Assembly and Immunological Properties of Newcastle Disease Virus-Like Particles Containing the Respiratory Syncytial Virus F and G Proteins[▽]

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Human respiratory syncytial virus (RSV) is a serious respiratory pathogen in infants and young children as well as elderly and immunocompromised populations. However, no RSV vaccines are available. We have explored the potential of virus-like particles (VLPs) as an RSV vaccine candidate. VLPs composed entirely of RSV proteins were produced at levels inadequate for their preparation as immunogens. However, VLPs composed of the Newcastle disease virus (NDV) nucleocapsid and membrane proteins and chimera proteins containing the ectodomains of RSV F and G proteins fused to the transmembrane and cytoplasmic domains of NDV F and HN proteins, respectively, were quantitatively prepared from avian cells. Immunization of mice with these VLPs, without adjuvant, stimulated robust, anti-RSV F and G protein antibody responses. IgG2a/IgG1 ratios were very high, suggesting predominantly T_H1 responses. In contrast to infectious RSV immunization, neutralization antibody titers were robust and stable for 4 months. Immunization with a single dose of VLPs resulted in the complete protection of mice from RSV replication in lungs. Upon RSV intranasal challenge of VLP-immunized mice, no enhanced lung pathology was observed, in contrast to the pathology observed in mice immunized with formalin-inactivated RSV. These results suggest that these VLPs are effective RSV vaccines in mice, in contrast to other nonreplicating RSV vaccine candidates.

Human respiratory syncytial virus (RSV) is the single most important cause of acute respiratory disease in infants and young children worldwide (22). Elderly and immunocompromised populations are also at significant risk for serious RSV disease (43, 52). Yet despite this very substantial disease burden, there are no vaccines available. Previous as well as current vaccine candidates are essentially modifications of vaccines developed by classical approaches, and none have resulted in a licensed vaccine (reviewed in references 10, 25, 38, and 41).

In general, inactivated viruses or purified protein vaccines are safer than any form of live virus vaccine, particularly for infants or immunocompromised populations. However, the first inactivated RSV vaccine candidate, a formalin-inactivated RSV preparation (FI-RSV), resulted in life-threatening disease upon subsequent exposure to infectious RSV (reviewed in references 8, 25, 37, and 38). This enhanced respiratory disease (ERD) has long been thought to be due to elimination of protective epitopes by formalin treatment (reviewed in references 8 and 9). More recently, it has been reported that FI-RSV, as well as other nonreplicating RSV vaccines, including purified protein vaccines or UV-inactivated RSV (UV-RSV), results in low-affinity, poorly neutralizing antibodies and a biased T_H2 immune response to the RSV fusion (F) protein compared to the response to infectious virus (12). The pre-

dominant T_H2 responses to these nonreplicating antigens correlated with enhanced lung pathology upon live virus infection (12).

While both soluble and cell-mediated immune responses are thought to be important for protection from RSV infection (2, 4, 5, 8, 15, 25, 45), antibodies, particularly antibodies to the F protein (8, 14, 47), are clearly sufficient for protection. The only currently effective prophylaxis for RSV disease is a humanized monoclonal antibody specific for RSV F protein (3, 6, 40). This reagent clearly demonstrates that serum antibodies specific to RSV F protein can be protective and underscores the importance of humoral immune responses to this virus in protection. The role of the G protein, the other major RSV surface glycoprotein, in stimulating protective immune responses is less clear, although recent studies have suggested that antibodies specific to the G protein are also protective in animal models (35, 46, 54) and prevent ERD stimulated by FI-RSV (44).

Virus-like particles (VLPs) are increasingly recognized to be safe, effective vaccines for viral diseases (21). VLPs are virus-sized particles composed of repeating structures on their surfaces and, in their cores, structures that mimic those of infectious viruses and that account, in part, for the very potent immunogenicity of viruses (21, 36). VLPs are formed by the assembly of the structural proteins and sometimes lipids without the incorporation of the viral genome. Thus, VLPs are incapable of multiple rounds of infection, yet they retain the superb antigenicity of virus particles. Two VLP vaccines, the papillomavirus vaccine and the hepatitis B virus vaccine, are licensed for use in humans, and a number of other VLP vaccines are in testing (21).

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We have recently described a novel RSV virus-like particle that stimulates, in mice, protective immune responses, responses similar to those observed with RSV infection (34). Furthermore, VLP immunization did not result in ERD upon exposure to live virus (34). These VLPs were formed with the structural core proteins, nucleocapsid protein (NP) and matrix (M) protein, of Newcastle disease virus (NDV) and the ectodomain of the RSV G protein fused to the transmembrane (TM) and cytoplasmic tail (CT) sequences of the NDV hemagglutinin-neuraminidase (HN) protein. These VLPs stimulated anti-G-protein-specific IgG antibodies (34).

Here we describe the assembly and immunological properties of VLPs that contain the ectodomains of both the RSV F and G proteins as well as the NDV NP and M protein. VLPs composed of NDV core proteins assembled with RSV glycoprotein ectodomains were characterized because, as we show here, VLPs composed entirely of RSV proteins are produced at extremely low levels, levels that were inadequate for their preparation as immunogens, in contrast to VLPs assembled with NDV core proteins. NDV-based VLPs containing the RSV F and G glycoprotein ectodomains stimulated both anti-G and anti-F protein IgG serum antibody responses at levels as good as or better than the levels resulting from RSV infection. Antibodies were predominantly the IgG2a isotype, typical of a more T_H1 -biased response, in contrast to the antibodies stimulated by other nonreplicating vaccines, FI-RSV or UV-RSV. Serum antibodies were neutralizing, and in contrast to neutralizing antibodies stimulated by RSV infection, neutralization titers were stable for over 4 months after a single immunization. Immunization with these VLPs completely protected mice from RSV replication in lungs upon virus challenge and did not result in ERD upon RSV infection.

MATERIALS AND METHODS

Cells, virus, and plasmids. ELL-0 cells (avian fibroblasts), Vero cells, and COS-7 cells were obtained from the American Type Culture Collection, while 293T cells were obtained from the laboratory of Frank Ennis. ELL-0 cells were maintained in Eagle's minimal essential medium (EMEM; Gibco) supplemented with 10% fetal calf serum (FCS). COS-7 cells were grown in Dulbecco minimal essential medium (DMEM) supplemented with penicillin, streptomycin, and 10% fetal calf serum. Vero cells were grown in DMEM supplemented with penicillin, streptomycin, and 5% fetal calf serum. RSV, A2 strain, was obtained from R. Tripp.

The RSV G, N, and M protein genes as well as codon-optimized F protein genes were synthetic genes obtained from Novavax, Inc., in pFastbac plasmid. The pFastbac N and M protein DNAs were digested with BamHI/HindIII and KpnI/BamHI, respectively, blunted with T4 DNA polymerase, and cloned into pCAGGS digested with MscI. NDV NP and M, F, and HN protein genes as well as the RSV genes were inserted into the pCAGGS expression vector as previously described (28, 29). The RSV F gene was cloned by two-step reverse transcription (RT)-PCR using RNA extracted (RNeasy; Qiagen) from RSV-infected Vero cells (RT primer, GGTCTACTATCTGGCCATAGACAACCC; forward PCR primer, GAATCAAAATAACTCGAGGGCAATATACA; reverse PCR primer, GGGTTGTCTATGGCCAGATAGTAAACC). The resulting clone was inserted into pCAGGS using the XhoI and MscI sites.

The construction and expression of the NDV HN protein-RSV G (H/G) protein chimera gene, inserted into pCAGGS, were previously described (34). The RSV F protein/NDV F (F/F) protein chimera gene was constructed by ligation of PCR products derived from pCAGGS-NDV F protein and pCAGGS-RSV F protein DNAs. The RSV sequences encoded amino acids 1 through 524, and the NDV F protein sequences encoded amino acids 501 to 554. The primers used to generate DNA encoding the RSV F protein ectodomain were CATAA TGTTGTAGTACTCTCCCGCGGTAACATTGTG (which introduced a ScaI site without changing the amino acid sequence) and GGTTATTGTGCTG TCGACTCATTTTGGC. The product was digested with ScaI and XhoI. Primers

used to generate a DNA encoding the NDV F protein CT and TM domains were GTCAATGTCAAACCTGACCAGTACTACTAATCTCATTACCTATAT CGCT (which introduced a ScaI site without changing the amino acid sequence) and CACACAGGAAACAGCTATGAC. This product was digested with HindIII and ScaI. The two DNA fragments were ligated into an XhoI-HindIII-digested pGAGGS vector. The resulting plasmid containing the chimera protein gene was sequenced in its entirety to verify the gene junctions (illustrated in Fig. 1A) and to ensure that no additional changes were introduced during the PCRs.

Antibodies. Polyclonal rabbit anti-NDV antibody was raised against UV-inactivated, purified NDV as previously described (31). Polyclonal goat anti-RSV antibody (Biosdesign) was used in Western blots to detect the RSV G protein. RSV F protein monoclonal antibody (clone 131-2A; Chemicon) was used in plaque assays and immunofluorescence of fixed cells. Polyclonal rabbit anti-RSV F protein antibody was produced by expressing the HR2 domain of the RSV F protein (amino acids 484 to 525) as a glutathione S-transferase fusion protein, purifying the fusion protein from *Escherichia coli* extracts as described by the manufacturer (Novagen). The purified protein was used as immunogen in rabbits to generate anti-RSV HR2 antibody. Anti-NDV Ftail is a polyclonal antibody raised against the cytoplasmic tail domain of the NDV F protein as previously described (31). Secondary antibodies utilized were anti-goat antibody (Sigma), anti-mouse antibody (Sigma), and anti-rabbit antibody (Sigma). NDV anti-HR2 is a polyclonal antibody specific for the NDV F protein and has been previously described (13).

Transfections. Small-scale transfections were accomplished using Lipofectamine (Invitrogen) as recommended by the manufacturer and as previously described (34). For quantitative preparations of VLPs, large-scale transfections of cells growing in T-150 flasks were utilized. Protocols have been previously described (34).

PAGE, silver staining, and Western analysis. Proteins in extracts, virus, or VLPs were resolved on 8% polyacrylamide gels (SDS-PAGE) as previously described (39). Silver staining of proteins in the polyacrylamide gels was accomplished as recommended by the manufacturer (Pierce). For quantification of NP and M and H/G proteins in the polyacrylamide gels, different concentrations of bovine serum albumin (BSA) were electrophoresed on the same gel in the presence of reducing agent. A standard curve based on the staining of the BSA (using a Bio-Rad densitometer to measure the intensity of staining) was used to determine the concentrations of each of the proteins in the purified VLPs or virus. For quantification of F protein, different concentrations of purified F protein (obtained from Novavax, Inc.) were electrophoresed on the same gel as the VLP proteins in the absence of reducing agent. The F proteins were detected by Western analysis using anti-F protein antibody (anti-RSV F-HR2). A standard curve based on the Western analysis of the pure F protein was used to determine the concentrations of F protein in VLPs. For Western analysis, proteins in the polyacrylamide gels were transferred to polyvinylidene difluoride membranes (PerkinElmer) using dry transfer (iblot; Invitrogen). Proteins were detected in the blots as previously described (24).

VLP purification. At 24 h posttransfection, heparin was added to the cells at a final concentration of 10 μ g/ml as previously described (34). At 72 h posttransfection and again at 96 h posttransfection, cell supernatants were collected and VLPs were purified as previously described (30, 34). The lipopolysaccharide (LPS) content of the preparations was determined using *Limulus* amoebocyte lysate endotoxin assay kits from GenScript Corporation and Lonza. The maximal amount of LPS per dose of VLP ranged from 0.0025 ng to 0.001 ng, which is well below maximum limits for preclinical studies and vaccine formulations (1, 26).

Preparation of RSV, UV-RSV, and FI-RSV. RSV, grown in Vero cells, was prepared as previously described (34). Preparation and quantification of UV-RSV have been previously described (34). FI-RSV was prepared by a modification of the method of Prince et al. (42) and has been previously described (34). The amount of virus adsorbed to the alum was calculated to be equivalent to 6.1×10^8 PFU/ml. This estimate was based on the known virus titer prior to formalin treatment, the volume of the virus prior to formalin inactivation, the percent protein bound to the alum, and the concentration factors for each step in the procedure.

RSV immunoplaque assays and antibody neutralization. RSV plaque assays on Vero cells (1.5×10^5 /well) were accomplished as previously described (34).

For antibody neutralization assays, mouse sera were complement inactivated and then diluted in DMEM without serum. RSV stocks were diluted to approximately 75 to 150 PFU in 100 μ l. These dilutions of mouse sera in 100 μ l were added to the virus and the mixture was incubated for 1 h at 37°C. The mixture was then added to prewashed, confluent monolayers of Vero cells growing in 24-well tissue culture dishes, and the cells were incubated at 37°C for 1 h. The antibody-virus mixture was removed and 1 ml of methylcellulose overlay was

FIG. 1. Expression of wild-type RSV F and F/F chimera proteins. (A) Ectodomain, TM, and CT domains of the NDV F and RSV F proteins as well as the domains in the F/F chimera protein. The sequences at the junction of the ectodomains and TM domains of the NDV F protein, RSV F protein, and the chimera F/F protein are shown below the diagram. (B) Surfaces of avian cells transfected with cDNAs encoding Fwt or F/F proteins were biotinylated as described in Materials and Methods. Biotinylated proteins were precipitated from cell extracts with neutravidin-agarose, and precipitated proteins containing RSV F protein sequences were detected by Western analysis using anti-RSV F protein HR2 antibody. V, vector DNA-transfected cells; Fwt, wild-type F protein; F/F, RSV F/NDV F chimera protein; RSV, total cell extracts of RSV-infected Vero cells. The predicted molecular weights of the NDV F₁, the RSV F₁, and the F₁ forms of F/F protein are 55,634, 50,921, and 51,676, respectively, accounting for glycosylation. Lanes 1 to 3 and lanes 4 and 5 were on different portions of the same gel. (C) Avian cells were transfected with identical concentrations of vector DNA or cDNAs encoding the RSV F wild-type or F/F proteins. At 48 h posttransfection, cells were methanol fixed and RSV F protein sequences were detected by immunofluorescence using mouse anti-F protein monoclonal antibody. Images were acquired with a 3.5-s exposure and processed through Photoshop software using the same settings for all three images. (D) Avian (lanes 2 and 3) and COS-7 (lanes 4 and 5) cells were transfected with cDNA-encoding vector DNA (V), and the F/F chimera protein (F/F) in cell extracts was detected by Western analysis using anti-NDV F tail antibody. The predicted molecular weights of the NDV F₀ protein and the F₀ form of the F/F protein are 68,408 and 76,576, respectively, accounting for glycosylation and signal sequence cleavage. NDV F, NDV-infected avian cell extracts; F₀, uncleaved fusion protein; F₁, cleaved fusion protein.

added to each well as described above. Plates were incubated for 3 to 4 days, and plaques were stained as previously described (34).

Biotinylation of cell surfaces. Biotinylation of RSV F and F chimera proteins was accomplished as previously described by Jain et al. (18, 19). Biotinylated molecules were precipitated from cell lysates using neutravidin-agarose as previously described. Precipitated F proteins were detected by Western analysis using anti-RSV F HR2 or anti-RSV antibodies.

Animal immunization and challenge protocols. Four-week-old BALB/c mice from Jackson Laboratories or Taconic Laboratories were housed (in groups of five) under pathogen-free conditions in microisolator cages at the University of Massachusetts Medical Center animal quarters. All protocols requiring open cages were accomplished in biocontainment hoods. Mice were immunized by intramuscular (i.m.) inoculation of 10 to 40 μ g total VLP protein in 0.05 ml of phosphate-buffered saline (PBS) containing 30% sucrose. For infection with RSV, mice were lightly anesthetized with isoflurane and then infected by intranasal (i.n.) inoculation of RSV (6×10^5 to 30×10^5 PFU/mouse in 50 μ l). Mice that received an immunization boost were injected i.m. with 10 μ g of total VLP protein or 6×10^5 to 30×10^5 PFU/mouse of RSV (i.n.). Mice challenged with live RSV were lightly anesthetized as described above and infected i.n. with 1×10^6 to 3×10^6 PFU of virus.

Detection of virus in lung tissue. Mice were anesthetized with isoflurane and exsanguinated after the right caudal artery was severed. Lungs were removed aseptically, placed in 0.5 ml of 30% sucrose in PBS, and stored at -80°C . After they were thawed, the lungs were weighed and then homogenized using a pestle (Kontes). The homogenate was centrifuged at 12,000 rpm for 15 min, and the virus titer in the supernatant was determined by plaque assay as described above.

Determination of antibody titers by ELISA. Antigen used as the G protein target in enzyme-linked immunosorbent assay (ELISA) were extracts from 293T cells transfected with pGAGGS-G as previously described (34). The amounts of transfected extracts added to each well were adjusted so that the amounts of G protein were comparable from experiment to experiment, as determined by Western blot analysis. Antigen used as F protein target was purified F protein (obtained from Novavax, Inc.). Each well contained 25 ng F protein. All antigens were placed in carbonate buffer, pH 9.6, and added to microtiter plates (Costar),

and the plates were incubated overnight at 4°C . After binding of the target antigen, wells were blocked in 50 μ l PBS containing 1% BSA at room temperature for 1 to 2 h, washed three times in PBS, and drained. Different dilutions of mouse sera were added to the microtiter wells in 50 μ l of PBS-BSA, and the plates were incubated for 1 h at room temperature. After the mouse sera were removed and the wells were washed three times, a biotinylated anti-mouse IgG antibody (1:4,000 dilution; Sigma), anti-mouse IgG1 antibody (1:2,000 dilution; Southern Biotechnology), or anti-mouse IgG2a antibody (1:2,000 dilution; Southern Biotechnology) in 50 μ l of PBS-BSA was added and the microtiter plates were incubated for 1 h at room temperature. The microtiter plates were then washed three times in PBS and a horseradish peroxidase-conjugated neutravidin (1:4,000 dilution; Pierce) was added in 50 μ l of PBS-BSA (1:4,000 dilution). The microtiter plates were incubated for 1 h at room temperature and washed four times in PBS. 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (Sigma) in 50 μ l was added to each well, and the plate was incubated for 15 to 20 min. The reaction was stopped with 50 μ l 1N H_2SO_4 , and the optical density (OD) (420 nm) was read in a plate reader (Molecular Devices). The titers of anti-G protein antibodies were defined as the reciprocal of the serum dilution that gave an OD of threefold over the background (34). Titers for anti-F protein antibodies were defined as the reciprocal of the serum dilution that gave an OD of 0.2 since the background values for this target antigen were zero.

Standard curves for IgG2a and IgG1 were derived by using as target antigen increasing amounts of pure antibody (0.5 to 64 ng; Southern Biotechnology) in an ELISA using anti-mouse IgG2a and IgG1 as described above.

Pulmonary histology of RSV-infected mice. For histological analysis of lung tissue, mice were anesthetized with isoflurane and exsanguinated after the right caudal artery was severed. The lungs were then fixed via infusion through the trachea with 4% formalin, removed, immersed in 4% formalin for 24 h, embedded in paraffin, sectioned, and stained with hematoxylin-eosin (H&E) by the University of Massachusetts Diabetes Endocrinology Research Grant-supported Histology Core Facility. Four sections per mouse were obtained. Sections from each mouse were scored, blindly, for the degree of inflammation of blood vessels, airways, or interstitial spaces on a scale of 0 to 3 as previously described (33, 34).

Statistical analysis. Statistical analyses of the data were accomplished using GraphPad Prism (version 5) software.

RESULTS

Construction and expression of RSV F protein/NDV F protein chimera gene. To incorporate the RSV F protein into ND VLPs, the sequences encoding the ectodomain of the RSV F protein were fused to sequences encoding the NDV F protein TM and CT domains to create a chimera RSV F/NDV F (F/F) protein. The RSV and NDV sequences at the junction of the F/F chimera protein are shown in Fig. 1A.

To compare cell surface expression of the chimera F/F protein with that of the wild-type RSV F protein in avian cells, proteins expressed on surfaces of intact transfected cells were labeled by biotinylation, and biotinylated molecules were precipitated from cell extracts with neutravidin-agarose. The RSV sequences in the precipitates were detected by Western analysis using anti-RSV antiserum (Fig. 1B). Surprisingly, the surface expression of the wild-type protein was significantly less than that of the chimera F/F protein. Total expression of the wild-type RSV F protein was also less than that of the F/F protein, as detected by Western analysis of total cell proteins (data not shown) as well as by immunofluorescence of fixed, permeabilized cells using a different anti-F protein antibody (Fig. 1C). Similar differences in expression levels of wild-type and chimera proteins were also observed in transfected HEp-2 cells and Vero cells (data not shown).

The chimera F/F protein was similar in size to the wild-type RSV F protein in infected Vero cell extracts; these molecules are likely primarily the cleaved F_1 form of the protein (Fig. 1B, lanes 4 and 5). Indeed, the predicted molecular weights of the F/F and the RSV F protein F_1 polypeptides are very similar (51,675 and 50,921, respectively, accounting for the glycosylation sites in the two molecules). Figure 1D shows that the major F/F polypeptide detected migrated slightly faster than the F_1 form of the NDV F protein, a result consistent with the larger predicted molecular weight of the NDV F_1 (55,634). Thus, the majority of the chimera protein synthesized in avian cells is likely fully cleaved, typical of the F protein expressed in mammalian cells. Figure 1D also shows that total cell expression of the F/F chimera protein was similar in avian (lanes 2 and 3) and COS-7 cells (lanes 4 and 5).

Requirements for assembly of RSV F protein into VLPs. We next determined if the chimera F/F protein could be assembled into ND VLPs. The F/F protein was coexpressed with the NDV NP and M protein in three cell types: avian, COS-7, and 293T cells. Particles released from these cells were purified, and the protein content of the purified particles was determined by Western analysis. The F/F chimera protein was incorporated into ND VLPs in the presence of the NDV NP and M protein in all three cell types (Fig. 2, lanes 4, 10, and 16), but comparisons of the ratios of NP and F/F protein in the VLPs show that F/F protein incorporation was significantly more efficient in avian cells (Fig. 2, lane 4). We also asked if expression of the H/G chimera protein had any effect on F/F protein assembly into VLPs. Strikingly, the presence of the H/G chimera protein in all three cell types significantly increased the incorporation of F/F protein into particles, although the levels of F/F protein

were again the highest in VLPs released from avian cells (Fig. 2, lanes 5, 11, and 17).

We have previously shown that avian cell-expressed H/G chimera protein was efficiently incorporated into ND VLPs containing the NDV NP and M protein (illustrated in Fig. 2, lane 3). Here we show that the cell type used to produce ND VLPs has little effect on H/G protein incorporation. Similar levels of H/G chimera protein, relative to the VLP-associated NP, were incorporated into VLPs released by all three cell types (as shown in Fig. 2A and B, lanes 3, 9, and 15). Furthermore, coexpression of the F/F protein with the H/G chimera protein had little effect on the incorporation of the H/G protein into ND VLPs in the three cell types (Fig. 2B). The differences in migration of the H/G protein or the wild-type G protein in different cell types has been previously noted (32, 34). It has been suggested that these differences relate to cell type variations in glycosylation.

To determine if VLPs composed entirely of RSV wild-type proteins could be detected under any of these conditions, the three cell types were transfected with cDNAs encoding the RSV M, RSV N, wild-type RSV G, and wild-type RSV F proteins. As previously reported (48), RSV VLPs were released, as detected by the presence of N and M proteins in purified particles (Fig. 2B, lanes 7, 13, and 19). There were also detectable levels of the two glycoproteins in released particles, but their detection required significantly increased exposure times (Fig. 2D and E). The levels of the RSV G and F proteins in these particles were dramatically reduced compared to the levels detected in ND VLPs prepared in parallel from similar numbers of cells, and this lower level of release was similar in all three cell types. Because of this inefficient release of VLPs containing only RSV proteins, it was impossible to prepare these RSV VLPs in quantities sufficient for further characterization. The reasons for inefficient release of RSV VLPs are unknown. The requirements for RSV VLP assembly and release have yet to be fully characterized. However, one possibility is that inefficient release could be related to inefficient F protein expression.

Quantitative preparation of ND VLPs containing RSV F protein ectodomain. Preparation of ND VLPs containing the RSV F protein ectodomain for use as an immunogen was accomplished by transient transfection of avian cells with cDNAs encoding the NDV NP and M protein as well as cDNA encoding the F/F chimera protein. Because assembly of the F/F protein was enhanced by the presence of the H/G protein and because an ideal immunogen should stimulate immune responses to both RSV glycoproteins, the H/G protein cDNA was also included. Particles released from these cells were purified as described in Materials and Methods. The total protein content of the purified VLPs was determined by silver staining of polyacrylamide gels containing the VLP proteins (Fig. 3A, lanes 2 to 4, and Fig. 3B, lanes 2 and 3). NDV was included as a marker for NDV NP and M protein, as were molecular weight markers. The proteins were electrophoresed in the presence (Fig. 3A) and absence (Fig. 3B) of reducing agent in order to detect the cleaved form of the F/F protein and the disulfide-linked F_1 - F_2 complex (Fnr), respectively. The presence of the RSV F protein sequences was verified by Western analysis of the VLP-associated proteins using anti-RSV F antibody (Fig. 3B, lanes 5 and 6), while the presence of the H/G

FIG. 2. Assembly of RSV F protein sequences into VLPs in different cell types. Avian cells, COS-7 cells, or 293T cells, growing on 35-mm dishes, were transfected with the cDNAs indicated at the bottom of the figure (H/G and F/F, chimera proteins; NP, NDV nucleocapsid protein; Mn, NDV M protein; HN, NDV hemagglutinin-neuraminidase protein; Fn, NDV F protein; N, RSV nucleocapsid protein; Mr, RSV M protein; G, RSV G protein; Fr, RSV F protein). At 96 h posttransfection, cell supernatants were harvested, VLPs were purified, and proteins in purified VLPs were resolved on duplicate polyacrylamide gels. The proteins present were detected by Western blot analysis (WB). NDV sequences (A) were detected using anti-NDV antibody (anti-NDV antibody does not efficiently detect NDV M protein [30]). RSV G sequences were detected using anti-RSV antiserum (which detects RSV G protein but does not detect RSV F) (B and D). RSV F protein sequences were specifically detected using anti-RSV F protein HR2 antisera (C and E). (D and E) Lanes 5, 6, and 7 in panels B and C exposed for five and four times longer than the lanes shown in panels B and C, respectively. The molecular weights of the proteins were determined by benchmark proteins included in the polyacrylamide gels as well as the NDV (A, lane 1) and RSV (B and C, lane 20) proteins included as markers. The molecular weights of NDV and RSV proteins are shown on the left side of the figure (K, thousands).

chimera protein was verified by Western analysis using anti-RSV antibody (Fig. 3A, lanes 6 to 8).

Densitometer analysis of silver-stained gels of four different VLP-H/G+F/F preparations showed that, on average, the levels of H/G protein incorporated were approximately 30% of the levels of M protein incorporated. Similarly, the amounts of F/F protein in these ND VLPs, using purified RSV F protein as a standard, as described in Materials and Methods, were, on average, approximately 30% of the levels of M protein.

Murine antibody responses to ND VLPs containing the RSV G and F protein ectodomains. To determine if VLP-H/G+F/F could stimulate anti-RSV F protein immune responses, we characterized immune responses in mice. Although antigens

that result in protective responses in mice are often not effective in humans, mice are the most widely used for initial characterization of the antigenic properties of RSV vaccine candidates (41). Groups of five mice were immunized with purified VLPs by i.m. inoculation. As positive controls, another group of mice received infectious RSV by i.n. inoculation. Anti-F protein IgG antibody levels in sera from these mice at various times after a primary immunization and after a boost immunization were determined by ELISA using purified RSV F protein as the target antigen. Figure 4A demonstrates that anti-F protein IgG antibody levels increased with time and that a boost immunization increased the titer of anti-F protein antibodies. The serum IgG responses were comparable to the

FIG. 3. Quantitative preparation of ND VLPs containing the F/F and H/G chimera proteins. Proteins present in increasing amounts of VLPs purified from large-scale transfections of avian cells expressing NDV NP and M protein as well as the F/F and H/G chimera proteins were separated on polyacrylamide gels in the presence (A) or absence (B) of reducing agent (β -mercaptoethanol [β ME]). NDV was electrophoresed in parallel (lanes 1 and 5, panel A; lanes 1 and 4, panel B). Lanes 1 to 4 (A) or lanes 1 to 3 (B) were silver stained. RSV G protein sequences were detected by Western blot analysis (WB) of lanes 5 to 8 in panel A using anti-RSV antibody. RSV F protein sequences were detected by Western analysis of lanes 4 to 6 in panel B using anti-F protein HR2 antibody. Marker NDV proteins are shown on the left of panel A, lane 1, and serve as marker proteins for lanes 1 to 8, which were derived from the same gel (K, thousands). Similarly lanes 1 to 6, panel B, were derived from the same gel, and the NDV proteins (lane 1) serve as marker proteins.

responses after i.n. immunization with RSV. Results in Fig. 4B demonstrate that these VLPs also stimulated anti-RSV G protein IgG antibodies and that the levels were comparable to the levels after RSV i.n. infection. The anti-G protein antibody titers were very similar to those previously reported after immunization with VLPs containing only the RSV G protein ectodomain, the H/G chimera protein (34).

Protective responses to VLP-H/G+F/F. We previously reported that immunization with VLP-H/G resulted in neutralizing antibody responses, but the titers were modest, between 3 and 4 ($\log_2$ of a dilution resulting in a 60% reduction in titer) (34). We therefore determined if inclusion of the RSV F protein ectodomain in the VLPs improved neutralizing antibody responses in immunized mice. Sera from five mice immunized

with a single inoculation of VLP-H/G+F/F were pooled, and the neutralization titer of these preboost sera was determined in an *in vitro* plaque reduction assay as described in Materials and Methods. Similarly, the neutralization titers in sera from the same five mice after a boost immunization were determined. As a positive control, the neutralization titers of pooled sera from another group of five mice immunized and boosted with an i.n. inoculation of infectious RSV were determined. Figure 5A shows that the preboost titer was approximately 5.5 and the postboost titer was 6 for both sets of mice. Thus, inclusion of RSV F protein sequences in the ND VLPs improved RSV neutralizing antibody responses to the VLPs.

Because there were significant levels of neutralizing antibody in sera from mice after a single immunization with VLP-H/G+F/F, we asked if a single immunization could protect mice from RSV replication in the lungs after an RSV challenge. Figure 5B shows that mice were completely protected. This result was expected since a single immunization of mice with VLP-H/G also fully protected mice from RSV replication in the lungs upon virus challenge (34).

Durability of antibody responses to VLP-H/G+F/F. Nonreplicating, protein antigens often result in short-lived immune responses. In addition, protective immune responses in humans after a primary RSV infection are often short-lived since some individuals can experience repeated RSV infections within the same season (reviewed in reference 41). To determine the durability of immune responses to RSV antigens presented in VLPs, two groups of five mice were given a single i.m. inoculation with 10 or 30 μ g total VLP protein, and the antibody responses were followed for 125 days. Figure 6 shows that both the anti-F protein (Fig. 6A) and anti-G protein (Fig. 6B) IgG antibody responses were robust and did not diminish during the course of the experiment. Note that the antibody titers after i.n. inoculation with RSV also did not decrease with time.

We next asked if the neutralizing antibody titers in these sera declined with time. Sera from each group of mice were pooled, and the titers (Fig. 6C) were determined as described above. Immunization with a higher dose of VLPs resulted in higher titers, at all times, than immunization with a lower dose, even though the total anti-F protein antibody levels determined by ELISA were very similar for the two groups of animals by 99 days. The neutralizing antibody titers after VLP immunization with either dose were relatively stable with time after 30 to 60 days. In contrast, while the total antibody levels after RSV immunization did not decrease (Fig. 6A and B), the neutralizing antibody titers dropped significantly with time (Fig. 6C).

Characterization of subtypes of IgG antibodies. The ratios of IgG subtypes during infection or immunization have been used as indicators of T_H1- or T_H2-biased immune responses (12, 27). It has been reported that immunization with the nonreplicating RSV vaccines, UV-RSV, FI-RSV, or purified F protein results in primarily T_H2 responses (25, 37), reflected in very low anti-F protein antibody IgG2a-to-IgG1 ratios (12). Since VLP-H/G+F/F is a nonreplicating RSV immunogen, we determined the ratios of IgG subtype responses to both the F and G proteins after immunization with this VLP. We compared these ratios to those obtained after intranasal RSV infection or after FI-RSV or UV-RSV immunization.

FIG. 4. Titers of anti-RSV F and anti-RSV G protein antibodies after immunization with VLP-H/G+F/F. Groups of five mice were immunized i.m. with VLP-H/G+F/F (4.5 μ g F/F protein/per mouse), RSV (2×10^6 PFU/mouse), or PBS (50 μ l). Mice received a second dose of immunogen (VLP-H/G+F/F containing 1.8 μ g F/F protein, 2×10^6 PFU of RSV, or 50 μ l PBS) 43 days after the first immunization. (A) Anti-RSV F protein IgG antibody titer at increasing times after the initial immunization and after a boost. Purified RSV F protein was the target antigen. (B) Anti-RSV G protein IgG antibody titers before and after the boost immunization. The target antigen was RSV G protein expressed in 293T cells transfected with cDNA encoding the RSV G protein. Titers are defined in Materials and Methods.

Indeed, as previously reported (12), the anti-F protein IgG responses to FI-RSV were primarily IgG1 (Fig. 7A). The IgG2a/IgG1 ratio was less than 0.1. In addition, we found that anti-G protein IgG responses after FI-RSV immunization were also primarily IgG1 (Fig. 7B), with ratios being below 0.3. In contrast, i.n. infection with RSV resulted in serum IgG ratios of 1 to 1.5 for both anti-F and anti-G protein antibodies (Fig. 7A and B). In addition, immunization with UV-RSV resulted in ratios more similar to those observed after RSV infection, particularly ratios of anti-F protein IgG subtypes.

VLP immunization resulted in anti-G protein IgG2a/IgG1

ratios of 1 to 2.5 in two separate sets of mice (Fig. 7D), results more similar to those after RSV infection than after FI-RSV immunization. However, VLP immunization resulted in anti-F protein IgG subtype ratios very different from the responses to either FI-RSV or RSV. In three separate sets of mice, initial antibodies were almost exclusively IgG2a (Fig. 7C). With time, IgG1 levels did increase somewhat, although the ratios remained very high. However, IgG1 levels significantly increased after a boost immunization, resulting in ratios of approximately 2, ratios very similar to those after a primary RSV infection.

FIG. 5. Protective responses after VLP immunization. (A) Neutralizing antibody titers in sera preboost and postboost from mice immunized with VLP-H/G+F/F (the initial immunization contained 6.4 μ g F protein, the boost contained 2 μ g F protein) or infectious RSV (6×10^5 PFU for both initial and boost immunizations). Aliquots of sera from mice at day 35 were pooled for preboost titers. Mice were boosted at day 43, and aliquots of sera harvested at day 56 were pooled for postboost titers. Neutralizing antibody titers, $\log_2$ of the dilution resulting in 60% reduction in virus titer, were determined as described in Materials and Methods. Results shown are the average of duplicate plaque assays and are representative of results of two separate mouse immunization experiments. (B) Virus titers in lungs of mice at 4 days postchallenge with infectious RSV. Mice were immunized with VLP-H/G+F/F (6 μ g F protein/mouse) i.m. or infectious RSV (6×10^5 PFU/mouse) i.n. Two other sets of mice were not immunized. At day 52, mice were challenged with RSV (1.5×10^6 PFU/mouse). Four days postchallenge, lungs were harvested and virus titers were determined by plaque assay. One set of mice was not immunized or challenged and represents the negative control.

FIG. 6. Durability of antibody responses after immunization. Two groups of five mice were immunized i.m. with VLP-H/G+F/F. Each mouse in one group received 10 µg total VLP protein, while the second group received 30 µg total VLP protein/mouse. A third group of five mice received RSV (2.25×10^6 PFU/mouse) i.n., while a fourth group of five mice received PBS (50 µl/mouse). (A) Anti-RSV F protein IgG antibody titers in VLP-H/G+F/F- or RSV-immunized mice at increasing times after immunization. Purified RSV F protein was the target antigen. (B) Anti-RSV G protein IgG antibody titers in sera from the mice described for panel A with time after immunization. The target antigen was RSV G protein expressed in 293T cells transfected with cDNA encoding the RSV G protein. No anti-F or anti-G protein antibodies in mice injected with PBS were detected (data not shown). (C) Neutralization titers of sera. Sera obtained at each time point were pooled, and neutralization titers were determined as described in the legend to Fig. 5. Each point is the average of three (days 20 to 99) or two (day 123) separate plaque assays, each done in duplicate. The standard deviations are shown.

Lung pathology after RSV challenge. The enhanced lung pathology resulting from RSV challenge of FI-RSV-, UV-RSV-, or purified F protein-immunized animals has been attributed to a T_H2 -biased immune response (reviewed in references 8 and 9). The IgG2a/IgG1 ratios after VLP immunization suggest that VLPs stimulate a T_H1 -biased immune response. To determine if VLP-H/G+F/F immunization resulted in increased lung pathology after RSV challenge, VLP-immunized mice were challenged with RSV and lungs were assessed for enhanced pathology as previously described (34). Included were three control groups of mice: (i) unimmunized, unchallenged mice, (ii) mice subjected to a primary RSV infection, and (iii) mice immunized, boosted, and then challenged with infectious RSV. Positive controls for ERD were mice immunized i.m. and boosted with FI-RSV. Lung sections from all mice were scored, blindly, for inflammation around blood vessels, around the airways, and in interstitial spaces, as described in Materials and Methods and previously (34). The results of two separate experiments were combined and are shown in Fig. 8A to C, respectively. While immunization with FI-RSV recapitulated previously documented abnormal histology of lungs after infectious virus challenge (for example, see reference 12), the lungs from mice immunized with VLP-H/G+F/F did not show this abnormal pathology. Rather, the inflammation scores for

the VLP-H/G+F/F-immunized mice showed no statistical differences from the scores for mice immunized and then challenged with infectious RSV or mice subjected to a primary RSV infection alone. In contrast, the differences between the scores of lungs from FI-RSV- and VLP-H/G+F/F-immunized mice were statistically significant. Thus, the lungs of RSV-challenged mice indicate that VLP-H/G+F/F-immunized mice do not show the abnormal pathology associated with the FI-RSV vaccine.

DISCUSSION

Virus-like particles can be effective, very safe vaccines, although their potential has not been fully exploited. The study described here characterized the assembly and immunological properties of a virus-like particle containing the major surface glycoproteins of respiratory syncytial virus. Because, as shown here, VLP release from cells expressing only RSV structural proteins was extremely inefficient, we focused on assembling RSV proteins into Newcastle disease VLPs (ND VLPs), which are very efficiently produced (30, 34). We have previously reported that a chimera H/G protein composed of the entire ectodomain of the RSV G protein fused to the TM and CT domains of the NDV HN protein could be efficiently assem-

FIG. 7. IgG subtype in sera of VLP-H/G+F/F-immunized mice. Mice were immunized with RSV, FI-RSV, UV-RSV, or VLP-H/G+F/F, as indicated at the bottom of each panel. (A and B) Mice immunized with RSV received in both prime and boost immunizations 6×10^5 PFU/mouse i.n. Mice immunized with FI-RSV received the equivalent of 1.5×10^7 PFU/mouse i.m. in both prime and boost immunizations. Mice immunized with UV-RSV received 30 µg total virion protein in the initial immunization and 10 µg in the boost. (C and D) Amounts of VLP-H/G+F/F in the initial immunizations are shown at the bottom of the panels. Boost immunizations were 10 µg total VLP protein. Sera were obtained at the times indicated, and antibody levels were determined by ELISA as described in Materials and Methods. The nanogram amounts of IgG2a and IgG1 in pooled sera were calculated on the basis of standard curves of purified IgG2a and IgG1. Each assay was done at least two times. The graphs plot the ratios of ng of IgG2a to ng of IgG1 present in the pooled sera with time after immunization. (A and C) Ratios of anti-F protein IgG2a/IgG1; (B and D) ratios of anti-G protein IgG2a/IgG1.

bled into ND VLPs. Results presented here demonstrate that the ectodomain of the RSV F protein fused to the TM and CT domains of the NDV F protein can also be incorporated into ND VLPs. Our results further showed that the presence of the H/G chimera protein considerably enhanced the incorporation of the F chimera protein into these particles. The immunological properties of these VLPs were assessed in a murine system. Immunization of mice with VLPs containing the ectodomains of both the RSV F and the RSV G proteins resulted in IgG antibody responses specific for the RSV F and G proteins that were as good as or better than the serum antibody responses after RSV infection. These IgG responses were primarily IgG2a. Furthermore, a single intramuscular injection of these VLPs stimulated durable, neutralizing antibody responses that persisted much longer than the neutralizing antibody responses after RSV infection initiated by intranasal inoculation, the natural route of infection of this virus. Differences in antibody responses to VLPs and RSV may be related to the form of the antigen, the route of immunization, or both. Lastly, immunization with VLPs containing both the F and G protein ectodomains did not result in enhanced lung pathology upon RSV challenge.

Assembly of ND VLPs containing RSV F protein. Assembly of RSV F protein sequences into VLPs required robust expression of the F protein from plasmid expression vectors. The

expression of RSV F protein cDNA from plasmid vectors has been reported to be very low (49). This restriction of expression has been attributed to internal poly(A) addition signals as well as other undefined sequences in the carboxyl-terminal half of the F protein open reading frame that were corrected by codon optimization (49). In contrast, we were unable to detect expression of a codon-optimized RSV F protein gene. However, we were able to detect expression of the wild-type F protein from a cDNA clone prepared using virion RNA as the template, even though this clone retained the internal poly(A) addition sites. Interestingly, the expression levels significantly increased by replacing the RSV F protein TM and CT domains with the analogous domains from the NDV F protein. Thus, the significant restriction of RSV F protein expression from expression plasmids is related primarily to sequences in the TM and CT domains. We do not yet know if restriction of wild-type protein expression is at the level of transcription, translation, or protein stability.

Both the expressed wild-type and the chimera proteins were efficiently cleaved and transported to cell surfaces in both avian and mammalian cells, indicating that the folding of the F protein ectodomain of the chimera protein is likely comparable to RSV F protein folding.

Requirements for incorporation of chimera glycoproteins into ND VLPs. To determine if there was a cell type depen-

FIG. 8. Scores of pulmonary inflammation after RSV challenge of immunized mice. Four groups of five mice each were immunized with RSV i.n. (6×10^5 PFU/mouse), 10 or 30 μ g total VLP-H/G+F/F protein, or FI-RSV (equivalent of 1.5×10^7 PFU/mouse). Two groups were injected with PBS (50 μ l). After 38 days, mice were boosted with RSV (6×10^5 /mouse), VLP-H/G+F/F (10 μ g total protein/mouse), FI-RSV (equivalent of 1.5×10^7 PFU/mouse), or PBS. Seven days postboost immunization, mice treated as indicated at the bottom of the figure were challenged with RSV (1.6×10^6 /mouse). Six days post-challenge, lungs were harvested as described in Materials and Methods. Tissue sections stained with H&E from each mouse were scored for inflammation on a scale of 0 to 3 as described in Materials and Methods. Results from two separate experiments are combined. (A) Blood vessels; (B) airways; (C) interstitial spaces. Bars indicate groups compared for statistical analysis, using GraphPad Prism software (*t* tests), with *P* values shown above the bar.

dence on release of ND VLPs containing RSV glycoprotein ectodomains, we characterized the release of VLPs in avian, primate, and human cells. The most efficient release of ND VLPs containing both chimera glycoproteins was from avian cells. This result is likely a property of NDV proteins, since VLPs with only NDV proteins are also most efficiently released from avian cells. Interestingly, release of particles containing one or the other chimera protein was comparable in all three cells types, as detected by the levels of NDV NP, but addition of both F/F and H/G glycoproteins or addition of both NDV HN and F proteins depressed particle release in mammalian cells. The reasons for these observations are unknown. It is

possible that there are cell type interactions of glycoprotein tails with the cellular machinery involved in particle assembly and release. Alternatively, there may be a cell type difference in glycoprotein protein localization in specific cellular domains important for virus assembly.

Most interesting is the observation that coexpression of the H/G chimera protein with F/F protein significantly enhanced incorporation of the F/F protein into particles in all three cell types. The mechanisms involved are unclear. Expression of the H/G protein does not increase expression of the F/F protein (unpublished observations). Perhaps there are interactions between the G and F protein ectodomains that enhance F/F protein assembly into particles. Alternatively, the H/G protein may change the localization of the F/F protein into cell domains important for particle assembly.

Since expression of the H/G protein enhanced the incorporation of the F/F protein into VLPs and since antibody responses to both F and G proteins can be protective, VLP-H/G+F/F was chosen for use in analysis of the immunological properties of VLPs containing RSV F protein sequences. Quantitative amounts of VLPs containing NDV NP and M protein and the two chimera proteins were readily prepared in avian cells.

Antibody responses to VLP-H/G+F/F. The effectiveness of a humanized monoclonal antibody specific for RSV F protein as a prophylaxis (3, 6, 40) demonstrates that serum antibodies specific to RSV F protein can be protective. Anti-G protein antibodies are also protective. It has been suggested that the soluble form of G protein acts as a fractalkine antagonist and is thus involved in immune evasion by the virus, ultimately resulting in disease (16, 50, 51, 54). Tripp and colleagues have shown that antibodies specific to the fractalkine CX3C motif in the G protein will block the binding of the G protein to the CX3C receptor and that immunization with a peptide encoding the CX3C motif protected mice from RSV challenge and decreased pulmonary inflammation (44, 54). Thus, stimulation of antibodies to the RSV G protein is likely also an important component of protection from RSV.

VLP immunization resulted in IgG antibody responses to both the RSV F and the RSV G proteins that were as good as or better than the serum IgG responses after RSV infection. While antibody responses were increased by an order of magnitude upon a boost immunization, a single immunization resulted in high levels of anti-F and anti-G protein antibodies that did not significantly decrease over 125 days. These results are consistent with T cell-dependent immune responses and provide strong circumstantial evidence for development of long-lived plasma cells in response to these VLPs. We have not yet directly characterized T cell responses to VLP-H/G+F/F. However, we have reported that ND VLPs containing the NDV HN and F proteins activated CD4⁺ and CD8⁺ T cells (30).

VLP immunization also resulted in primarily IgG2a responses. IgG2a is the subtype that is the most prevalent during virus infections (11) and the subtype that is the most effective in providing protection from virus infections (27). A similar predominance of the IgG2a subtype has been reported after simian immunodeficiency virus (SIV) VLP immunization (53) as well as after immunization with VLPs composed of Q β proteins and single-stranded RNA or CpG (20). This IgG

subtype can be the result of B cell isotype switching stimulated by T_H1 cell-derived cytokines, while switching to IgG1 has been attributed to T_H2 cell-derived cytokines (7). Thus, the ratio of IgG2a to IgG1 has been used as a marker for T_H1 versus T_H2 responses (12, 27).

B cell isotype switching can also be induced by direct interaction of an antigen with Toll-like receptors (TLRs) expressed on B cells (20). Jegerlehner et al. (20) have reported that direct stimulation of B cell TLR7 or TLR9 by VLPs composed of Q β proteins and single-stranded RNA or CpG is responsible for the B cell class switch recombination to IgG2a. Delgado et al. (12) have proposed that nonreplicating RSV vaccines such as FI-RSV and UV-RSV fail to protect because of inefficient direct TLR activation of B cells which leads to a lack of affinity maturation of antibodies. The mechanism for induction of class switching to predominantly IgG2a after VLP-H/G+F/F immunization is not yet known. However, these VLPs may activate TLRs, either in T_H cells or in B cells, due to the reported interaction of RSV F protein with TLR4 (23). Alternatively, paramyxovirus VLPs are reported to contain random host cell RNAs due to their interactions with NP (reviewed in reference 17). Thus, these single-stranded RNAs could stimulate immune cells via a TLR7 pathway.

Protective immune responses to VLPs. Antibody responses to VLP-H/G+F/F also included potent neutralizing antibody responses, and, importantly, the neutralization titers did not decrease with time. In contrast, while serum IgG titers did not decrease with time after RSV intranasal infection, neutralizing antibody titers did diminish to undetectable levels. This result suggests that after RSV infection, in contrast to VLP immunization, murine B cells producing neutralizing antibody responses to RSV are not recruited into the long-lived plasma cell pool, which indicates differences between B cell responses to RSV i.n. infection and VLP i.m. immunization in murine systems.

We have previously shown that immunization with a single dose of VLP-H/G completely protected mice from RSV challenge (34). Not surprisingly, a single immunization with VLPs containing not only the G protein but also the F protein ectodomain similarly protected mice from RSV replication. Thus, inclusion of the F protein ectodomain in the VLPs had no negative effect on the protective responses observed with VLP-H/G.

Failure of the FI-RSV vaccine to protect and its stimulation of ERD have been attributed to a T_H2 -biased immune response (8, 9, 25, 37). Indeed, as previously reported (12), anti-F protein antibodies stimulated by FI-RSV were primarily IgG1, as were antibodies specific for the G protein. This result, as well as negligible neutralizing antibody responses after FI-RSV immunization (34), correlated with the ERD after RSV challenge of FI-RSV-immunized animals observed here and previously (34). The predominant IgG2a responses to VLP-H/G+F/F, indicative of T_H1 -biased immune responses, and the potent neutralizing antibody titers are consistent with the absence of enhanced respiratory disease after RSV challenge of VLP-H/G+F/F-immunized mice.

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