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Development of an Adenovirus-Based Respiratory Syncytial Virus Vaccine: Preclinical Evaluation of Efficacy, Immunogenicity, and Enhanced Disease in a Cotton Rat Model

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ABSTRACT

The lack of a vaccine against respiratory syncytial virus (RSV) is a challenging and serious gap in preventive medicine. Herein, we characterize the immunogenicity of an adenovirus serotype 5-based RSV vaccine encoding the fusion (F) protein (Ad5.RSV-F) and the protection provided following immunization with Ad5.RSV-F and assess its potential for producing enhanced disease in a cotton rat (CR) model. Animals were immunized intranasally (i.n.) and/or intramuscularly (i.m.) and subsequently challenged with RSV/A/Tracy (i.n.) to assess protection. Robust immune responses were seen in CRs vaccinated with Ad5.RSV-F given i.m. or i.n., and these responses correlated with reduced replication of the virus in noses and lungs after challenge. Neutralizing antibody responses following immunization with a single dose of Ad5.RSV-F at 1×10^{11} viral particles (v.p.) elicited antibody titers 64- to 256-fold greater than those seen after natural infection. CRs boosted with Ad5.RSV-F i.n. 28 days after an i.m. dose also had significant increases in neutralizing antibody titers. Antibody affinity for different F-protein antigenic sites revealed substantial differences between antibodies elicited by Ad5.RSV-F and those seen after RSV infection; differences in antibody profiles were also seen between CRs given Ad5.RSV-F i.m. and CRs given Ad5.RSV-F i.n. Ad5.RSV-F priming did not result in enhanced disease following live-virus challenge, in contrast to the histopathology seen in CRs given the formalin-inactivated RSV/A/Burnett vaccine.

IMPORTANCE

Respiratory syncytial virus (RSV) is the most common cause of acute lower respiratory infection in infants and young children and a serious health threat in the immunocompromised and the elderly. Infection severity increased in children in an immunization trial, hampering the over 4-decade-long quest for a successful RSV vaccine. In this study, we show that a genetically engineered RSV-F-encoding adenoviral vector provides protective immunity against RSV challenge without enhanced lung disease in cotton rats (CRs). CRs were vaccinated under a number of different regimens, and the immunity induced by the recombinant adenoviral RSV vaccine administered by use of an intramuscular prime-intranasal boost regimen may provide the best protection for young infants and children at risk of RSV infection, since this population is naive to adenoviral preformed immunity. Overall, this report describes a potential RSV vaccine candidate that merits further evaluation in a phase I clinical study in humans.

The first attempts to produce a vaccine against respiratory syncytial virus (RSV) began 5 decades ago. The most notable program culminated in a pediatric clinical trial in the 1960s in which RSV was inactivated with formalin and administered to RSV-naive infants; unfortunately, the product exacerbated disease when vaccine recipients were subsequently infected with RSV (1). It is now proposed that this vaccine elicited little neutralizing antibody and may have induced an imbalanced T-cell production of interleukins (2). The association of humoral responses with safe protection has since been demonstrated by a number of passive protection studies using RSV-neutralizing immune globulin and humanized monoclonal antibodies (MAbs) (1). In past decades, numerous RSV vaccine candidate studies have been conducted. Research has largely focused on subunit and live viral vaccines. Among the live vaccines, cold-passaged (cp), temperature-sensitive (*ts*), and xenotropic viruses have been studied the most. cp and *ts* RSV vaccines have advanced from preclinical to clinical trials, including those involving children, but none have progressed toward licensure (3).

Recombinant DNA vaccines show incredible promise for the prevention of human disease by their capacity to effectively induce both humoral and cellular immune responses. Among the available DNA technologies for generating recombinant vaccines, adenovirus is one of the most appealing. In the gene therapy and vaccine fields, recombinant human adenoviral vectors based on adenovirus serotype 5 (Ad5) have been studied extensively. Ad5-vectored vaccines induce potent and protective immune responses against several pathogens in a variety of animal models (4–9). On the basis of encouraging preclinical results, this vaccine

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vehicle has progressed into large-scale clinical trials (8, 10, 11). Although results from these studies echo the results obtained from studies with mice (12–15) and rhesus monkeys (16), they also suggest that the high prevalence of preexisting anti-Ad5 immunity might be a major limitation (10) to their implementation in adolescent and adult populations. Nevertheless, a potential target population for an Ad5-vectored RSV vaccine still exists among infants 4 to 23 months old who present with limited adenoviral preformed active immunity (since Ad5 infections are uncommon early in life) and who have little or no passive immunity (since infants in this age group have already lost most or all of the anti-Ad5 antibodies acquired from their mothers) (17, 18). The seroprevalence of human adenovirus serotypes 2 and 5 circulating in different age groups supports the knowledge that antiadenoviral neutralizing immunity is present in newborns and tends to decline after 6 months of age (19). Thus, recombinant adenovirus may represent an excellent platform for vaccines against RSV and other members of the paramyxoviridae (human parainfluenza virus type 1 [hPIV1] to hPIV4, metapneumovirus) within this young pediatric population.

The RSV fusion glycoprotein (RSV-F) is a major target antigen for induction of humoral and cellular protective immunity. F protein is highly conserved between RSV subtype A and B strains. In previous studies, different serotypes of replication-competent adenoviruses encoding the wild-type cDNA of RSV-F and RSV G glycoprotein antigens were tested *in vivo*. The results showed that intranasal (i.n.) administration of Ad4- or Ad5-based RSV vaccines in ferrets induced specific immune responses (20). Likewise, recombinant adenovirus serotype 5 containing codon-optimized RSV-F was immunogenic when tested in mice and protected animals against live-virus challenge. However, ferrets and mice are not very effective animal models for human RSV due to the limited permissiveness of human RSV infection. Cotton rats (CRs) have been used for years as an excellent small-animal model of RSV vaccine-enhanced disease and are considered the model of choice for early RSV vaccine testing. Herein, we describe the development of a recombinant adenoviral vector expressing codon-optimized RSV-F as a vaccine candidate (Ad5.RSV-F), investigate its ability to induce neutralizing immune responses, and assess its ability to protect against live-virus challenge and potentiate enhanced disease in a CR model (21).

MATERIALS AND METHODS

Replication-defective adenovirus vaccine preparation. The RSV recombinant A2cp F (rA2cp F) gene (GenBank accession no. AAC14902.1) was codon optimized for optimal expression in mammalian cells by the UpGene codon optimization algorithm (22) and synthesized by GenScript. pAd/RSV-F was generated by subcloning the codon-optimized RSV-F gene into the shuttle vector pAdlox (GenBank accession no. U62024) at SalI/NotI sites. Subsequently, replication-defective human adenovirus serotype 5 expressing RSV-F, designated Ad5.RSV-F, was generated by *loxP* homologous recombination and subsequently purified and stored as described previously (6, 23, 24).

Immunocytochemistry. For detection of RSV-F expression in cells transfected with pAd/RSV-F, cells were fixed with cold methanol for 36 h following transfection and were incubated with the RSV-F monoclonal antibody 131-2A (Millipore). After washing, the cells were incubated with horseradish peroxidase-coupled antimouse secondary antibody (Invitrogen), and RSV-F was visualized by use of avidin/biotin complex solution (Vector).

Fluorescence-activated cell sorter (FACS) analysis. HEK293 cells were transfected with pAd/RSV-F or pAd using the Lipofectamine reagent (Invitrogen), or A549 cells were seeded in six-well plates and infected with Ad5.RSV-F or Ad5 expressing enhanced green fluorescent protein (Ad5.eGFP) (10^{10} viral particles [v.p.] per cell). After 24 h at 37°C, cells were harvested, trypsinized, washed with phosphate-buffered saline (PBS), and stained with monoclonal antibody against RSV-F (131-2A) or mouse antiserum against Ad5.RSV-F, followed by a phycoerythrin (PE)-conjugated antimouse secondary antibody (Jackson ImmunoResearch). Data acquisition and analysis were performed using FACScan and CELLQuest software.

IFN-γ ELISPOT assay. Splenocytes were isolated from BALB/c mice immunized intraperitoneally once with 5×10^{10} v.p. of Ad5.RSV-F or Ad5. A gamma interferon (IFN-γ) enzyme-linked immunosorbent spot (ELISPOT) assay was performed according to the manufacturer's instructions (BD Biosciences). Briefly, 96-well plates were coated overnight with anti-IFN-γ monoclonal antibody at 4°C. On the next day, plates were blocked and 5×10^5 cells were seeded per well in 100 μl culture medium (RPMI, 10% fetal calf serum, L-glutamine, penicillin-streptomycin) and supplemented with H-2K^d-restricted RSV-F-derived restimulation peptide (KYKNAVTEL, consisting of the F-protein epitope from residues 85 to 93 [F/85-93]) at a final concentration of 5 μg/ml. After 12 h of restimulation at 37°C, cells were removed, the plates were washed, and the IFN-γ trapped on the plates was visualized using biotinylated monoclonal anti-IFN-γ antiserum. Spots were counted using a zoom stereomicroscope. For the mouse immunization study, an approved protocol by the University of Pittsburgh Animal Care and Use Committee was followed.

Immunizations and RSV challenge in cotton rats. CRs (five or six animals per group) were injected with various doses of the replication-defective adenovirus vaccine via the intramuscular (i.m.) or i.n. route. For i.n. immunizations, CRs were lightly anesthetized with isoflurane prior to applying 100 μl of vaccine containing 1×10^{11} v.p. to the nostrils. i.m. immunization was performed by injection of 100 μl of vaccine containing 1×10^{11} v.p. into the area of the left tibialis anterior leg muscle using a tuberculin syringe. For the prime-boost regimen, 100 μl of vaccine containing 1×10^{11} v.p. was administered i.n. 4 weeks after an i.m. dose. CRs in the control groups received Ad5 expressing parainfluenza virus type 3 (PIV3) hemagglutinin-neuraminidase (HN) (Ad5.PIV3-HN) or were left untreated (not immunized). CRs in the RSV control group were inoculated i.n. on day 0 with 100 μl of 2.25×10^5 PFU RSV/A/Tracy. For the pulmonary histopathology, CRs were immunized by the i.m. route only with one (day 0) or two (day 0 and 28) doses of Ad5.RSV-F. Formalin-inactivated (FI) RSV/A/Burnett in 100 μl of 1:10-diluted vaccine was injected i.m. as a positive histopathology control. At 4 weeks following the last boost immunization, all CRs were challenged i.n. with 2.25×10^5 PFU of RSV/A/Tracy. CRs were euthanized on day 4 or 7 postchallenge, and lung tissue was collected for virus isolation (day 4) or histopathology (day 7).

RSV preparation. Preparation and isolation of RSV/A/Tracy from HEp-2 cells were performed as previously described (25). A master seed, working seed, and working pools were made in HEp-2 cells, which were snap-frozen and stored at −70°C to −80°C. FI RSV/A/Burnett was made in Vero cells as previously described (25, 26). Briefly, stock RSV/A/Burnett, the original virus used in the 1960s FI RSV vaccine studies, was grown in Vero E6 cells using 0% fetal bovine serum (FBS)–minimal essential medium (MEM) and then sucrose purified. Vero E6 cells were inoculated with RSV/A/Burnett (~10^5 PFU/ml). Once a cytopathic effect of 50 to 75% (+3) was visualized, usually after 3 to 4 days, the cell supernatant fractions were collected and maintained on ice. Following centrifugation and filtration, filtered formaldehyde was diluted and added to the RSV suspension. The suspension was rotated at 37°C for 72 h and then pelleted at 22,000 rpm at 4°C for 30 min. The pellet was resuspended with MEM to 1/25 of the original volume (FI RSV/A/Burnett 25×). Aluminum hydroxide (4 mg/ml) was added to the FI RSV 25×, the mixture was gently rotated for 12 h at 4°C, the contents were pelleted by centrifugation, and

the pellet was resuspended with MEM to 1/100 of the original volume to make the final product, FI RSV/A/Burnett 100×. Aliquots of the alum-absorbed FI RSV/A/Burnett 100× were made and stored at 4°C.

Serum neutralization assay. Neutralizing antibody (Nt Ab) titers were determined by using plaque-purified RSV/A/Tracy and RSV/B/18537 in 96-well microtiter plates on HEp-2 cell monolayers as previously described (25). Ad5 serum neutralizing antibody titers were determined as previously described (27).

RSV titers in nasal wash and lung lavage specimens. Following CO_2 euthanasia, the left lung and one of the large lobes of the right lung were removed and rinsed in sterile water to remove external blood contamination. The lung lobes were transpleurally lavaged using 3 ml of Iscove's medium with 15% glycerin mixed with 2% FBS–MEM (1:1, vol/vol). For nasal washes, the jaws were disarticulated, the head was removed, and 1 ml of Iscove's medium with 15% glycerin mixed with 2% FBS-MEM (1:1, vol/vol) was pushed through each naris (total of 2 ml). Fluids were collected and stored on ice until titrated.

Plaque assays were performed using 24-well tissue culture plates as previously described (25). The plaques in wells containing between 20 and 100 plaques were enumerated, the average was obtained, and virus titers were calculated as the total $\log_{10}$ number of PFU for nasal wash fluid or $\log_{10}$ number of PFU/g of tissue for lungs. The lower limit of detection by this method was approximately 0.40 $\log_{10}$ total PFU in nasal wash fluid specimens and 1.00 to 1.20 $\log_{10}$ PFU/g in lung lavage fluid specimens.

Pulmonary histopathology. Following CO_2 euthanasia, both lungs were isolated intact, rinsed, and perfused with 10% neutral buffered formalin (at a 27- to 30-cm pressure for 1 to 1.5 h) (25). Sections of paraffin-embedded inflated lungs were stained with hematoxylin-eosin and subsequently scored without awareness of group assignment by an experienced veterinary pathologist. The histopathology score was based on the total area of involvement and severity for the following parameters: (i) peribronchial mononuclear inflammatory cell infiltrates, (ii) bronchiolitis, (iii) bronchiolar eosinophilia, (iv) alveolitis, (v) alveolar eosinophilia, and (vi) perivascular mononuclear inflammatory cell infiltrates. A scale from 0 to 4+ was used for each score. The summary value for each of the six parameters was added together to arrive at a single summary score for each animal. Summary scores for each animal in a group were expressed as the arithmetic mean ± standard deviation (SD).

RSV-F BMAb competitive enzyme-linked immunosorbent assay (ELISA). Competitive binding of CR pooled sera versus biotin-labeled monoclonal antibodies (BMAbs) was performed using a panel of MAbs mapped previously, including anti-RSV-F antibodies 1121 (site A), 1129 (site A), 1107 (site AB), 1269 (site B), 1243 (site C), and 104-5 (anti-G MAb) (28, 29). Homologous unlabeled MAbs were used as positive controls, and anti-PIV3-F b108 was used as a negative control in this assay. Mouse monoclonal antibodies were biotinylated using the Enzotin reagent per the manufacturer's protocol (Enzo Life Sciences). BMAbs were titrated on monolayers of LLC-MK2 cells infected with RSV A2, and 50% binding concentrations were determined from dose-response curves as previously described (28).

The RSV ELISA described in reference 28 was modified and used in a competitive binding assay as follows. On the assay day, antigen plates were blocked with 20% goat serum–5% nonfat dried milk (NFDM) with PBS–Tween (PBST) at room temperature for 2 h using 100 μl/well. In a separate plate, each BMAb was mixed 1:1 either with PBS or with cotton rat serum pooled from each group and serially diluted 4-fold in triplicate, resulting in final serum concentrations ranging from 1:20 to 1:5,120 and BMAb concentrations at 50% saturation. Each BMAb was also tested against serial dilutions of homologous, unlabeled MAb and against an anti-PIV3-F MAb as positive and negative controls, respectively. These antibody mixtures were added to blocked and washed antigen plates and incubated overnight at room temperature. On the following morning, the plates were washed with PBST, streptavidin-horseradish peroxidase was added, and the plates were incubated at 37°C for 3 h prior to washing and addition of ABTS [2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid)] to each well. The plates were then incubated at room temperature for up to 30 min, and optical density (OD) readings at 405 nm (OD_{405}) were obtained using an ELISA plate reader (maximum reading, 4.0). For data analysis, the mean value for blank wells (i.e., antibody-free wells with no competitor antibody and/or no BMAb) was subtracted from OD values obtained for all antibody-containing wells. The mean OD signals for BMAb control wells in the absence of competitor were determined, and competition in the presence of unlabeled competitor was calculated as follows: (OD_{405} in the presence of the unlabeled competitor/mean OD_{405} in the absence of competitor) × 100. Values are expressed as a percentage of the value for the control.

Statistical analysis. Statistical analysis was performed using Prism software, version 5.04 (GraphPad, San Diego, CA). The quantity of RSV in nasal wash and lung lavage fluid specimens is presented as the mean ± SD of the $\log_{10}$-transformed titers. Statistical analyses were performed using a nonpaired two-tailed Student *t* test. For neutralizing antibody titers, $\log_2$-transformed values were analyzed using one-way analysis of variance and Tukey-Kramer multiple-comparison tests. Statistical significance was determined at a *P* value of ≤ 0.050. The sums of the areas of involvement and severity scores calculated from the five parameters per animal were used for analysis of the histopathology data.

RESULTS

Robust expression of F protein by adenoviral vector. The limited efficiency of expression of recombinant RSV-F in eukaryotic cells is due to a premature polyadenylation site within the F-protein open reading frame (ORF) (30). To overcome this limitation, we generated adenovirus (Ad5)-based vectors with E1/E3 deletions encoding a codon-optimized RSV rA2cp F protein using the UpGene algorithm. In our studies, we compared the expression of wild-type F protein and codon-optimized F protein. As expected, the expression level of F protein was much lower than that of the codon-optimized ORF (data not shown). Initially, we examined RSV-F expression by immunocytochemistry (Fig. 1A) and flow cytometry (Fig. 1B) after transfection into HEK293 cells with pAd/RSV-F or a control. Moreover, the recombinant adenoviral vaccines were evaluated in A549 cells after infection of Ad5.RSV-F by examining F-antigen expression levels using flow cytometry analysis (Fig. 1C). Ad5.RSV-F could induce a higher level of cell surface F-antigen expression in infected cells than that in cells infected with Ad5 without the insert.

Induction of humoral and cellular immune responses in mice. We next examined whether recombinant adenoviral vaccines could elicit an antigen-specific immune response *in vivo*. BALB/c mice were inoculated intraperitoneally with 5×10^{10} v.p. of Ad5.RSV-F. At 3 weeks after immunization, sera from all mice were pooled and tested for RSV-F-specific antibodies using FACS analysis of A549 cells infected with Ad5.RSV-F or the Ad5 control. Ad5.RSV-F-immunized mice were found to have developed RSV-F-specific antibodies when their sera were tested on cells infected with Ad5.RSV-F, whereas their sera lacked reactivity when they were tested on cells infected with Ad5 without an insert (Fig. 2A).

To determine T-cell responses specific to RSV-F in immunized mice, ELISPOT assays measuring IFN-γ secretion were performed. Splenic lymphocytes were harvested at week 5 postimmunization and stimulated with a previously described H-2K^d-restricted F/85-93 epitope for IFN-γ production (31). As shown in Fig. 2B, an F/85-93 epitope-specific CD8 T-cell response was detected in mice vaccinated with Ad5.RSV-F, while lymphocytes from mice given Ad5 without the insert did not respond when stimulated with this peptide. Taken together, our results suggest that immunization with the Ad5.RSV-F vaccine elicits both RSV-

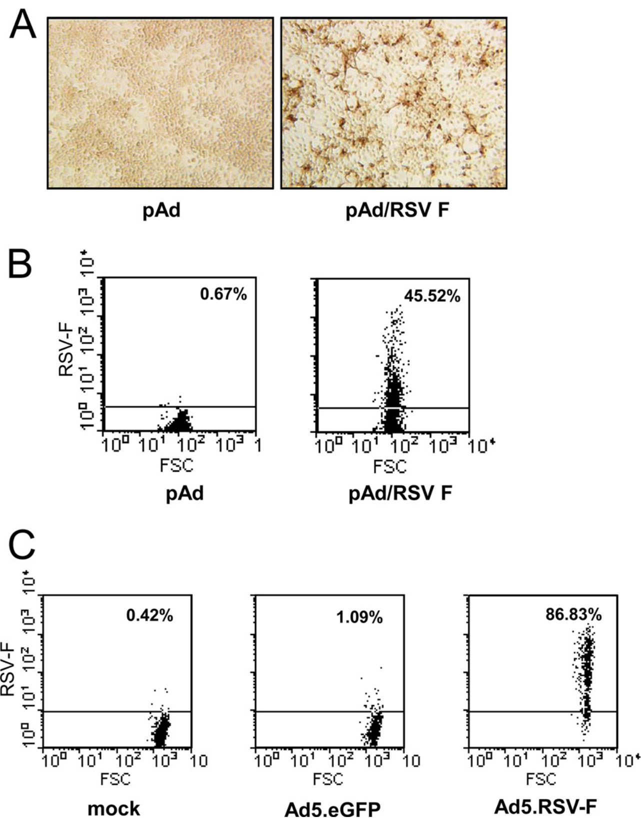

FIG 1 Expression of RSV-F recombinant antigen. (A and B) Detection of RSV-F by immunocytochemistry (A) or flow cytometry (B) on HEK293 cells transfected with pAd/RSV-F or pAd as a control. (C) A549 cells were infected with Ad5.RSV-F or Ad5.eGFP. At 24 h postinfection, monolayers were analyzed for RSV-F immunostaining with anti- RSV-F monoclonal antibody, followed by staining with PE-conjugated anti-mouse IgG and flow cytometry. Numbers represent the percentage of cells positive for RSV-F. FSC, forward scatter.

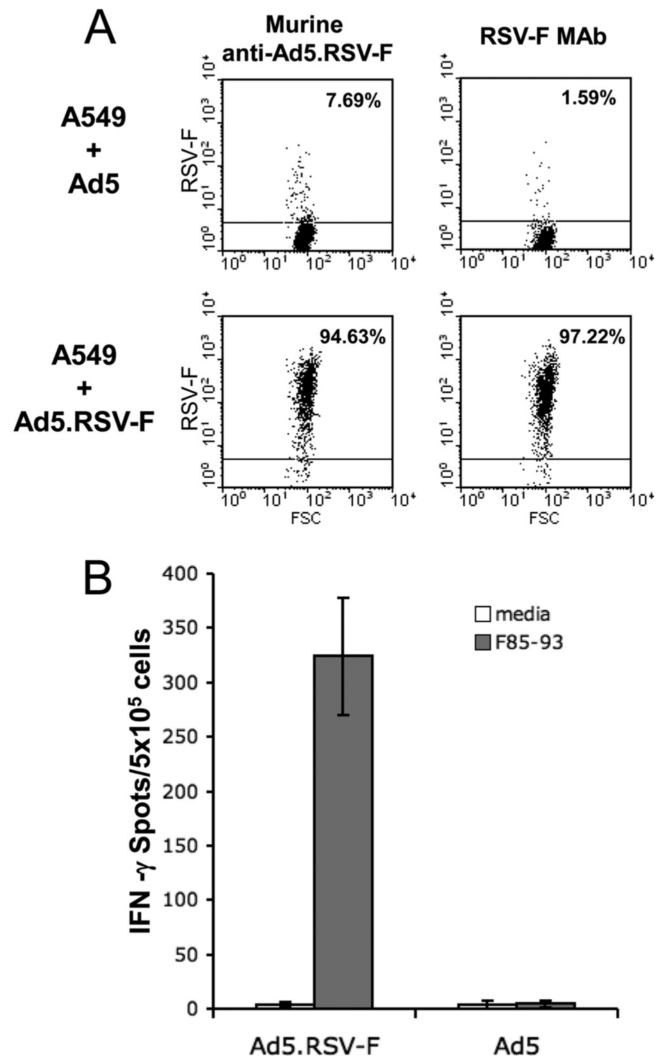

FIG 2 Immunization with Ad5.RSV-F elicits humoral and cellular immune responses in mice. BALB/c mice were immunized intraperitoneally with 5×10^{10} v.p. (A) RSV-F expression in A549 cells transduced with Ad5.RSV-F or Ad5. Pooled mouse sera and a monoclonal antibody against RSV-F (313-2A) were used for detection of RSV-F expression at the cell surface by flow cytometric analysis. The percentages of RSV-F-positive cells are also shown. (B) Spleens were isolated and restimulated *ex vivo* with F85-93 peptide, and F85-93 epitope-specific splenocytes were counted by IFN-γ ELISPOT assay. Control groups received 5×10^{10} v.p. of Ad5. Bars represent the average number of spots.

specific humoral immune responses and RSV-specific cellular immune responses.

Ad5.RSV-F-induced immune response in the cotton rat. We then investigated the immunogenicity of the Ad5.RSV-F vaccine candidate in CRs. On day 0, experimental CR groups were immunized i.m. with Ad5.RSV-F (groups 4 and 5) or PBS (group 6), and on day 28, the animals were immunized i.n. with Ad5.RSV-F (groups 5 and 6). Additional control groups included animals immunized with Ad5.PIV3-HN (group 3) or animals that received no treatment (group 1). The RSV infection positive control (group 2) received a single i.n. dose of 2.25×10^5 PFU of the RSV/A/Tracy strain on day 0. CRs immunized with Ad5.RSV-F i.m. and/or i.n. developed serum antibodies that neutralized RSV subtype A and RSV subtype B strains (Fig. 3A and B), and the Ad5.RSV-F vaccine used at 1×10^{11} v.p. per dose induced neutralizing antibody responses that were 64- to 256-fold greater than those seen following experimental infection. Surprisingly, i.n. boosting with Ad5.RSV-F on day 28 significantly increased the neutralizing antibody responses on day 56 in CRs primed by i.m. injection (group 5; $P = 0.0022$, unpaired Mann-Whitney nonparametric test, two-tailed). A single i.n. dose of Ad5.RSV-F (group 6) also produced a robust serum neutralizing response on day 56 that was approximately 32-fold greater than that produced by experimental infection. As expected, untreated CRs and CRs given Ad5.PIV3-HN (group 3) had no detectable neutralizing antibody response to RSV.

Cotton rat challenge studies. To investigate the ability of Ad5.RSV-F to protect against RSV challenge, we assessed viral replication in nasal turbinates and lung lavage fluid specimens from CRs challenged with 2.25×10^5 PFU RSV/A/Tracy on study day 56. While there was active RSV replication in the lungs of untreated control animals and in the group given Ad5.PIV-HN, all three Ad5.RSV-F immunization regimens effectively and significantly reduced virus replication in the lungs after challenge (Fig. 3C). Experimental live RSV i.n. infection on day 0 reduced the levels of virus in the noses and lungs, as expected. Single i.m.

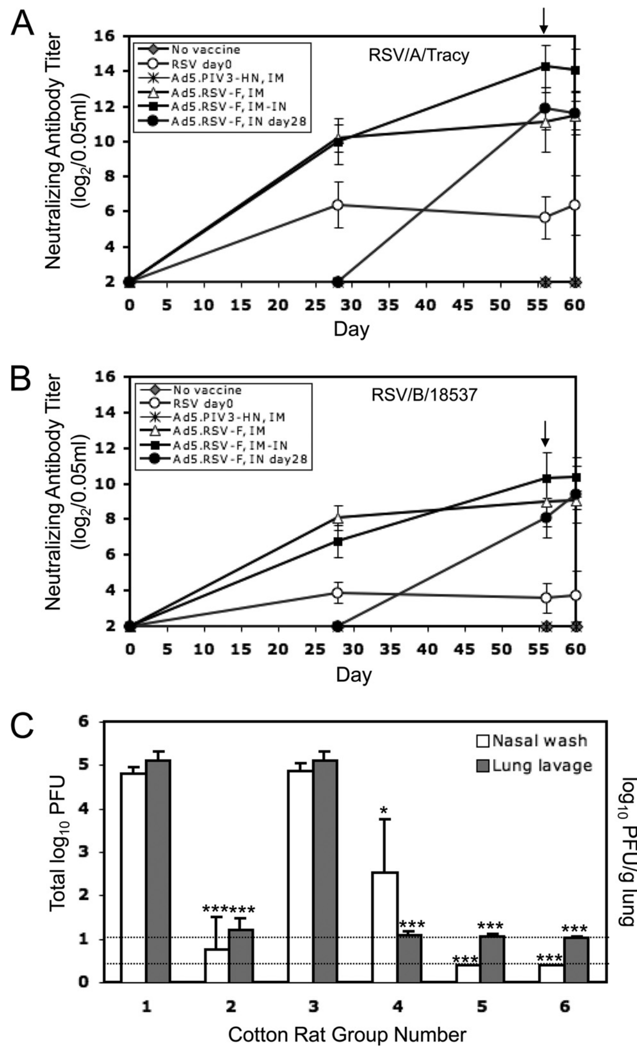

FIG 3 Protection from RSV challenge after vaccination with Ad5.RSV-F. Cotton rats were challenged intranasally with 1.22×10^5 PFU RSV/A/Tracy 28 days after the second vaccination and sacrificed 4 days later to assess protection. RSV serum neutralizing antibody titers against RSV/A/Tracy (A) and RSV/B/18537 (B) were measured by determining the highest serum dilution that inhibited RSV infection by more than 50%. Results are expressed as geometric mean titers and SDs ($\log_2$). The minimal detection level is 2.5, and for statistical analysis, a value of <2.5 was counted as 2. Arrows, RSV challenge on day 56. (C) Infectious RSV/A/Tracy quantitated as the total number of PFU per nasal wash specimen and the number of PFU per gram of lung tissue from immunized cotton rats (group 1, no vaccine; group 2, live RSV i.n. on day 0; group 3, Ad5.PIV3-HN i.m.; group 4, Ad5.RSV-F i.m. only; group 5, Ad5.RSV-F i.m. on day 0 and i.n. on day 28; group 6, Ad5.RSVF i.n. only on day 28). Nasal wash and lung lavage fluid specimens were prepared 4 days after challenge with RSV/A/Tracy. RSV titers were determined on HEp-2 cells by standard plaque assay. The results represent the mean ± SD for six animals per group. Zero plaques in an undiluted sample were counted as 0.40 $\log_{10}$ total PFU in nasal wash fluid specimens and ~1.00 to 1.20 $\log_{10}$ PFU/g in lung lavage fluid specimens. The dotted lines indicate these values. Statistically significant differences (Student *t* test) are marked by asterisks. *, $P < 0.002$; ***, $P < 0.0001$.

immunization with Ad5.RSV-F resulted in only a partial but a statistically significant reduction of RSV titers in the nasal turbinate (group 4) ($P = 0.0013$). However, i.n. administration of the Ad5.RSV-F vaccine as either a single dose (group 6) or a boost immunization (group 5) was able to induce RSV sterilization of the nasal turbinates ($P < 0.0001$). These results indicate that mucosal immunization with the Ad5.RSV-F vaccine candidate is highly effective in preventing RSV/A/Tracy virus replication in both the upper and lower respiratory tract following challenge of cotton rats.

Ad5.RSV-F vaccine dose escalation. To investigate the dose-response activity of the Ad5.RSV-F vaccine, a dose escalation regimen was tested in the CR model. On day 0, animals were vaccinated i.m. with 1×10^8, 1×10^9, 1×10^{10}, and 1×10^{11} v.p. of Ad5.RSV-F per CR. Additional groups included the RSV infection control group, which received a single i.n. dose of 2.25×10^5 PFU of the RSV/A/Tracy strain on day 0, and the untreated control group. On day 28, all animals were challenged with 2.25×10^5 PFU of RSV/A/Tracy and sacrificed at 4 days postinfection for analysis of lung and nose viral titers. While the RSV/A-neutralizing antibody responses obtained with doses of 1×10^8 and 1×10^9 v.p. per CR were statistically significantly similar to those on day 0 of experimental infection and greater than those for the nonvaccinated group (group 1) ($P < 0.0001$), they were significantly lower than the neutralizing antibody responses seen in CRs given 1×10^{10} or 1×10^{11} v.p. per dose ($P < 0.05$). No statistically significant difference in the responses between the two highest doses was detected (Fig. 4A). A similar dose-response result was seen when anti-Ad5 serum neutralizing antibodies were measured, with no adenovirus-neutralizing antibodies being detected at doses of 1×10^8 or 1×10^9 v.p. (Fig. 4B); however, detectable responses were observed in CRs immunized at the two high doses. RSV replication in nasal washes and lung lavage fluid specimens was reduced irrespective of the Ad5.RSV-F dose (Fig. 4C). Overall, a single i.m. dose of Ad5.RSV-F vaccine at any potency tested produced protective levels of serum neutralizing antibody and effectively reduced RSV/A/Tracy replication in the lungs and, to a lesser extent, in the nose.

Effect of Ad5.RSV-F vaccine on lung histopathology following RSV/A/Tracy challenge. We next questioned whether immunization with Ad5.RSV-F would predispose CRs to disease exacerbation following an early challenge with RSV/A/Tracy. To assess the effect of various doses of the Ad5.RSV-F vaccine on pulmonary histopathology, CRs received 1×10^9 or 1×10^{11} v.p. of Ad5.RSV-F i.m. in either one (day 0) or two (day 0 and day 28) vaccinations. The FI RSV/A/Burnett vaccine was used as a positive control for enhanced lung pathology following RSV challenge.

CRs given Ad5.RSV-F vaccines produced robust neutralizing antibody responses against both RSV/A/Tracy and RSV/B/18537 when measured 7 days after live viral challenge. At doses of 1×10^9 and 1×10^{11} v.p., antibody responses were statistically significantly higher than those seen following experimental infection. The serum neutralizing antibody titers against subtype A virus were significantly higher in CRs given a single dose of 1×10^{11} v.p. than in CRs immunized twice. In contrast, RSV/B/18537 antibody levels were comparable in CRs given 1×10^{11} v.p. after challenge irrespective of the number of doses given (Fig. 5). Interestingly, serum neutralizing antibody titers against both subtype A and B viruses in CRs immunized with the FI RSV/A/Burnett vaccine were low when measured 7 days after RSV challenge.

For the histopathological assessment, lungs from CRs immunized with the FI RSV/A/Burnett vaccine were used as the positive control. All Ad5.RSV-F vaccine groups had lower histopathology area scores than the FI-RSV/A/Burnett vaccine group (Table 1).

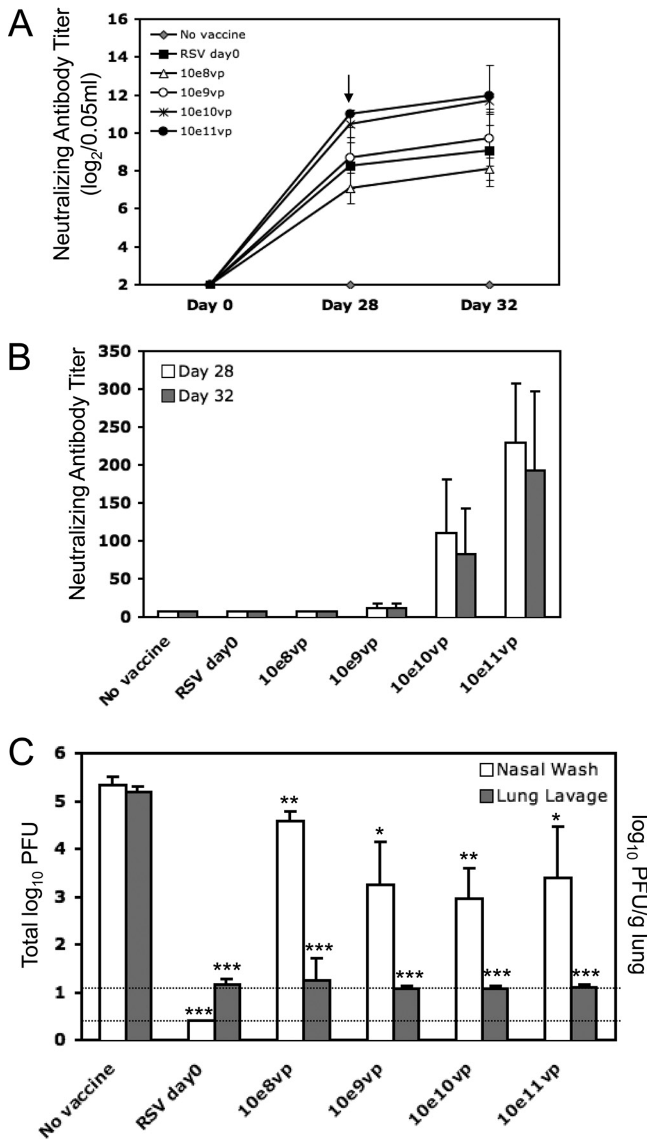

FIG 4 Dose-dependent efficacy study. The dose dependency of the Ad5.RSV-F vaccine was tested by using different doses of Ad5.RSV-F (1×10^8 to 1×10^{11} v.p./ml) administered once by intramuscular injection for immunization. Immunized cotton rats were challenged with 1.22×10^5 PFU RSV/A/Tracy intranasally at 28 days after immunization. (A) RSV serum neutralizing antibody titers against RSV/A/Tracy were measured by determining the highest dilution that inhibited RSV infection by ≥50%. Results are expressed as the geometric mean titer and standard deviation (log_2). Arrow, RSV challenge on day 28. (B) The Ad5 serum neutralizing antibody titer is the reciprocal of the serum dilution which inhibited cell transduction by Ad5.eGFP by ≥50%. (C) The levels of RSV replication in the nasal wash and lung lavage fluid specimens of each group were determined by plaque assay at day 4 postchallenge, as described in the Fig. 3 legend. *, $P < 0.01$; **, $P < 0.001$; ***, $P < 0.0001$.

The same general pattern was observed when severity scores were compared, except for the group receiving a single dose of the Ad5.RSV-F vaccine at 1×10^{11} v.p. (severity score, 2.67). However, two animals in this group (which received 1×10^{11} v.p. Ad5.RSV-F once) had inhaled bedding (plant material), and it was not possible to determine the contribution of this material to the overall score. It should be noted that the same vaccine given twice (group 5) was not associated with any detectable histopathology 7

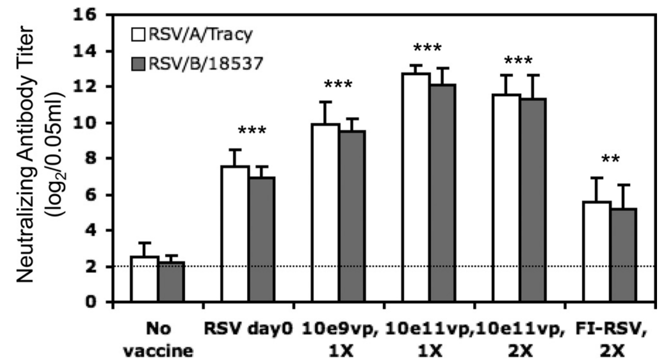

FIG 5 Efficacy of the Ad5.RSV-F vaccine. The RSV-neutralizing activity of sera from cotton rats immunized i.m. with 1×10^9 and 1×10^{11} v.p. once or 1×10^{11} v.p. and FI RSV/A/Burnett twice i.m. is shown. RSV-F neutralizing antibody titers against RSV/A/Tracy and RSV/B/18537 were measured on day 63 by determining the highest dilution inhibiting RSV infection by ≥50%. Results are expressed as the geometric mean titer and standard deviation (log_2). Dashed line, limit of detection. **, $P < 0.001$; ***, $P < 0.0001$.

days after RSV challenge. Thus, it does not appear that the Ad.5RSV-F vaccine is associated with enhanced histopathology in CRs challenged soon after immunization.

RSV antibody response repertoire. Palivizumab, a humanized RSV-neutralizing antibody targeting antigenic site A, is a Food and Drug Administration (FDA)-approved prophylactic antibody that prevents severe RSV disease. To identify the epitope- and site-specific antibody responses induced by immunization with Ad5.RSV-F, including antibodies that mimic palivizumab, we performed a competitive binding assay using anti-F-protein BMAbs B1121 (site A), B1129 (palivizumab parent, site A), B1107 (site AB), B1269 (site B), and B1243 (site C), which bind antigenic sites A, B, and C involving amino acids 255 to 275, 389, and 422 to 438, respectively (28). Sera from individual animals from each group were pooled and tested for the ability to inhibit the binding of biotinylated anti-F-protein or anti-G-protein monoclonal antibodies to antigens expressed on the surface of RSV-infected cells. Data are shown for sera diluted 1:20, since blocking of BMAbs was not detected at higher serum dilutions. Competition was not observed in sera pooled from CRs given PBS (group 1) or Ad5.PIV3-HN (group 3) or by the use of pooled preimmune serum samples (group 7). Interestingly, inhibition of BMAb binding was not observed with sera obtained from CRs infected on day 0 with RSV/A/Tracy (group 2). Pooled sera from animals in group 4 given Ad5.RSV-F i.m. did not significantly block binding of any of the anti-F-protein BMAbs tested, albeit binding of BMAb 1129 was decreased by 40%; however, this decrease was not significant (Fig. 6). In contrast, sera from group 5 (given Ad5.RSV-F i.m. followed by Ad5.RSV-F i.n.) significantly inhibited binding of BMAbs 1129, 1107, and 1269, while sera from group 6 (given Ad5.RSV-F i.n. only) significantly inhibited binding of BMAbs B1129 and B1269. The significance of these observations is addressed in the Discussion section.

DISCUSSION

In the present study, we have shown that immunization of CRs with a recombinant human adenovirus serotype 5 strain encoding the codon-optimized ORFs of the fusion protein of RSV (Ad5.RSV-F) leads to a protective immune response without vaccine-related en-

TABLE 1 Pulmonary histopathology scores after RSV challenge[a]

Animal group	Total score for each histological parameter													
	Peribronchial mononuclear inflammatory cell infiltrates		Bronchiolitis		Bronchiolar eosinophilia		Alveolitis		Alveolar eosinophilia		Perivascular mononuclear inflammatory cell infiltrates		Mean for all parameters	
	Area	Severity	Area	Severity	Area	Severity	Area	Severity	Area	Severity	Area	Severity	Area	Severity
No vaccine	0	0	0	0	0	0	0	0	0	0	0	0	0.00	0.00
RSV infection, day 0	0	0	0	0	0	0	1	4	0	0	0	0	0.17	0.67
1×10^9 v.p. given once	0	0	0	0	1	3	0	0	0	0	0	0	0.17	0.50
1×10^{11} v.p. given once	1	2	0	0	3	6	2	4	3	3	1	1	1.67	2.67[b]
1×10^{11} v.p. given twice	0	0	0	0	0	0	0	0	0	0	0	0	0.00	0.00
FI RSV/A/Burnett given twice	8	6	0	0	5	6	5	5	7	12	7	6	5.33	5.83

[a] Lung histopathology scores from cotton rats ($n = 6$ per group) immunized intramuscularly with the indicated dose of Ad5.RSV-F once or twice, challenged with RSV/A/Tracy, and examined 7 days later are shown. Lung tissues were subjected to histopathology, and blinded samples were scored for area of involvement and severity, each on a scale from 0 to 4+. The total score of all animals in each group and the mean of all measures per animal for each histological parameter are given.

[b] Two animals in this group had inhaled bedding (plant material). The adjusted mean area and mean severity score for this group without the two mice with bedding in the lungs are both 0.25.

hanced respiratory disease following an early experimental RSV challenge. This study did not assess whether Ad5.RSV-F induced a prolonged duration of protection. RSV-F is an attractive vaccine target antigen due to its considerable conservation among RSV isolates. However, the expression of wild-type RSV-F from cDNA via RNA polymerase II in eukaryotic cells is impaired due to a premature polyadenylation site within the protein ORF (30). To overcome this obstacle and increase its expression level, we and others have synthesized a codon-optimized version of RSV-F (32–35). Codon optimization is a technique that maximizes protein expression in living organisms by increasing the translational efficiency of the gene of interest by altering DNA codon triplet sequences without altering the wild-type protein sequence. The optimal eukaryotic codon usage for RSV-F coincidentally abolishes the premature polyadenylation site upregulating F-protein expression. By evaluating several different codon-optimized RSV-F constructs (data not shown), we were able to not only select a recombinant with a high level of F-protein expression in mammalian cells but also identify a recombinant adenoviral vector able to provide satisfactory production yield in HEK293 cells and to provide the level of RSV-F expression *in vivo* necessary to elicit potent immune responses.

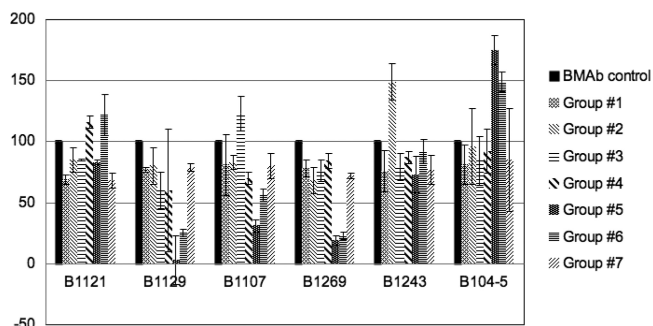

FIG 6 Competitive binding antibody assay. BMAbs B1121 (anti-F protein, site A), B1129 (palivizumab parent, anti-F protein, site A), B1107 (anti-F protein, site AB), B1269 (anti-F protein, site B), B1243 (anti-F protein, site C), and B104-5 (anti-G protein subtype A) were used for competition at 50% saturation. Each BMAb was mixed 1:1 either with PBS or with cotton rat serum (final dilution, 1:20 after mixing) pooled from each group and tested in triplicate on RSV-infected LLC-MK2 cells. Binding of BMAbs was detected following addition of streptavidin-conjugated horseradish peroxidase, followed by addition of ABTS substrate and reading at an optical density of 405 nm. The signal obtained for each BMAb in the absence of competitor was set at 100%, and the signal obtained in the presence of competitor was expressed as a percentage of that for the control. Group 1, no vaccine; group 2, live RSV i.n. on day 0; group 3, Ad5.hPIV3 i.m.; group 4, Ad5.RSV-F i.m. only; group 5, Ad5.RSV-F i.m. on day 0 and i.n. on day 28; group 6, Ad5.RSV-F i.n. only on day 28; group 7, prevaccination sera on day 0.

Previous studies with adenovirus carrying codon-optimized full-length RSV-F or soluble F antigen showed a protective immune response to RSV in a mouse model (32, 33). Here, our work differs from previously published studies, because we used the CR model to investigate the immunogenicity, efficacy, and enhanced disease potential of the Ad5.RSV-F vaccine candidate. Vaccine safety is an important point to consider when developing an RSV vaccine candidate for use in infants. The pediatric clinical trials performed in the 1960s with the FI RSV 100× vaccine impeded RSV vaccine development because of the exacerbated clinical disease that occurred when the youngest vaccinees were subsequently infected with RSV, resulting in increased hospitalization and two vaccine-related deaths (26). CRs are more susceptible to infection with human RSV than mice and have been used for years as an excellent small-animal model for RSV vaccine-enhanced disease (21).

In the studies presented here, we show that the Ad5.RSV-F vaccine administered to CRs is capable of inducing high levels of RSV-specific neutralizing antibodies that are comparable to those induced by experimental RSV infection. Comparing two different routes of administration or by combining them in a prime-boost regimen, we also demonstrate that i.n. delivery of the vaccine may be more effective than delivery by the i.m. route. These findings are similar to those in previous reports where an adenovirus-based RSV vaccine encoding codon-optimized F antigen induced after a single i.n. dose strong mucosal IgA responses associated with protection against experimental RSV challenge in mice (32). However, further investigations are necessary to evaluate i.n. versus i.m. immunization on day 0 with challenge on day 56 to show that the protection seen following i.n. immunization on day 0 is robust and that the immunity seen following dosing on day 28 is not due

to the shorter interval between immunization and challenge. Furthermore, in a dose escalation study, we determined that a single dose at a low v.p. concentration of Ad5.RSV-F vaccine (10^8 or 10^9 v.p.) was able to induce a humoral response comparable to that seen following experimental RSV infection, which is at least the minimum level of humoral response desired for a candidate RSV vaccine. Moreover, antibodies elicited by Ad5.RSV-F immunization exhibited cross-neutralization against the RSV subtype A and RSV subtype B strains, even though the F-protein sequence was derived from a subtype A RSV strain, as expected.

The humoral response was further characterized to determine if antibodies elicited following Ad5.RSV-F immunization bound to previously identified neutralization epitopes on RSV-F and to see if epitope-specific responses *in vitro* would correlate with protection *in vivo* (36). Interestingly, our studies showed that the route and/or regimen of immunization might influence the repertoire of the humoral response. We observed that while a single i.m. immunization protected CRs from RSV challenge, the humoral response generated did not compete with monoclonal antibodies specific for site A, AB, B, or C on RSV-F expressed on the surface of infected cells. Conversely, when the same vaccine was administered i.n. as a single dose or as a boost following i.m. priming, the humoral response competed with anti-RSV-F monoclonal antibodies 1129 (site A) and 1269 (site B). Significantly, 1129 (site A) is the palivizumab parent whose effectiveness and safety are well established in the clinical setting. Moreover, the i.m. prime-i.n. boost regimen also elicited immunity to antigenic site AB that was not measurable in sera from animals given a single dose of Ad5.RSV-F i.m. or i.n. These observations suggest induction of epitope spreading and somatic maturation of the antibody response following prime-boost immunization. However, further investigations are necessary to better define the effect of sequential immunization on the antibody repertoire and somatic maturation. It is also important to remember that RSV-F is a type I fusion protein that rearranges itself from a metastable prefusion form into a highly stable postfusion structure. The antigenic sites examined in the competition assay are known to exist on the postfusion form of RSV-F (37). The palivizumab/motavizumab binding site is similarly present on the prefusion form of RSV-F, albeit the binding affinity to pre-F protein appears to be significantly lower than the affinity to postfusion F antigen (36). Natural RSV infection in humans induces humoral neutralizing activity directed primarily against the prefusion conformation of RSV-F, and a number of prefusion-specific neutralizing antibodies have shown higher potency than palivizumab or motavizumab (36). This might explain why the humoral response induced in the CR group experimentally infected with RSV/A/Tracy i.n. failed to compete with the monoclonal antibodies with high specificity for postfusion neutralizing sites, although this group was completely protected from RSV challenge. Since our competition assay was not optimized to detect antibodies binding to the newly identified antigenic site Ø that is present only on prefusion F antigen (36), we intend to further investigate the repertoire of antibodies elicited by Ad5.RSV-F immunization, once prefusion antigen and prefusion blocking antibodies become available.

It has been suggested that an optimal RSV vaccine is needed to elicit both high titers of neutralizing anti-RSV antibodies and RSV-specific-CD8$^+$ T-cell responses (38). Viral clearance from the lungs occurs once a potent T-cell response is induced (39). Fatal or severe lower respiratory tract RSV infections are characterized by robust viral replication and the near absence of CD8$^+$ lymphocytes or lymphocyte-derived cytokines (40). Our studies of the Ad5.RSV-F vaccine in mice demonstrated elicitation of both the B-cell response and an F/85-93 epitope-specific T-cell response. While the contribution of B-cell/T-cell activity was not dissected in the current CR model studies, it is likely that each contributed to the protective effect, and perhaps due to the presence of balanced neutralizing B-cell and T-cell activity, protection was conferred in the absence of enhanced lung pathology after RSV challenge.

In conclusion, our studies show that recombinant adenovirus expressing the codon-optimized RSV-F gene provides protective immunity against RSV challenge without enhanced lung pathology in a CR model. We believe that immunity induced by Ad5.RSV-F administered by use of an i.m. prime-i.n. boost regimen may provide the best protection for young infants and children at risk of RSV infection, since this population is naive to adenoviral preformed immunity. Thus, we have confidence that Ad5.RSV-F represents a promising RSV vaccine candidate that merits further evaluation in a phase I clinical study in humans.

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