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Vaccination with Recombinant Alphavirus or Immune-Stimulating Complex Antigen Against Respiratory Syncytial Virus[1]

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Respiratory syncytial virus (RSV) causes severe respiratory diseases in infants and young children. Inappropriate immunity to the virus can lead to disease enhancement upon subsequent infection. In this study, we have characterized the antiviral immunity elicited by the recombinant Semliki Forest virus (SFV) encoding the RSV fusion (F) and attachment (G) protein, and compared with that induced by the immune-stimulating complex (ISCOM)-incorporated FG proteins. Antiviral immunity against RSV elicited nasally or parentally by either of the immunogen having divergent profiles could reduce lung RSV titers upon challenge. However, resistance to RSV without disease enhancement was only observed in those vaccinated with SFV recombinants via nasal route. Presence of postvaccination pulmonary IFN-γ response to the H-2K^d-restricted T cell epitope ($F_{85–93}$; KYKNAVTEL) was found to be associated with absence of enhanced pulmonary disease and goblet cell hyperplasia as well as reduced Th2-cytokine expression. This result demonstrates that the SFV recombinants can result in enhanced clearance of RSV without enhancing the RSV-associated disease, and underlines the importance in priming pulmonary MHC class I-restricted T cells when RSV FG-based vaccines are used. ***The Journal of Immunology,*** **2002, 169: 3208–3216.**

Respiratory syncytial virus (RSV)[3] causes annual epidemics worldwide. There is a wide spectrum of clinical illness and symptoms in RSV-infected infants and children. Symptoms can range from a mild cold-like illness to severe respiratory distress and death, even in previously healthy children. RSV infection is responsible for a large proportion of hospitalizations of infants in the winter months and consumption of tremendous health care resources. Significant morbidity is associated with RSV bronchiolitis, and a link to development of asthma has been proposed (1). RSV is a negative sense RNA paramyxovirus in which the major virus neutralization Ags are the virus spike proteins, the glyco (G) and the fusion (F) proteins that mediate attachment and fusion to the host cell during virus infection (2). Attempts to vaccinate children using a Formalin-inactivated RSV absorbed to alum adjuvant (FI-RSV) led to enhanced lung pathology upon subsequent exposure to natural RSV infection (3, 4). Enhanced lung pathology during RSV infection is also observed in FI-RSV-vaccinated BALB/c mice, which thus has become a useful model for RSV pathogenesis. There is strong evidence indicating that this lung disease is a T cell-mediated immunopathological condition (5). Vaccination of BALB/c mice with the G or F protein of RSV can increase disease severity during clearance of RSV, due to Th2-driven lung eosinophilia and Th1-driven pulmonary infiltration, respectively (6–9). To date, few RSV vaccine candidates have shown promising result in preclinical studies (10), and no vaccine has yet been proven safe and efficacious in humans.

In search of new RSV vaccine candidates, we have compared two RSV vaccine strategies in the murine RSV model. The first immunogen is based on an alphavirus vector, the Semliki Forest virus (SFV) particles, which are self-abortive vector particles that express RSV F and G proteins from an RNA replicon. One feature that makes SFV vectors attractive is that SFV vaccine particles are designed to undergo one round of infection without giving rise to production of new virus progeny, thereby eliminating the safety concerns that may hinder the use of other viral recombinants. Replication of SFV RNA allows expression of the inserted foreign gene in infected cells, which has been shown to induce strong humoral and cell-mediated immunity to the heterologously expressed protein in several disease models in rodents and primates (11–15). The SFV infection induces production of type I and II IFNs, and infected cells undergo apoptotic death within a few days, which may be important in molecular mechanisms involving cross-priming of professional APCs (16–18).

The second immunogen is the immune-stimulating complex (ISCOM)/FG particles that consist of RSV F and G proteins incorporated in ISCOM, which is composed of *Quillaja* saponin, lipids, and Ags incorporated in the same particle that has inbuilt adjuvant property (19, 20). When delivered by ISCOM, Ags exhibit enhanced immunogenicity by inducing a wide range of immune effector responses, including Ab production, T cell proliferation, and MHC class I-restricted responses. Efficient induction of RSV-specific and neutralizing IgG and IgA responses by ISCOM/FG has been demonstrated previously (21); however, the efficacy of the ISCOM/FG to elicit RSV-specific T cell responses

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[3] Abbreviations used in this paper: RSV, respiratory syncytial virus; β-gal, β-galactosidase; BAL, bronchoalveolar lavage; DC, dendritic cell; F, fusion protein of RSV; FI-RSV, Formalin-inactivated RSV; G, attachment protein of RSV; i.n., intranasal; ISCOM, immune-stimulating complex; PAS, periodic acid-Schiff; SFV, Semliki Forest virus; Tc, cytotoxic T cell.

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and to protect against RSV infection or its disease has not been evaluated.

In this study, we demonstrate that nasal vaccination with SFV/F or SFV/FG could elicit immunity against RSV, including IFN-γ responses to the recently identified CTL epitope in the F protein (22), and resistance to RSV infection did not lead to exacerbation of the disease. But, in contrast to SFV recombinants, the IFN-γ response to the F CTL epitope was omitted from the antiviral immunity elicited by ISCOM/FG, and enhanced postchallenge lung pathology with goblet cell hyperplasia was observed. Our result suggests that local immunity to RSV provided by SFV recombinants seems best in enhancing RSV clearance and the associated disease enhancement. This result also underlines the importance in priming CTLs when using FG-based RSV vaccines.

Materials and Methods

Preparation of RSV stock

RSV (subtype A, Long strain) was propagated in MA104 cells in DMEM medium (Life Technologies, Gaithersburg, MD) supplemented with 3% heat-inactivated FCS, 2 mM glutamine, 20 mM HEPES, nonessential amino acid, penicillin, and streptomycin. A high-titer RSV stock purified by ultracentrifugation through a 20% sucrose gradient was used in this study. Virus titration was conducted before each challenge experiment by plaque assay with agarose overlay on MA104 and Vero cells (23).

Preparation of rSFV particles expressing SFV/F and SFV/FG

The plasmids pSFV/F and pSFV/FG were made by inserting the genes encoding the F or G protein of RSV, kindly provided by J. A. Melero (Instituto de Salud Carlos III, Madrid, Spain), into the pSFV-4.2 expression vector. rRNAs were synthesized in vitro and then packaged into SFV particles in BHK-21 cells using the SFV two-helper RNA system (24, 25). rSFV particles were harvested and purified by ultracentrifugation through a 20% sucrose cushion. The protein expression was verified in infected BHK-21 cells by metabolic labeling with [^{35}S]methionine and further confirmed by immunoprecipitation. In SFV/FG, the F and G proteins are expressed in approximately equal amount (data not shown). Immunoprecipitation and indirect immunofluorescence assays were performed with mAbs 47F and 021/1G, which were gifts of J. A. Melero.

Preparation of ISCOM/F and FI-RSV

ISCOM/FG was prepared from sucrose gradient-purified virus (Long strain). The preparation procedure and biochemical characterization of ISCOM/FG were performed, as previously described (21). The FI-RSV absorbed directly to an aluminum hydroxide gel adjuvant (Superfos Biosector a/s, Kvistgaard, Denmark) was prepared using the purified RSV by procedures used for the 1969s vaccine trial (4).

Mice and immunization

Age- and sex-matched BALB/c mice (6–10 wk old) were obtained from the breeding unit at MTC (Karolinska Institutet). Groups of mice were anesthetized with Metofane (Pitman-Moore, Mundelein, IL) and immunized through intranasal (i.n.) route or s.c. route in the right flank. Immunization was given on wk 0 and 4. ISCOM/FG was given according to a previously described immunization protocol (5 μg for i.n. and 1 μg for s.c. route) (21). rSFV particles were administered at 10^6 infectious units per dose. Control mice were given SFV/β-galactosidase (β-gal), ISCOM/matrix (empty ISCOM), or PBS. FI-RSV was given i.m., as previously described (3, 4). Blood and bronchoalveolar lavage (BAL) were collected 2 wk after the second immunization, as previously described (11).

Titration of IgG Abs specific to the RSV proteins

Sucrose gradient-purified RSV prepared from infected MA104 cells was passively adsorbed overnight at +4°C onto the wells of microtiter plate (F96 Maxisorp, Nunc Immunoplates, Roskilde, Denmark) at concentration of 5 μg/ml in 30 mM sodium carbonate, 68 mM sodium bicarbonate, pH 9.4. The plates were blocked with 2% BSA/PBS. Serially diluted serum samples were added to wells to allow Ab binding. Goat anti-mouse IgG alkaline phosphatase conjugate (Sigma-Aldrich, St. Louis, MO) was used to indicate bound Abs. To determine IgG-specific isotype titers, HRP-labeled mAbs to the mouse IgG1, IgG2a, IgG2b, and IgG3 subclasses (Southern Biotechnology Associates, Birmingham, AL) were used. The enzymatic reactions were developed with *p*-nitrophenyl phosphate substrate or tetramethylbenzidine substrate, respectively. The assay cutoff value was calculated by adding three times the SD to the mean OD of sera from control mice.

Virus neutralization assay

Serum samples were tested in duplicates for virus-neutralizing activity on RSV. The procedure for end-point neutralization has been described previously (26). Briefly, serial 4-fold dilutions of mouse sera in a volume of 0.15 ml were mixed with an equal volume of 300 tissue culture-infective $dose_{50}$ RSV (Long strain). The mixtures were shaken and incubated at room temperature for 1 h. Ag-Ab mixtures were added in duplicates in a volume of 0.1 ml to monolayer of MA104 cells grown in tissue culture tubes, and incubated at 37°C in a roller incubator. The cells were inspected for cytopathic effect, and final readings were made on day 7.

In vitro restimulation and cytokine measurement

Spleens and lungs were removed the day before RSV challenge, and processed through cell strainers. Single cell suspension was cultured at 6×10^6/ml in RPMI supplemented with 2% heat-inactivated FCS, 2 mM glutamine, 50 mM 2-ME, 1 mM sodium pyruvate (Life Technologies), and various concentrations of UV and heat-inactivated RSV (sucrose gradient purified). Supernatants were collected after 24 and 48 h and analyzed for presence of IFN-γ, IL-4, and IL-5 by capture ELISA. The ELISAs were performed using paired Abs purchased from Endogen (Woburn, MA). Ab pairs were MM-700 and MM-700-B (murine IFN-γ), MM-450C and MM-450D-B (murine IL-4), and MM-550D and MM-550C-B (murine IL-5). To identify the phenotype of IFN-γ-producing cells, mAbs anti-CD4 (clone GK1.5) or anti-CD8 (clone 53-6.7) purchased from BD PharMingen (San Diego, CA) were added in the restimulation culture at 10 μg/ml. Captured cytokines were detected with HRP-conjugated streptavidin and tetramethylbenzidine substrate. Serially diluted recombinant cytokine protein was used as standard reference. The ELISA detection limit was 12.3 pg/ml for IFN-γ, 4.6 pg/ml for IL-4, and 12 pg/ml for IL-5.

Enzyme-linked immunospot

Spleen and lungs (three mice per group) were removed for enumeration of IFN-γ-secreting cells by cytokine ELISPOT system (murine IFN-γ; U-CyTech, Utrecht, The Netherlands), according to manufacturer's recommendation. Briefly, spleens and lungs were removed and processed through sterile cell strainers (100 μM). Purified single cell suspensions were plated overnight in RPMI medium (5% FCS, 1 mM sodium pyrovate, 2 mM L-glutamine, and penicillin streptomycin) with or without 1 μg/ml purified UV and heat-inactivated RSV, and synthetic peptides $F_{85–93}$ KYKNAV TEL or $G_{183–197}$ WAICKRIPNKKPGKK that correspond to sequences of H-2^d-restricted T cell epitopes (22, 27, 28). The peptides were purchased from Innovagen (Lund, Sweden) and have 80% purity. The cells were plated at concentrations of 4×10^6 and 1.3×10^6 cells/ml with or without RSV Ag. Con A (4 μg/ml) was used as positive control. After overnight incubation, spots formed from IFN-γ-producing cells were developed and detected following manufacturer's recommendation, and counted under a light microscope.

Live RSV challenge and lung cytokine mRNA analysis

Three weeks after the second immunization, mice were challenged i.n. with 2×10^6 PFU sucrose gradient-purified RSV (Long strain) diluted in cold sterile PBS. Four days later, lungs were removed, weighted, and homogenized in 10% (w/v) suspension of complete DMEM (Life Technologies; 10% FCS, 2 mM glutamine, 20 mM HEPES, nonessential amino acid, penicillin, and streptomycin sulfate). Cleared homogenates were serially diluted in complete DMEM and assayed for RSV titer by plaque assay. For lung cytokine analysis, lungs were snap frozen immediately after removal from sacrificed animals and stored at −70° until use. Total RNA was isolated and subjected to analysis of cytokine mRNA by RT-PCR. Reverse-transcriptase was conducted by adding 1 μg RNA to reaction cocktail consisting of 10 μg/ml oligo$(dT)_{15}$, 0.5 mM of each dNTP, 10 mM DTT, 30 U RNasin (Promega, Madison, WI), and 200 U Moloney murine leukemia virus reverse transcriptase (Life Technologies). The reaction mixture was incubated at 37°C for 1 h and 95°C for 5 min. Aliquots of cDNA were used to amplify cytokine mRNAs using following oligonucleotides: IL-4, ATGGGTCTCAACCCCCAGCTA and CGAGTAATCCATTTGCATG AT; IL-5, ATGAGAAGGATGCTTCTGCAC and TCAGCCTTCCATT GCCCACTC; IFN-γ, ATGAACGCTACACACTGCAC and GCAGCG ACTCCTTTTCCGCTT; IL-12p40, CCACTCACATCTGCTGCTCCACA AG and CAGTTCAATGGGCAGGGTCTCC, IL-13, TCTCCCCAGCAA AGTCTGAT and CTGGATTCCCTGACCAACAT; β-actin, ATCGTGC GTGACATCAAAGA and TGGAAGGTGGACAGTGAGGC. To keep

Table I. *Anti-RSV responses in BALB/c mice given i.n. or s.c. vaccination*

Vaccine	Immunization Route	Serum IgG[a] (log_{10})	IgG2a:IgG1 Ratio	Neutralizing[b] (log_2)	BAL IgA (log_{10})	RSV-Negative[c] Animals (%)	Lung RSV[c] Titer (log_{10})
ISCOM/FG	i.n.	5.45 ± 0.72	0.44	4.0 ± 0.8	3.53 ± 0.4	5/6 (83)	3.56 ± 0
SFV/F	i.n.	5.36 ± 0.25	7.10	4.7 ± 0.47	3.12 ± 0	6/6 (100)	<1.87
SFV/FG	i.n.	4.71 ± 0.25	18.7	4.0 ± 0	3.20 ± 0	8/8 (100)	<1.87
ISCOM-matrix	i.n.	<2 ± 0	n.t.	<2 ± 0	n.t.	0/4 (0)	4.53 ± 0.11
SFV/β-gal	i.n.	<2 ± 0	n.t.	<2 ± 0	n.t.	0/4 (0)	4.65 ± 0.21
ISCOM/FG	s.c.	5.52 ± 0.39	0.85	5.3 ± 0.5	<1.69	6/6 (100)	<1.87
SFV/F	s.c.	4.80 ± 0.36	2.73	3.5 ± 0.5	<1.69	6/6 (100)	<1.87
SFV/FG	s.c.	3.26 ± 0.63	5.24	3.0 ± 0.5	n.t.	3/8 (37.5)	3.35 ± 0.27
ISCOM-matrix	s.c.	<2 ± 0	n.t.	<2 ± 0	n.t.	0/4 (0)	4.74 ± 0.05
SFV/β-gal	s.c.	<2 ± 0	n.t.	<2 ± 0	n.t.	0/4 (0)	4.67 ± 0.09
PBS	i.n.	<2 ± 0	n.t.	<2 ± 0	<1.69	0/10 (0)	4.48 ± 0.26
FI-RSV	i.m.	5.47 ± 0.54	<0.01	n.t.	<1.69	5/5 (100)	<1.87

[a] Groups of mice were immunized at wk 0 and 4. Sera and BAL were collected and analyzed at wk 6 to determine levels of RSV-specific IgG and IgA by RSV ELISA. Results are expressed as geometric mean ± SD; n.t., not tested.

[b] Mean end-point serum dilution that can completely inhibit cytopathic effect following incubation with 300 tissue culture-infective $dose_{50}$ RSV in an in vitro neutralization assay.

[c] Number of RSV-negative animals 4 days after RSV challenge (2.2×10^6 PFU/mouse), and the mean lung RSV titer ± 1 SD in RSV-positive animals determined by RSV plaque assay. The assay detection level was 75 PFU/g lung.

PCR in a linear range of amplification, the number of cycles used for each primer set was determined empirically. For all primer sets except β-actin, 30 amplification cycles were required. The β-actin cDNA was amplified for 25 cycles. Final PCR products were analyzed on agarose gels. A semi-quantitative measurement of PCR products has been conducted on a densitometer using the ImmageMaster program (Pharmacia Biotech, Uppsala, Sweden).

Lung histopathology

Five days after RSV challenge, the animals were killed by CO_2. Lungs were perfused via trachea using Bouin's Solution (Sigma-Aldrich). Histopathology of H&E-stained lung sections was evaluated according to the lung pathology index described previously (29). Individual slides were then read blindly, and 30 areas in each slide were scored. In brief, inflammatory infiltrates of bronchioles and pulmonary vessels were scored from 1 to 6: 1, surrounding space free of infiltrating cells; 2, surrounding space contains few infiltrating cells; 3, surrounding space contains focal aggregates of infiltrating cells; 4, surrounding space contains single uninterrupted layer of infiltrating cells; 5, surrounding space contains two uninterrupted layers of infiltrating cells; 6, surrounding space contains three or more uninterrupted layers of infiltrating cells. The presence of goblet cells was examined on periodic acid-Schiff (PAS)-stained lung sections.

Collection of BAL fluid and measurement of BAL leukocyte subsets

The animals (four mice per group) were killed and bled on day 5 post-challenge. Lungs were perfused via trachea with 1 ml 12 mM Lidocaine in PBS. Collected BAL fluids were centrifuged and resuspended in 100 μl PBS, of which 5 μl was subjected to cytological analysis. Duplicates of BAL preparations were air dried and stained with May-Grunwald Giemsa. The number of BAL leukocytes was counted, and the number of monocytes/macrophages, eosinophils, and lymphocytes was identified and registered by microscopic examination.

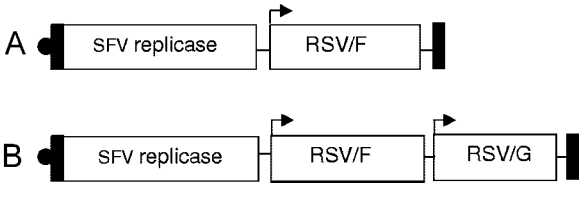

FIGURE 1. Genomic structures of encapsidated rSFV RNA. *A*, The SFV/F construct encodes the SFV replicase, followed by the RSV F gene. *B*, The SFV/FG construct encodes the SFV replicase, followed by the RSV F and G with individual SFV 26S subgenomic promotor to allow expression of F and G separately. Arrow indicates the site of SFV 26S subgenomic promotor that controls the heterologous gene expression.

Results

Ab responses following immunization with ISCOM/FG or SFV recombinants

We have previously demonstrated that the serum IgG response elicited by ISCOM/FG showed no quantitative difference after i.n. or s.c. immunization, and that BAL IgA could be induced by i.n. immunization (21). In this study, a qualitative difference regarding the serum IgG response was observed. In the s.c. group, this response was characterized of a balanced IgG2a-IgG1 ratio with RSV-neutralizing activity, and consists of high levels all IgG isotypes, while this response in the i.n. group showed reduction in IgG2a-IgG1 ratio and neutralizing activity, as well as loss of IgG3 (Table I and Fig. 2).

rSFV particles encoding the F or F and G of RSV were made and designated as SFV/F and SFV/FG, respectively (Fig. 1). In this study, we show that the SFV/F and SFV/FG, but not SFV/β-gal, elicited RSV-specific Ab responses (Fig. 2 and Table I). Serum IgG end-point titers were in general 4–5 log_{10}, and consisted of all four IgG isotypes, except the group that was given SFV/FG via s.c. route in which lower response was observed. It was noticed that

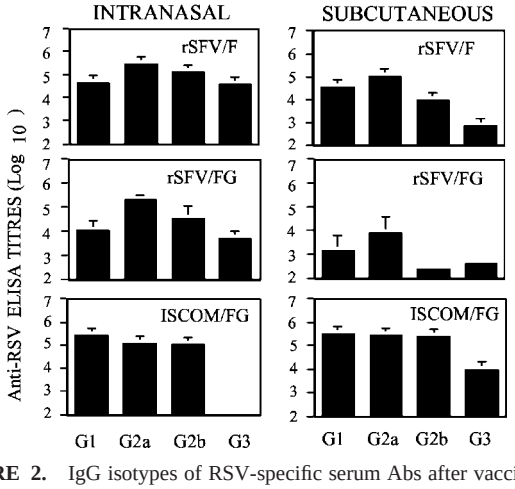

FIGURE 2. IgG isotypes of RSV-specific serum Abs after vaccination. Mice were immunized i.n. or s.c. with ISCOM/FG, SFV/F, or SFV/FG at 0 and 4 wk. Sera were collected at wk 6 and end point titrated by RSV ELISA for IgG isotype-specific Abs. Data are expressed as log_{10} mean Ab titers ± SD.

nasal immunization generated higher Ab response with better neutralizing activity than s.c. immunization. Also, irrespective of the immunization route, SFV recombinants elicited Abs with high IgG2a-IgG1 ratios, indicating that a Th1 response was elicited, which differed from the result of ISCOM/FG. Higher IgG2a-IgG1 ratios were generally observed following nasal immunization, also a result that differs from ISCOM/FG. The BAL IgA response was induced at similar level as i.n. ISCOM/FG immunizaiton.

RSV-specific cytokine response in spleen following i.n. immunization

Spleen cells were prepared after i.n. immunization and cultured with increasing concentration of Ag (inactivated RSV particles). As shown in Fig. 3*A*, splenocytes from animals primed with ISCOM/FG, SFV/F, or SFV/FG produced IFN-γ in the presence of RSV Ag in a dose-dependent manner. However, IFN-γ production in splenocytes primed with ISCOM/FG required less RSV Ag than those primed with SFV-based vaccines (1 μg, $p < 0.0001$ and 0.0001; 5 μg, $p = 0.00014$ and 0.00024, comparing ISCOM/FG with SFV/F and SFV/FG, respectively; Fig. 3*A*). Secreted IFN-γ was blocked by anti-CD4 Ab treatment, but not by anti-CD8 Ab (data not shown). IL- 4 or IL-5 could not be detected in any of the culture supernatant collected from 24- and 48-h cultivation (data not shown).

In a separate immunization experiment, spleen and lung were obtained and single cell suspensions were tested in an IFN-γ ELISPOT assay. Again, ISCOM/FG-vaccinated animals had more splenic and pulmonary IFN-γ-secreting cells reacting to the inactivated RSV Ag (Fig. 3*B*) than SFV/F- or SFV/FG-vaccinated animals (spleen, $p = 0.0013$ and 0.0057; lung, $p = 0.0004$ and 0.024, comparing ISCOM/FG with SFV/F and SFV/FG, respectively). From these results, we conclude that both ISCOM and SFV vaccines administered at mucosal sites are able to prime RSV-specific $CD4^+$ T cells. Furthermore, the result also indicates that less RSV Ag is required to recall this response following ISCOM/FG vaccination.

Lung and splenic IFN-γ responses to immunodominant epitopes within F and G proteins

H-2^d-restricted T cells with cytotoxic and helper effector function directed to the $F_{85–93}$ and $G_{183–197}$ peptides, respectively, have recently been described (22, 27, 28). We used these two peptides in an IFN-γ ELISPOT assay to determine the frequency of the peptide-specific memory effector cells in spleen and lungs before RSV challenge. In the spleen, the number of IFN-γ-secreting cells responding to $F_{85–93}$ was highest in SFV-F- or SFV/FG-vaccinated animals (Fig. 4). The $G_{183–197}$-specific IFN-γ-secreting cells were also detected, although at lower frequency. In the lung, the $F_{85–93}$-specific response was elevated only in the groups receiving SFV/F or SFV/FG by i.n. route. Lung $G_{183–197}$-specific response was rarely detected. The ISCOM/FG-vaccinated animals, despite robust Ab and T cell responses against inactivated RSV Ag, had low to no detectable IFN-γ-secreting T cells responding to either $F_{85–93}$ or $G_{183–197}$. The 48-h culture supernatants tested by cytokine ELISA further confirmed a higher IFN-γ concentration in the SFV vaccine group. IL-4 and IL-5 were not detected in any of the culture supernatants (data not shown).

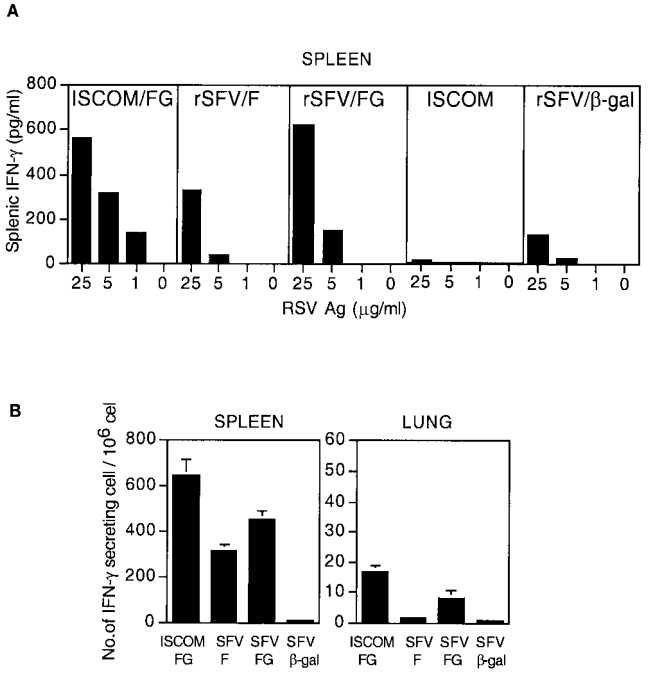

FIGURE 3. Splenic IFN-γ response to RSV Ag is generated after nasal immunization with ISCOM/FG, SFV/F, or SFV/FG. *A*, Spleen cells from mice immunized with indicated immunogen (three mice per group) were pooled and restimulated in triplicates with various concentrations of purified heat/UV-inactivated RSV. Result shows the concentration of IFN-γ in the 48-h culture supernatants measured by cytokine ELISA. *B*, Cells from spleen or lung of mice immunized with indicated immunogen (three mice per group) were cultured overnight with or without purified heat/UV-inactivated RSV at 2 μg/ml. Result shows the numbers of IFN-γ-secreting cells determined by ELISOT assay. Data are expressed as mean response ± 1 SD obtained from two independent experiments.

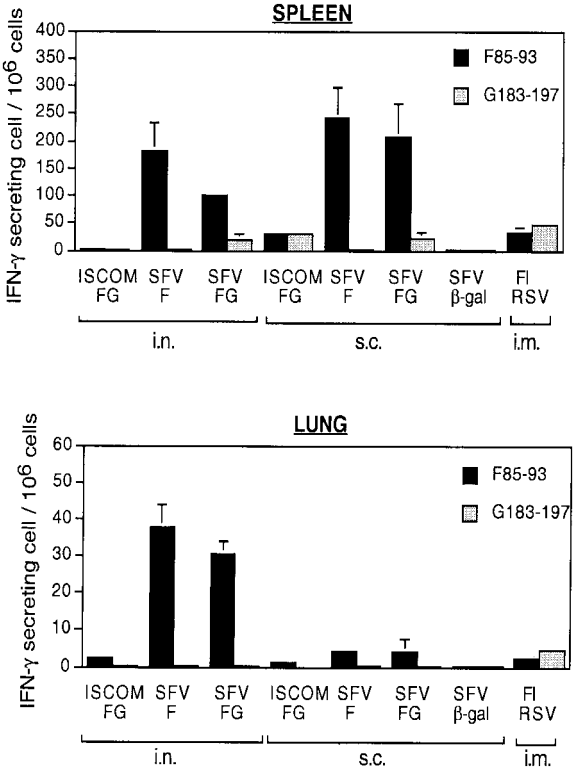

FIGURE 4. Increased F85–93-specific IFN-γ responses in lung and spleen after i.n. immunization with SFV/F or SFV/FG. Spleens or lungs from indicated group (three mice/group) were pooled and analyzed by IFN-γ ELISPOT assay after restimulation with the immunodominant F85–93 CTL or G183–197 Th peptide. The experiment was repeated twice with similar results. Data are expressed as mean number ± 1 SD of IFN-γ-secreting cell per million cells.

RSV challenge and postchallenge lung cytokine profile

We next asked whether the immune response elicited by the two types of immunogens could enhance clearance of RSV infection. The levels of lung RSV on day 4 postinfection were at ~4 $\log_{10}$ PFU/g lung in sham-immunized mice receiving SFV/β-gal, ISCOM/matrix, or PBS (Table I). In contrast, RSV was not detected in lungs in any of the mice immunized with SFV/F or SFV/FG i.n., or in those given ISCOM/FG or SFV/F s.c. Mice immunized with ISCOM/FG by i.n. or SFV/FG by s.c. route were not all RSV negative; however, their viral titers were significantly reduced compared with control mice. The outcome of the challenge result for animals given SFV recombinants correlates with the Ab levels shown in Table I.

Because the FI-RSV-enhanced immunopathology is associated with production of Th2 cytokines in lungs (30, 31), the cytokine profile in lung tissues after RSV challenge was analyzed by a semiquantitative RT-PCR method. Compared with control mice (sham infected or naive), FI-RSV-immunized mice had increased levels of mRNA encoding Th2-associated cytokines IL-4, IL-5, IL-13, and Th1 cytokine IL-12 (Fig. 5*A*). Mice immunized with ISCOM/FG showed increased IL-13 and IFN-γ, but no IL-4 or IL-5 expression. Increased IL-12 expression was also observed in mice i.n. immunized with ISCOM/FG. The SFV/F i.n. and s.c. immunized mice showed expression of IL-12 and IFN-γ; however, the s.c. immunized mice also showed a modest increase of IL-13 expression. Similar as SFV/F, the SFV/FG i.n. immunized animals showed an increased Th1, but not Th2 cytokine expression (Fig. 5*B*). An increased variation was observed in unvaccinated animals. This variation most likely reflects the difference in the kinetics of cytokine response at this early time point, as the peak T cell response during primary RSV infection occurs not until several days later (22).

Because SFV/FG vaccination, when administered s.c., could only enhance RSV clearance from the lungs (in this study referred to as protected) in <50% of animals, the protected (sc-p) and nonprotected (sc-np) animals were analyzed as two separate groups. There was a significant increase of IL-13 in the protected mice (sc-p). However, in the nonprotected mice (sc-np), increase in several cytokines, including IL-4, IL-13, IL-12, and IFN-γ, was observed (Fig. 5*B*).

The results indicate that upon RSV challenge, SFV/F or SFV/FG i.n. vaccinated animals can develop lung cytokine profiles similar to those undergoing a primary RSV infection, which involves a mainly Th1-type cytokine response. On the contrary, SFV/F and SFV/FG given by s.c. route gave similar postchallenge cytokine pattern as ISCOM/FG, which consists of both Th1 and Th2 cytokine response, and the latter involves mostly IL-13. Morevover, the SFV/FG given s.c. could lead to an increase in Th2 cytokine transcripts in particular when lung RSV replication was not fully restricted.

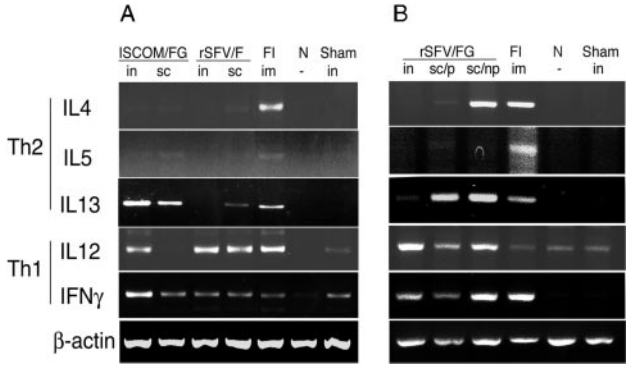

FIGURE 5. Absence of lung Th2 cytokines during RSV infection in mice previously immunized with SFV/F or SFV/FG via i.n. route. On day 4 postchallenge, RNA was extracted from lungs and analyzed for expression of cytokine and housekeeping (β-actin) genes. *A*, Postchallenge lung Th2 cytokine expression was not detected in mice i.n. immunized with SFV/F, but was detected in those immunized with ISCOM i.n. and s.c., SFV/F s.c., or FI-RSV i.m. (FI). *B*, Lung Th2 cytokine expression was lower in SFV/FG i.n. immunized animals compared with s.c. immunized animals. p, Protected; np, nonprotected. Sham-immunized mice were given SFV/β-gal or ISCOM/matrix at the time of immunization and challenged as above. Naive control mice (N) were not immunized or exposed to RSV infection. The experiment was performed using pooled lung RNA from two mice each time from three independent experiments.

Postchallenge lung histopathology and BAL leukotypes

Lungs were examined for signs of pathology at day 5 postchallenge. At this time point, PBS- or SFV/β-gal-immunized mice showed similar lung RSV titers ($4.3\text{-}4.7 \times 10^4$ PFU/g lung) as the previous experiment shown in Table I. H&E- or PAS-stained lung sections (Fig. 6) in these mice showed mild to moderate peribronchial inflammation and moderate increase of PAS-positive mucus-producing goblet cells (group D), not found in naive control mice (group N). In contrast, mice primed with FI-RSV showed augmented interstitial disease (group FI), with massive layers of dense leukocyte infiltration that completely surrounded the bronchioles and arterioles and a marked goblet cell hyperplasia characterized by higher goblet cell density and larger cell size. Immunostaining of lung sections showed that the infiltrating cells comprised predominantly of $CD3^+$ T cells (data not shown). Severe peribronchiolary and perivasculary inflammation, including goblet cell hyperplasia, was also seen in ISCOM/FG-immunized mice. The inflammation appears to be independent of the immunization route (groups A and E). Interestingly, goblet cell hyperplasia was absent in SFV/F and SFV/FG i.n. vaccinated mice (groups B and C), which, apart from occasionally observed focal leukocytic aggregates, rarely had any mucus-producing goblet cells. The lung histology resembles that of primarily RSV-infected mice, perhaps even more attenuated. However, there was an increased inflammatory response and marked goblet cell hyperplasia in animals that had received SFV/F or SFV/FG s.c. (groups F and G). A quantitative and qualitative assessment of BAL leukocytes was performed in a separate experiment. The result further demonstrates that there was an increased inflammatory response in the ISCOM/FG and FI-RSV groups, as their BAL leukocyte counts were considerably increased and contain significant number of eosinophils, while the SFV/F and SFV/FG i.n. groups had lower BAL leukocyte counts, which were dominated by monocytes, macrophages, and lymphocytes (Table II). When compared with the lung cytokine profile in Fig. 5, *A* and *B*, the degree of inflammation observed in this experiment was found to be associated with the level of lung IL-13 expression. For instance, SFV/F and SFV/FG i.n. groups had mild inflammation and low or no detectable IL-13 expression, whereas groups that had severe inflammation with goblet cell hyperplasia also had high IL-13 expression. These data, combined with the result shown in Table I, indicate that the nasal vaccination with SFV replicons, among the vaccination approaches tested in this study, was effective in enhancing the clearance of RSV infection, and that resistance to infection did not lead to exacerbation of the disease. The histopathology result is given in Table III together with the results of Th, cytotoxic T cell (Tc), and cytokine profiles (summarized from Table I and Figs. 4 and 5).

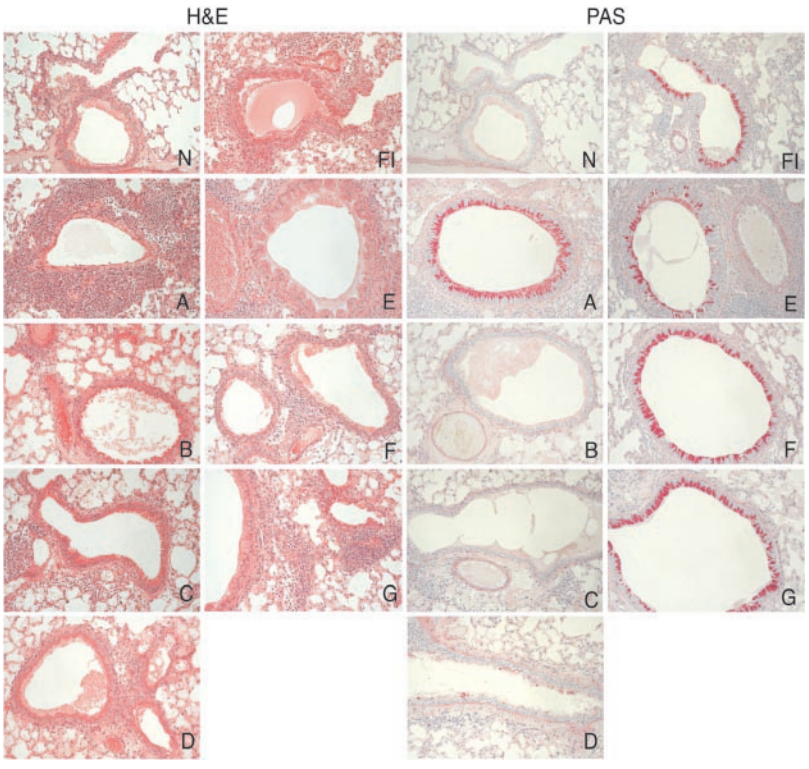

FIGURE 6. The severity of lung pathology in RSV-infected animals on day 5 after RSV challenge. Formalin-fixed lung sections were stained with H&E to determine the degree of inflammatory reaction, or PAS to identify the mucus-producing goblet cells. Lungs were isolated from RSV-challenged mice previously vaccinated with ISCOM (*A*), SFV/F (*B*), SFV/FG (*C*), SFV/β-gal or PBS (*D*) by i.n. route, or ISCOM (*E*), SFV/F (*F*), SFV/FG (*G*) by s.c. route. Lungs from naive uninfected (N) and RSV-challenged FI-RSV (FI)-vaccinated animals were included as controls.

Discussion

RSV infection causes severe morbidity that is closely related to symptoms of asthma, and is responsible for a significant mortality rate in infants. Immunity against RSV can either mitigate or exacerbate the RSV disease. Therefore, there is a need of an efficacious RSV vaccine that can induce a protective immunity that does not exacerbate the disease. To date, no vaccine has yet been proven safe and efficacious in clinical studies. With this aim in mind, we examined the immunogenicity and immunopathogenicity of RSV envelope proteins delivered by two diverse vaccine vehicles, the ISCOM and the virus-like SFV replicon in an experimental mouse model. We found that SFV/F or SFV/FG vaccine applied i.n. could elicit a broad, systemic, and local RSV-specific immunity that consists of a full spectrum of RSV-binding and neutralizing IgG, mucosal IgA, splenic as well as pulmonary T cells recognizing several RSV Ags, including the recently identified $F_{85–93}$ CTL epitope located within the F protein (22). Moreover, resistance to RSV infection did not lead to exacerbation of the disease. The result is encouraging because nasal vaccination is an appreciated needleless approach if efficient priming of the respiratory lymphoid tissues can be achieved. Intranasal immunization with ISCOM/FG, however, led to an enhanced pathology following RSV challenge. The immunization induced an elevated production of Th2-associated IgG1, a strong $CD4^+$ T cell-dependent IFN-γ response limited to the inactivated RSV Ag, and a poor response to the $F_{85–93}$ CTL peptide. This result indicates that ISCOM/FG predominantly primed the $CD4^+$, but not the $CD8^+$ T cells. Poor response to $F_{85–93}$ peptide and subsequent disease enhancement in the animals s.c. immunized with ISCOM/FG further suggest that immunity to FG may enhance the disease when priming of $F_{85–93}$-specific CTLs is inefficient. The result is similar to the evidence that immunity to the G protein can cause immunopathology due to lack of CTL epitopes (32, 33). It also supports previous observation in which RSV M2-specific $CD8^+$ T cells, present at the time of initial G priming, can reduce the degree of lung pathology during RSV infection (5), thus further strengthening the importance in priming memory CTLs in RSV FG-based vaccination.

A readily established local immunity in the respiratory tract before exposure of RSV would be beneficial for the host, as the virus and virally infected cells could be rapidly and efficiently eliminated. Clearly, our result demonstrates that the animals that had significant number of pulmonary $F_{85–93}$-specific IFN-γ-secreting cells before challenge also exhibited less lung pathology after challenge (Fig. 4). If this local pre-existing immunity ($F_{85–93}$-specific T cells as well as RSV Abs) is not sufficient to eliminate all virally infected cells, the viral clearance from the airway may be delayed. RSV may mimick chemokine functions, activate monocyte/macrophage via binding to Toll-like receptor 4 and CD14, and block TNF-α signaling (34–36), which may alter the cytokine environment at the infection foci; the infection also suppresses lung $CD8^+$ T cell effector and memory activity in the respiratory tract (22, 37). A less efficient $CD8^+$ T cell-mediated viral clearance could lead to an overactivation of other effectors, for instance, the memory RSV-specific $CD4^+$ cells. This could explain why delayed RSV clearance in animals immunized s.c. with SFV/FG led to lung pathology with a goblet cell hyperplasia, as well as an increased lung Th2 cytokine production. To rule out the role of $F_{85–97}$-specific CTLs in protection against both the RSV and its disease, postchallenge responses following in vivo depletion of $CD8^+$ T cells, or vaccination with a mutated F construct lacking the $F_{85–97}$ epitope remain to be examined. In fact, we have preliminary result indicating that the lung histopathology is significantly increased in SFV replicon i.n. vaccinated mice depleted of $CD8^+$ T cells. However, the degree of pathology is still not at the same extent as the ISCOM- or FI-RSV-vaccinated animals (unpublished observation), indicating that other effectors, which remain to be identified, also contribute to a significant part in preventing the disease enhancement. Moreover, IFN-γ secreted from the effector $CD8^+$ T cells may contribute to viral clearance through a nonlytic mechanism (38, 39). Eliminating RSV-infected cells through a nonlytic

Table II. *Quantitative and qualitative determination of the postchallenge BAL leukocytes*[a]

Vaccine	Immunization Route	BAL Leukocyte Cell Counts (10^4/ml)	Subset of BAL Leukocytes (%)		
			Eosinophils	Neutrophils	Lymphocytes
ISCOM/FG	i.n.	82.5 ± 15.1*	37.5 ± 11.0*	31.5 ± 9.5	31.1 ± 11.3
SFV/F	i.n.	27.5 ± 10.8	2.0 ± 2.0	47.4 ± 11.2	50.2 ± 12.7
SFV/FG	i.n.	33.3 ± 10.8	3.6 ± 2.2	32.3 ± 6.3	64.0 ± 4.0
ISCOM/FG	s.c.	81.5 ± 10.2*	47.5 ± 12.8*	31.8 ± 6.7	20.7 ± 6.6*
SFV/F	s.c.	23.0 ± 10.1	5.8 ± 0.7	54.0 ± 5.3	43.7 ± 5.3
SFV/FG	s.c.	38.5 ± 9.4	8.0 ± 4.9	63.6 ± 1.1	28.4 ± 4.5*
PBS	i.n.	37.0 ± 11.5	3.9 ± 1.2	46.9 ± 4.8	49.2 ± 3.6
FI-RSV	i.m.	112.0 ± 13.3**	71.8 ± 1.4**	21.0 ± 1.7**	7.1 ± 2.4**

[a] BAL was performed on day 5 postchallenge, and duplicates of individual BAL smear were analyzed. Total number of BAL leukocytes and percentage of the indicated subset are expressed as mean ± SD. Statistical SD compared with the PBS control vaccines (*, $p < 0.05$; **, $p < 0.01$ by Student's *t* test).

pathway could potentially lessen the degree of immunopathology of essential tissues such as the airway, which deserves to be closely examined.

It is interesting that SFV, a nonspreading virus vaccine that per dose produces significantly less amount of Ag than that delivered by ISCOM, is capable of eliciting similarly strong humoral and cellular responses. Two central characteristics important for the immunogenicity of SFV are the viral dsRNA intermediate and the virus-induced apoptosis. The dsRNA produced during SFV RNA replication is recognized as a pathogen-associated molecular pattern similar to the LPS and CpG motif, is a potent activator of macrophages and dendritic cells (DCs) that via binding to Toll-like receptor 3 (40) activates the production of type I IFNs and expression of costimulatory molecules, and up-regulates expression of MHC class II molecules (41). Moreover, SFV infection induces apoptosis, and the generated cell-associated materials represent an attractive source of Ag for DCs, which are the most potent stimulator of naive T cells known today (42–44). DCs that have internalized cell-associated Ags mature and migrate from perpheral tissues to secondary lymphoid organs, in which they efficiently stimulate T cells that, in return, secrete inflammatory cytokines/chemokines, which can lead to the activation of neigboring APCs. Despite that SFV replication is abortive, the stated mechanism may enable Ag-specific responses to become amplified.

ISCOM-associated Ags have been shown to be presented under MHC class I-restricted pathway, and robust CTL responses elicited by other viral envelope proteins including influenza virus and HIV incorporated in ISCOMs have been demonstrated (45–48). Our result in which ISCOM/FG failed to induce significant response to $F_{85–93}$ CTL epitope contradicts those findings. One of the explanations would be an inefficient transport of F-derived peptides to MHC class I molecules in the cells that have internalized ISCOM/FG. Alternatively, it could be related to a selective APC targeting. It is known that nonviable Ags when captured by DCs via macropinocytosis or receptor-mediated endocytosis can be efficiently presented on MHC class I molecules to naive CD^+ T cells (49–51). Indeed, the size of ISCOM/FG is ~40 nm (21), which should enable uptake through macropinocytosis. However, the F and G proteins are responsible for cell fusion and attachment to RSV receptors, the multimeric F and G on the ISCOM/FG may facilitate uptake through receptor-mediated endocytosis. The latter is supported by results in which influenza virus envelope proteins incorporated in ISCOM are efficiently internalized by murine peritoneal cells and P815 mastocytoma cell line, but the OVA ISCOMs are not (48). If the uptake of ISCOM/FG is mediated preferentially by macrophages (52–54) and not by DCs, the presentation of ISCOM-delivered FG proteins could be skewed to the classical lysosomal pathway common for exogenous Ags, leading to the MHC class II molecule-rich post-Golgi compartment. The latter would explain why ISCOM/FG elicits prodominantly IFN-γ response to the RSV Ag, but not to the MHC class I-restricted $F_{85–93}$ peptide.

RSV infection results in peribronchial inflammation and airway mucus production (1). Goblet cell hyperplasia is a reflection of chronic small airway inflammation. Airway goblet cells discharge mucus in response to a wide variety of stimuli including inflammatory mediators, and develop hyperplastic and metaplastic changes as a result of a prolonged airway insult (55, 56). In this study, we observed that the number of PAS-positive mucus-producing goblet cells had increased in the mock-immunized animals on day 5 postchallenge. We show that immunity to RSV acquired

Table III. *Relationship among prechallenge Th, Tc profiles, postchallenge IL-12/IL-13 profiles, and lung pathology*[a]

	Group and Route		Prechallenge		Postchallenge	
			Th[b] IgG2a: IgG1 ratio	Tc[c] spleen:lung	IL-12:IL-13 ratio mean ± SE	Pathology score
N.	Naive uninfected		ND	0:0	ND	1.17 ± 0.38
F.	FI-RSV	i.m.	0.01	33:3	1.23 ± 1.20	5.38 ± 1.03
A.	ISCOM/FG	i.n.	0.44	0:2	0.65 ± 0.20	5.35 ± 1.03
B.	**SFV/F**	**i.n.**	**7.10**	**181:38**	**668.50 ± 171.5**	**2.30 ± 0.75**
C.	**SFV/FG**	**i.n.**	**18.70**	**102:31**	**622.20 ± 22.0**	**2.71 ± 1.11**
D.	PBS or SFV/β-gal	i.n.	ND	0:0	326.00 ± 233.0	3.60 ± 0.86
E.	ISCOM/FG	s.c.	0.85	30:1	0.05 ± 0.10	5.31 ± 0.96
F.	SFV/F	s.c.	2.73	242:4	61.20 ± 37.8	4.53 ± 0.9
G.	SFV/FG	s.c.	5.24	207:4	0.92 ± 0.27	4.72 ± 0.96

[a] Semiquantitative IL-12/IL-13 measurement and the pathology index used are described in *Materials and Methods.* The groups that had lowest postchallenge pathology scores are highlighted.
[b] Th, Ratio of RSV-specific IgG2a/IgG1 titers.
[c] Tc, Number of $F_{85–93}$-specific IFN-γ-secreting cells per million leukocytes in spleen and lung, respectively.

by nasal SFV/F or SFV/FG vaccination could prevent the increase of pulmonary goblet cells upon RSV challenge. However, immunity acquired by other vaccination protocols used in this study, e.g., FI-RSV, ISCOM/FG, or s.c. vaccination with SFV/F or SFV/FG, could result in a postchallenge pulmonary goblet cell hyperplasia (Fig. 6), which appears to be predominantly associated with pulmonary IL-13 production (Fig. 5). These results are in line with what was demonstrated by Tekkanat et al. (57), that after a primary RSV infection, mice can develop an increased mucus and IL-13 production in lungs, and that mucus production can be related to the presence of IL-13. Given that goblet cell hyperplasia combined with increased airway responsiveness are the principal causes of airway obstruction such as allergic asthma, and that IL-13 is a central mediator of allergic asthma (58, 59), our observation further supports the role of IL-13 as a pivotal cytokine in initiating RSV-related lung disease as well as its linkage to allergic asthma.

The reason SFV/FG was not as efficacious as SFV/F is not clear. It has been verified that the SFV/FG construct in vitro displays same capacity and kinetic as SFV/F regarding to RSV protein expression; we therefore did not expect this finding. Several reasons may account for this observation, including that the in vivo expressed G-Ag may modulate the response to SFV/FG vaccination (36), the simultaneous expression of F and G may lead to immunodominant responses different from only F, or that these two proteins when expressed by SFV are in close proximity at the cell surface and mask antigenic sites required for Ab recognition.

It cannot be excluded that the severity of RSV disease following RSV challenge observed in this and other studies could be influenced by several factors, such as the virus dose and strain. The challenge dose used in this study is 10-fold higher than used elsewhere for testing other RSV immunogens, which failed to detect lung pathology (9, 60, 61). Moreover, the RSV Long strain used in this study has been used elsewhere to study the pathogenesis of RSV infection (57, 62). Despite the fact that RSV is not a natural pathogen of mice and has limited infectivity in rodents when compared with humans, the lung pathology observed in this study in the RSV-challenged mice demonstrates its usefulness in the mouse RSV challenge model.

In conclusion, replicating agents such as live attenuated RSV and other viral/nonviral genetic vaccines expressing RSV Ags seem to have the best potential to protect against RSV infection and its disease. Successful RSV vaccination in the mouse model has been achieved by several approaches (10, 29, 63). The novelty of the SFV replicon strategy is that it has the advantages of live and nonlive virus vaccines, in that it is capable of inducing broad immune response from the mucosal site, and yet is not spreading. This supports current evidence that the optimal viral vaccines are those that mimic the ability of pathogens to activate the immune system.

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