunization	Histopathology score[a]		
	Alveolar tissue	Peribronchial tissue	Perivascular tissue
Control	0.2 ± 0.2	0.1 ± 0.1	0.1 ± 0.1
RSV	1.3 ± 0.4	1.3 ± 0.3	1.6 ± 0.2
VRP-RSV.F	1.1 ± 0.1	1.2 ± 0.2	1.7 ± 0.5
VRP-RSV.G	0.2 ± 0.2	0.8 ± 0.4	1.4 ± 0.3
FI-RSV	2.2 ± 0.2	2.2 ± 0.3	2.7 ± 0.1

[a] Lung sections were viewed and scored by a pathologist in a group-blind fashion. Scores ranged from 0 (normal) to 3 or 4 (severe), as described in Materials and Methods.

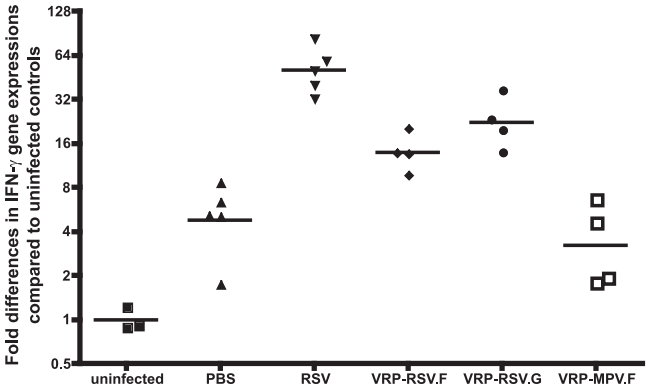

FIG. 7. IFN-γ gene expression levels 4 days after RSV challenge in the lungs of vaccinated BALB/c mice. IFN-γ gene expression levels were measured in lung lysates with real-time PCR and expressed as the mean change (fold) compared to the uninfected control.

Cytokine gene expression levels were measured in the same tissues by reverse-transcribed real-time PCR on purified cellular RNA. Only IFN-γ gene expression in the lungs was upregulated in RSV-challenged mice among all cytokines tested. None of the other cytokine genes tested (IL-2, IL-4, IL-5, IL-10, and IL-12) was statistically different from the uninfected controls (data not shown). Naïve animals and animals that received control replicons (VRP-MPV.F) had about a fourfold increase in IFN-γ gene transcription. Animals that were vaccinated with VRP or those previously infected with RSV had 16- to 50-fold increases in IFN-γ gene expression (Fig. 7).

DISCUSSION

In this study, we developed VEE replicon particles as vectors to deliver RSV surface glycoproteins and showed that when these vaccine candidates were delivered i.n., they induced immune responses comparable to, or greater than, those following wild-type virus infection.

VEE VRPs are attractive vaccine vectors for several reasons. First, they are less sensitive than most live viruses to type I interferons (74), which allows enhanced protein expression in replicon-infected cells in the draining lymph nodes. Translation of gene inserts from other alphaviruses, such as Sindbis virus, could be inhibited by such interferons (61). Second, parenteral or intradermal inoculation of VEE replicons induces mucosal responses directed toward the encoded antigens (28, 67), which are optimal for protecting against viruses at the respiratory mucosa. Although the mechanism underlying this unique mucosal immunogenicity of VRPs is not completely understood, protection and significant numbers of cells secreting antigen-specific IgA have been detected in the mucosa in immunized animals following VRP or VEE immunization via a nonmucosal route (18, 19, 28, 36, 60, 67). The study presented here focused on i.n. delivery as a first-step feasibility study with VRP vaccination for RSV. Other routes of nonmucosal delivery of VRP also were examined for the ability to generate immune responses and were found comparable.

Third, VRPs possess the ability to target specialized antigen-

presenting cells such as Langerhans cells in the dermis and human monocyte-derived dendritic cells (DCs) (44, 48). Compared to VEE replicons, other alphavirus vectors are not as effective in infecting DCs. Sindbis virus does target DCs, but protein expression is shut down rapidly by the innate immune response (61) and Semliki Forest virus does not infect DCs efficiently (32). Activation of DCs would greatly enhance both the innate and adaptive immune responses to vaccine antigens.

Finally, when VRPs were coadministered with microbial antigens, they exhibit adjuvant activity in the systemic and mucosal immune compartments (67). Although the mechanism of VRP-enhanced adjuvant activity is not well understood, the ability to enhance immune responses through adjuvant activity would likely play an important role in increasing vaccine efficacy in populations with immature immune systems, such as those of very young infants. Further study to develop RSV F protein vaccine with VRP as an adjuvant would be of interest.

Given the multiple advantages of VRPs over other viral vectors, we incorporated the genes for RSV fusion (F) and attachment (G) glycoproteins into the replicons and tested them in mice and cotton rats. F and G surface glycoproteins have been the targets for multiple experimental vaccines since these proteins are the targets for RSV neutralizing antibodies. Expression of RSV proteins from VRPs appeared authentic in every aspect. In BHK cells, VEE replicons expressed robust amounts of the encoded antigens. These antigens were expressed in a membrane-bound manner, which is consistent with published data on the distribution of F or G during RSV infection.

When inoculated i.n. in mice and cotton rats, VEE replicons induced RSV-specific binding and neutralizing antibodies in both the systemic and mucosal immune compartments. By inoculating VRPs via a mucosal site, we elicited a robust response against RSV in the respiratory tract and induced high levels of systemic RSV neutralizing antibodies. The RSV serum neutralizing titers induced by VRPs were directly proportional to vaccine dose, presumably due to an increase in antigen expression from higher numbers of VRPs. Remarkably, the serum neutralizing titers of VRP-RSV.F-vaccinated mice were higher than those following RSV infection, which demonstrates the potential of this vaccine. Similar serum neutralizing titers also were observed in mice vaccinated via the i.p. or i.m. routes. More importantly, mucosal IgA antibodies also were detected in the upper and lower respiratory tracts of i.n.-vaccinated animals. It should be noted that this comparison was performed with animals vaccinated with two doses of VRP-RSV.F versus animals vaccinated with a single dose of RSV. A single dose of RSV appeared to be equally effective in protecting animals from RSV challenge as two dose of VRP-RSV.F. In this study, we showed that vaccination with a single dose of VRP-RSV.F elicited a higher serum neutralizing titer at the 10^6-IU dose than RSV vaccination at 14 days postvaccination (Fig. 5). The dosing and immunogenicity of RSV vaccines in mouse models and human infants are not perfectly correlated, so that it is difficult to extrapolate from our current data to say whether or not one or two doses would be immunogenic in young infants. Immunogenicity for human infants would have to be determined in clinical trials.

Possible combination of VRP-RSV.F vaccination with VRP-RSV.G may also broaden the immune response to RSV and give benefit to young human infants.

Another issue of importance is the presence of maternal antibodies in very young infants that could potentially suppress the immune response and efficacy to the VRP vaccine. Passively transferred antibodies have been shown to mediate suppression of the immunogenicity and efficacy of both replication-competent as well as defective vaccinia virus-based vaccines in rodents and nonhuman primates (20, 23, 34). The effect of passively acquired RSV antibodies should be studied in future studies in VRP-vaccinated animals.

Although RSV-specific antibodies are shown to be effective in restricting viral replication during infection, cytotoxic T lymphocytes appeared to be required for resolution of infection and short-term protection against reinfection (11, 41). Both RSV-specific $CD4^+$ and $CD8^+$ T cells have been shown to confer protection to naïve animals against RSV challenge in adoptive transfer experiments (5, 55). Here, we demonstrated that vaccination with VRP encoding RSV F protein also induced F-specific $CD8^+$ T lymphocytes. Upon stimulation with *H-2K*^{d} MHC class I-restricted F epitopes, lung lymphocytes, or splenocytes from VRP-RSV.F-vaccinated mice secreted IFN-γ. In contrast, VRP-RSV.G replicons induced much lower humoral and cellular immune responses in comparison to those responses induced by VRP-RSV.F. This finding could be caused by several factors, such as a potential reduced expression level of G in vivo, the greater amount of glycosylation of G compared to F, and the need for complex processing of RSV G in vivo.

We used a homologous prime-boost strategy to evaluate the efficacy of VRPs in inducing neutralizing antibodies at various time points postimmunization. We found that a single prime boost was sufficient to induce a maximal level of neutralizing antibody responses. Further boosting with the same vectors had no significant effect on neutralizing titer, possibly due to the generation of antivector immunity.

When mice were challenged with RSV, only those that were vaccinated with VRP-RSV.F had viral replication reduced to undetectable levels in both the lungs and nasal turbinates. VRP-RSV.G-vaccinated mice that received a dose of 10^4 IU did not exhibit significant increases in neutralizing antibody titer, yet they were still protected in the lungs against RSV challenge. These mice may have produced low levels of neutralizing antibodies that could not be detected. In a semipermissive small animal model, such immune responses may be sufficient to restrict RSV in vivo; however, this level of immunogenicity is not likely to be effective in human subjects. RSV titers in the nasal turbinates of VRP-RSV.G-vaccinated mice remained high. This is consistent with the low levels of antibodies and lack of antigen-specific $CD4^+$/$CD8^+$ T cells, which had been shown to correlate with upper respiratory tract protection in RSV-infected mice (55).

This finding was supported by the real-time RT-PCR detection of relative quantity of RSV RNA following challenge in vaccinated animals. There was a greater reduction of RSV genome in the lungs of VRP-RSV.F-vaccinated or RSV-infected mice compared to those vaccinated with VRP-RSV.G, when using this sensitive detection approach.

One of the major hurdles to development of a RSV vaccine is concern over safety in RSV-naïve recipients. Increased mor-

tality rates and exacerbated disease were seen in infants vaccinated with formalin-inactivated RSV in the 1960s during subsequent natural infection (38, 40). Enhanced histopathology with excessive cellular influx and skewed Th2-dominant cytokine production was seen in animals vaccinated with formalin-inactivated RSV following viral challenge (58, 72). In these animals, a highly disproportionate number of cells of the Th2 subset of $CD4^+$ T cells was induced and found to be responsible for secreting IL-4 and IL-5, which in turn caused a pulmonary eosinophilic response (1). We performed multiple experiments to determine the profile of the type of responses in VRP-vaccinated mice pre- and postchallenge. The subclass distribution of antigen-specific IgG was determined after immunization, to evaluate the balance of Th1 versus Th2 responses. Mice immunized with VRP-RSV.F showed a balanced IgG1/IgG2a ratio (~0.7) compared to RSV-infected STAT1-deficient mice genetically predisposed to Th2 responses upon RSV infection (~3.7). A skewed Th2/Th1 response predisposes animals to develop vaccine-enhanced RSV disease, as seen in FI-RSV-immunized animals. In addition, we evaluated lung histopathology and cytokine gene expression in VRP-vaccinated mice after live RSV challenge. There was no evidence of enhanced lung histopathology in VRP-vaccinated animals upon RSV challenge when compared to animals that were previously infected with RSV. Vaccinated animals had moderate alveolar, peribronchiolar, and perivascular infiltrates and no significant airway mucus production. Unvaccinated animals did show minor increases in lung inflammation with mild lymphocytic infiltration with a histopathology score slightly lower than that of the immunized groups, possibly due to the delay of the appearance of pathogenic responses seen in primary infection in naïve animals compared to vaccinated animals. In animals vaccinated with formalin-inactivated RSV, severe inflammation and cellular infiltration were seen with a significant increase in histopathology scores, as has previously been reported (58).

Cytokine gene expression also was determined from lungs of these animals. Only IFN-γ gene expression was increased among all the cytokine genes tested. Infected groups had higher IFN-γ gene expression compared to uninfected controls. Interestingly, animals that had been vaccinated with VRP-RSV.F or VRP-RSV.G and those that were infected previously with RSV showed a dramatic increase in IFN-γ expression (~3 to 12 times greater depending on the groups) over groups that were not previously vaccinated or that were vaccinated with a heterologous VRP (VRP-MPV.F). This is probably due to the faster response times of T cells from vaccinated animals upon RSV challenge. This finding further suggests the development of properly balanced cellular immune responses in vaccinated animals upon RSV exposure.

In summary, we demonstrated that VEE VRPs encoding RSV F protein induced strong antigen-specific humoral and cellular responses on mucosal surfaces and protected animals against i.n. RSV challenge. This study provides strong feasible evidence for further development of this vaccine candidate for RSV.

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