Nonreplicating Vaccines Can Protect African Green Monkeys From the Memphis 37 Strain of Respiratory Syncytial Virus

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Background. We evaluated the immunological responses of African green monkeys immunized with multiple F and G protein–based vaccines and assessed protection against the Memphis 37 strain of respiratory syncytial virus (RSV).

Methods. Monkeys were immunized with F and G proteins adjuvanted with immunostimulatory (CpG) oligodeoxynucleotides admixed with either Alhydrogel or ISCOMATRIX adjuvant. Delivery of F and G proteins via replication incompetent recombinant vesicular stomatitis viruses (VSVs) and human adenoviruses was also evaluated. Mucosally or parenterally administered recombinant adenoviruses were used in prime-boost regimens with adjuvanted proteins or recombinant DNA.

Results. Animals primed by intranasal delivery of recombinant adenoviruses, and boosted by intramuscular injection of adjuvanted F and G proteins, developed neutralizing antibodies and F/G protein–specific T cells and were protected from RSV infection. Intramuscular injections of Alhydrogel (plus CpG) adjuvanted F and G proteins reduced peak viral loads in the lungs of challenged monkeys. Granulocyte numbers were not significantly elevated, relative to controls, in postchallenge bronchoalveolar lavage samples from vaccinated animals.

Conclusions. This study has validated the use of RSV (Memphis 37) in an African green monkey model of intranasal infection and identified nonreplicating vaccines capable of eliciting protection in this higher species challenge model.

Keywords. pathogen; immunization; adaptive immunity; mucosal; adjuvant; viral vector; intranasal; nonhuman primate; translational; CpG.

Respiratory syncytial virus (RSV), an enveloped RNA virus belonging to the Paramyxovirus family, is a significant human pathogen [1]. RSV-induced lower respiratory tract disease is a frequent cause of hospitalization for infants and there is increasing recognition that the pathogen also causes appreciable morbidity and mortality in older adults and individuals with underlying health issues [2–4]. Even outside these “high-risk” populations, the economic cost and healthcare burden associated with RSV epidemics can be significant [5–7]. Although reinfection occurs throughout life, experimental evidence indicates that circulating neutralizing antibodies afford some level of resistance to RSV, as shown by protection data from human challenge studies [8] and serological analyses during epidemics [3, 9, 10]. Similarly, passive immunoprophylaxis using fusion-inhibiting monoclonal antibodies can be an effective preventive measure for infants at risk of RSV-mediated lower respiratory tract disease [11]. Other studies highlight the importance of mucosal antibodies [12] and cell-mediated immunity [13, 14].

We evaluated novel immunization approaches in an African green monkey (AGM) model to understand
more about the critical components of vaccine-mediated immunity against RSV infection. Vaccines that significantly reduce viral loads in RSV-challenged AGMs have potential for disease prevention in humans; a correlation between viral loads and disease severity has been established in human challenge studies [15]. By selecting the Memphis 37 strain (M37) of RSV for our challenge experiments, we have been able to assess vaccine efficacy against a virulent clinical isolate that can also be used for human challenge studies [15]. AGMs were immunized with RSV-derived F and G proteins combined with novel adjuvants, including immunostimulatory Toll-like receptor 9 binding oligodeoxiribonucleotides (CpG) and nanoparticles containing a saponin complexed with cholesterol and phospholipid (ISCOMATRIX adjuvant) [16]. We also evaluated replication-incompetent recombinant viruses and DNA vectors capable of expressing RSV F and G proteins following in vivo administration. All approaches had proven efficacious in earlier studies using murine and/or cotton rat models of RSV infection (results not shown and [17]), yet demonstrated rather different and more differentiated outcomes in the AGM model.

**METHODS**

**Recombinant Vesicular Stomatitis Viruses**

Recombinant vesicular stomatitis virus (VSV) vectors were derived from complementary DNA encoding complete VSV<sub>IN</sub> genomes containing the RSV A2 F or G genes in the third genomic position [17] and a deletion in the G gene (Gstem) that truncated the majority of the extracellular domain of the protein, including the attachment and fusion domains [18–20]. These viruses were propagated on recombinant Vero cells expressing the full-length glycoprotein (G) from either the Indiana or New Jersey serotype of VSV [21], to complement the viruses and to circumvent antivector immune responses when boosting [22]. Recombinant VSV vectors were used at a dose of approximately 1 × 10<sup>7</sup> plaque-forming units (PFU) for each construct administered by either the intramuscular or intranasal route.

**Recombinant Adenoviruses**

Recombinant human adenovirus (serotype 5) genomes capable of expressing RSV F or G proteins were generated using an AdEasy-XL Kit (Biogene). In brief, polymerase chain reaction was used to clone DNA encoding full-length F protein (A2 sequence) or G protein ectodomain (A2 sequence) into pCR2.1. F and G protein sequences were then subcloned from this vector into pShuttle-CMV by restriction digest. Following ligation, bacterial transformation, and identification of positive clones, DNA constructs were sequence verified prior to homologous recombination with the adenovirus plasmid in Escherichia coli. Production and purification of the recombinant adenoviruses was outsourced to ViraQuest Inc. Recombinant adenovirus vectors were used at a dose of approximately 1 × 10<sup>9</sup> PFU for each construct administered by either the intramuscular or intranasal route.

**Purified Natural Proteins**

RSV fusion and attachment proteins were purified and characterized as described elsewhere [23–25]. F protein was extracted from Vero cells infected with a cold-passaged temperature-sensitive mutant strain, which was originally derived from RSV A2, and purified by ion exchange chromatography. Natural G protein was purified by immunoaffinity chromatography from Vero cells infected with RSV A2. Each immunizing dose contained 40 µg of F protein and 4 µg of G protein in phosphate-buffered saline (PBS). Two adjuvating strategies were used for the purified proteins: proteins were mixed either with 220 µg of Alhydrogel “85” (Alum; Brenntag Biosector) and 500 µg of CpG 24555 (5′ TCATTGTTTTTTTGTTTTTTTTT 3′) or with 45 ISCO units of ISCOMATRIX adjuvant (CSL Biotherapies Inc) and 500 µg of CpG 24 555. Both adjuvanted formulations were injected intramuscularly.

**DNA**

DNA plasmids encoding full-length F and G proteins from RSV A2 were generated by cloning the relevant sequences into the mammalian expression vector pPJV7563 [26] prior to scale-up using Top10 E. coli cells (Invitrogen). Plasmids encoding either the F or G protein were coprecipitated onto gold beads (1–3 µm) with DEI-LT pPVJ2012, a plasmid that expresses the A and B subunits of E. coli heat-labile enterotoxin, and were then filled into specialized cassettes prior to in vivo experimentation. Each cassette contained 1.8 µg of DNA encoding either F or G protein coprecipitated onto 1 mg of gold with 0.2 µg of DEI-LT pPVJ2012. Ten cassettes, 5 of each encoding F or G, were actuated into the shaved abdomen during each particle-mediated epidermal delivery (PMED) immunization event.

**Immunization**

Forty-two AGMs, genetically unselected, were assigned to this study. The animals were randomized to 7 study groups (Table 1) on the basis of RSV neutralizing antibody titer and body weight. For intramuscular delivery, 0.5 mL of a vaccine formulation was injected into the left quadriceps muscle. For intranasal delivery of adenoviruses, animals were anesthetized and 0.25 mL of the vaccine formulation was administered drop-wise into each nare. A proprietary “gene-gun” device was used for ballistic delivery of the DNA constructs into the epidermal skin layers using pressurized helium (PMED) [26–28]. One group of 6 animals (RSV intranasal) was infected, following the same procedure used for the live virus challenge, 10 weeks prior to the actual challenge. Animals were sedated with ketamine hydrochloride for immunization procedures (except...
intranasal inoculation when Telazol was used). All procedures were conducted in accordance with guidelines for the care and use of laboratory animals and with full approval of local Institutional Animal Care and Use Committee officials.

### Challenge

RSV M37 was purchased from Meridian Life Science Inc. At week 18 of the study, immunized and control animals were challenged intranasally with 7.5 × 10⁵ PFU of M37 suspended in 1.0 mL of PBS: animals were anaesthetized and 0.5 mL of virus suspension was administered dropwise into each nare. Copan-locked swabs with Universal Transport Media (Diagnostic Hybrids Inc) were used to sample the nasal passages and throats of challenged animals. Bronchoalveolar lavage (BAL) samples were collected by inserting a tracheal tube and infusing a suitable volume of isotonic saline, based on animal body weight. The total volume of BAL recovered was measured using a graduated pipette and diluted using an appropriate volume of concentrated sucrose-phosphate-glutamate buffer (0.2 M sucrose, 7.0 mM K₂HPO₄, 3.8 mM KH₂PO₄, 5.0 mM glutamic acid). Nasal swabs, throat swabs, and BAL were collected from all challenged animals. Bronchoalveolar lavage (BAL) samples were quantified by measurement at 405 nm using a Versamax plate reader (Molecular Devices). The antibody titer is expressed as the reciprocal serum dilution at which the optical density

### Antibody Analyses

Animals were bled periodically throughout the study to derive serum for analysis. Endpoint anti-F or G serum immunoglobulin G (IgG) titers were determined by enzyme-linked immunosorbent assay (ELISA). In brief, 0.2 µg/mL RSV F or RSV G protein was coated onto Immuno 96-well plates (Nunc) and serial dilutions of the antisera were added in duplicate to these coated plates. Goat antimonkey IgG (Fc)-biotinylated (Nordic Immunology) and streptavidin horseradish peroxidase (Invi- trogen) was added to amplify the detection of the antigen-antibody complexes. Following addition of 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (KPL), color changes were detected by measurement at 405 nm using a Versamax plate reader (Molecular Devices). The antibody titer is expressed as the reciprocal serum dilution at which the optical density
Antibody avidity was determined using a thiocyanate ELISA assay adapted from Pullen et al [30]. A plaque reduction neutralization test was used to quantify RSV neutralizing antibody titers in serum samples. Neutralization was measured by inoculating Vero cells with RSV M37 preincubated with serially diluted heat inactivated serum. The infected cells were counted per well and compared to a virus-only control. Neutralization was determined as the serum dilution factor resulting in a 60% reduction in infectious units. No additional source of complement was added to the serum samples tested in the assay. Antibody levels in BAL were determined as described for serum.

**T-Cell Response**

To analyze antigen specific T-cell responses, peripheral blood mononuclear cells (PBMCs) were isolated: diluted blood was centrifuged on a Histopaque (Sigma) gradient and the PBMCs were carefully removed and washed. Cells were counted and resuspended in culture media (RPMI w/Glutamax, penicillin 100 U/mL, streptomycin 100 µg/mL, MEM nonessential amino acid solution 0.1 mM, HEPES 0.01 M, and 0.055 mM Eagle’s basal medium all obtained from Invitrogen) containing 10% dimethyl sulfoxide (DMSO) (Sigma) and 20% fetal calf serum (PAA), at a concentration of approximately 5 × 10^6 cells/mL. PBMCs were cryopreserved at each blood draw.

Sequential peptides, 15mers overlapping by 10 amino acids, were ordered covering the entire sequences of RSV F and G proteins (JPT Peptide Technologies). Peptides were diluted in DMSO at 20 mg/mL and pooled as follows: 2 peptide pools containing F protein-specific peptides 1–56 and 57–113, respectively, and a single peptide pool containing G protein-specific peptides 1–58. An interferon gamma (IFN-γ) enzyme-linked immunospot (ELISpot) assay was performed as per the manufacturer’s instructions (Mabtech). Each test reagent was diluted to the required concentration in culture media (phytohemagglutinin [Calbiochem] 10 µg/mL, peptide pools 10 µg/mL/peptide) and plated, 100 µL per well, in duplicate, with 100 µL of PBMC sample. Plates were incubated (37°C, 5% CO2) for 16–18 hours prior to development and enumeration of spot-forming cells using an ELISpot plate reader (CTL) and Immunospot software.

**Statistical Analysis**

Significance was defined as $P < .05$ using nonparametric statistical testing (Kruskal–Wallis and Dunn test for multiple comparisons). All statistical calculations were conducted with Prism 5 (GraphPad Software).

**RESULTS**

**RSV Challenge**

All 6 control (naive) monkeys shed infectious virus particles into their respiratory tracts following an internasal challenge with RSV M37 (Figure 1 and Supplementary Figure 3). Conversely, no RSV was detectable in nasal and throat swabs and BAL from RSV (intranasal)-immunized AGMs, indicating that intranasal challenge with RSV M37 initiates infection throughout the respiratory tract in susceptible AGMs, whereas appropriate immunological effector responses elicited by prior infection confers immunity.

The peak load of RSV M37 detected in nasal and throat swabs from challenged AGMs previously immunized with F
### Table 2. Total and Differential Counts in Bronchoalveolar Lavage, Cells/mL

| Day | Cell Type | Median | IQR | Median | IQR | Median | IQR | Median | IQR | Median | IQR | Median | IQR | Median | IQR | Median | IQR |
|-----|-----------|--------|-----|--------|-----|--------|-----|--------|-----|--------|-----|--------|-----|--------|-----|--------|-----|--------|-----|
| -7  | Total     | 56 200 | 204 450 | 78 600 | 185 075 | 97 700 | 111 275 | 87 150 | 125 698 | 124 000 | 215 050 | 81 200 | 124 150 | 161 000 | 216 500 |
|     | Lymphocytes | 6040 | 11 075 | 6240 | 22 277 | 9417 | 5783 | 8369 | 9803 | 11 610 | 18 241 | 7346 | 16 295 | 9340 | 15 711 |
|     | Monocytes | 9588 | 30 552 | 12 438 | 38 537 | 18 656 | 19 687 | 16 301 | 24 196 | 20 820 | 31 635 | 20 256 | 31 156 | 14 162 | 26 562 |
|     | Macrophages | 36 767 | 78 233 | 56 743 | 119 340 | 66 707 | 79 849 | 60 911 | 70 018 | 89 780 | 154 553 | 53 012 | 76 923 | 120 320 | 148 152 |
|     | Neutrophils | 1283 | 7431 | 948 | 2618 | 911 | 1350 | 0 | 1269 | 615 | 2052 | 471 | 1082 | 1828 | 6072 |
| 4   | Total     | 187 500 | 61 000 | 220 000 | 253 675 | 193 700 | 411 150 | 249 000 | 193 000 | 457 575 | 184 000 | 253 250 | 213 500 | 336 275 |
|     | Lymphocytes | 17 880 | 8524 | 26 540 | 34 288 | 18 759 | 37 087 | 27 370 | 20 745 | 16 278 | 25 954 | 19 703 | 8719 | 17 535 | 40 973 |
|     | Monocytes | 30 395 | 11 746 | 35 255 | 46 211 | 32 383 | 72 449 | 47 420 | 25 720 | 27 555 | 50 634 | 29 665 | 15 904 | 29 173 | 83 034 |
|     | Macrophages | 117 555 | 44 669 | 134 315 | 164 276 | 113 017 | 294 881 | 127 905 | 78 170 | 139 233 | 142 645 | 126 040 | 105 053 | 145 793 | 157 013 |
|     | Neutrophils | 15 775 | 30 210 | 7000 | 9539 | 4676 | 14 462 | 7870 | 54 578 | 11 428 | 229 853 | 6245 | 15 209 | 10 138 | 55 982 |
| 7   | Total     | 101 000 | 211 450 | 215 000 | 128 700 | 182 000 | 209 750 | 222 000 | 203 950 | 141 500 | 368 270 | 116 500 | 45 975 | 189 500 | 236 300 |
|     | Lymphocytes | 7534 | 18 845 | 24 425 | 16 386 | 17 520 | 25 019 | 23 310 | 34 343 | 12 735 | 23 607 | 11 183 | 9028 | 24 768 | 21 258 |
|     | Monocytes | 19 488 | 39 001 | 39 938 | 28 088 | 29 345 | 45 209 | 45 085 | 39 408 | 21 828 | 80 250 | 21 100 | 14 302 | 32 525 | 36 635 |
|     | Macrophages | 60 320 | 119 944 | 115 473 | 75 744 | 105 780 | 111 131 | 111 330 | 109 025 | 85 308 | 235 423 | 63 465 | 26 375 | 93 423 | 161 231 |
|     | Neutrophils | 9516 | 44 730 | 3823 | 5425 | 11 340 | 37 926 | 5850 | 5039 | 6068 | 24 940 | 4300 | 11 192 | 10 710 | 16 011 |
| 21  | Total     | 166 500 | 149 750 | 116 700 | 120 575 | 148 500 | 123 925 | 121 500 | 90 750 | 42 500 | 71 200 | 88 500 | 99 550 |
|     | Lymphocytes | 27 458 | 37 330 | 13 136 | 21 580 | 13 398 | 11 975 | 9720 | 12 954 | 11 540 | 10 123 | 3296 | 10 764 | 9405 | 15 104 |
|     | Monocytes | 36 285 | 39 909 | 21 015 | 24 016 | 25 033 | 32 591 | 14 580 | 21 810 | 23 910 | 17 851 | 7325 | 15 907 | 20 678 | 20 089 |
|     | Macrophages | 78 523 | 27 349 | 57 085 | 62 459 | 81 223 | 69 664 | 50 760 | 68 792 | 75 008 | 70 758 | 23 733 | 20 465 | 48 285 | 49 890 |
|     | Neutrophils | 3248 | 4428 | 5226 | 10 378 | 7249 | 14 026 | 2277 | 2973 | 7285 | 13 009 | 1742 | 5948 | 461 | 2672 |
|     | Eosinophils | 22 108 | 33 278 | 7216 | 13 303 | 6952 | 14 209 | 7245 | 13 899 | 9565 | 11 786 | 827 | 5571 | 9175 | 14 018 |

Median total and differential cell counts with interquartile range in bronchoalveolar lavage (BAL). BAL samples were taken from all African green monkeys on −7, 4, 7, and 21 days following intranasal challenge with respiratory syncytial virus (RSV) M37 at week 18 of the experiment. Overall, there is no significant difference at any time point between cell numbers (total, lymphocytes, monocytes, macrophages, neutrophils, eosinophils) in BAL from vaccinated and unimmunized control (naive) or previously infected (RSV intranasal) groups. Basophils were not detected in any of the BAL samples, so these data were omitted. Significance was defined as $P < 0.05$ using nonparametric statistical testing (Kruskal–Wallis and Dunn test for multiple comparisons).

Abbreviations: AD5, recombinant adenovirus; Alum, Alhydrogel “85”; BAL, bronchoalveolar lavage; CpG, immunostimulatory oligodeoxyribonucleotides; IM, intramuscular; IN, intranasal; IMX, ISCOMATRIX adjuvant; IQR, interquartile range; PFU, plaque-forming units; PMED, particle-mediated epidermal delivery; RSV, respiratory syncytial virus; VSV, vesicular stomatitis virus.
and G proteins adjuvanted with CpG/ISCOMATRIX adjuvant or recombinant VSV and adenovirus (VSV & AD5) or recombinant adenovirus and PMED (AD5 & PMED) was similar to that detected in swabs from control (naive) animals (Figure 1). AGMs previously intranasally immunized with recombinant adenoviruses in a heterologous regimen with proteins (AD5 intranasal & proteins intramuscular), had no virus recovered from their upper respiratory tracts following challenge. Monkeys immunized by intramuscular injection of F and G proteins adjuvanted with CpG/Alum (proteins CpG/Alum) also had no virus recovered from their upper respiratory tracts following challenge. Monkeys immunized by intramuscular injection of F and G proteins adjuvanted with CpG/Alum (proteins CpG/Alum) were effectively protected from infection. Furthermore, 5 of 6 monkeys immunized by intramuscular injection of F and G proteins adjuvanted with CpG/Alum (proteins CpG/Alum) were protected as judged by undetectable levels of RSV in their BAL following challenge. Peak RSV loads in BAL from this group were significantly lower than in BAL from naive animals (P < .05).

Cell counts from BAL derived 7 days prior to challenge and 4, 7, and 21 days postchallenge confirmed a transient increase in total cell numbers in all the test groups after the RSV challenge (Table 2). Differential cell counts highlighted changes in neutrophil and eosinophil numbers in BAL taken from unimmunized (naive) animals prior to and 4 days after the challenge. Granulocyte numbers also increased in BAL from AGMs immunized with CpG/Alum-adjuvanted proteins. None of the immunization approaches elicited significantly increased

Figure 2. Respiratory syncytial virus (RSV) M37 neutralizing antibody levels in serum samples from individual animals (represented as the serum dilution required to achieve 60% inhibition of viral infectivity ex vivo) derived at weeks −1 (A), 2 (B), 10 (C), 18 (D), and 22 (E) of the experiment. African green monkeys were immunized according to Table 1, prior to intranasal challenge at week 18 with 7.5 × 10⁵ plaque-forming units of RSV M37. No additional source of complement was added to serum samples used in the microneutralization assay. Bar denotes group median. Significant differences between treatment groups are denoted by encircled numbers referring to comparator groups. Significance was defined as P < .05 using nonparametric statistical testing (Kruskal–Wallis and Dunn test for multiple comparisons). Abbreviations: ADS, recombinant adenovirus; Alum, Alhydrogel “85”; CpG, immunostimulatory oligodeoxyribonucleotides; IM, intramuscular; IN, intranasal; IMX, ISCOMATRIX adjuvant; PMED, particle-mediated epidermal delivery; RSV, respiratory syncytial virus; VSV, vesicular stomatitis virus.
concentrations of granulocytes in postchallenge BAL compared with unimmunized (naive) animals.

**Antibody Response to Vaccination**

Immunization of AGMs, as outlined in Table 1, elicited RSV M37 neutralizing antibodies (Figure 2). A single injection of adjuvanted proteins was sufficient to induce significant levels of neutralizing antibodies relative to (naive) controls (P < .05). Titers in all vaccination groups were improved by a second immunization event at week 8. At the time of challenge (week 18), AGMs immunized with proteins formulated with CpG/Alum had higher circulating neutralizing antibody titers than animals immunized using a combined viral vector-DNA approach (AD5 & PMED; P < .05). Although other immunization approaches (VSV & AD5, AD5 intranasal & proteins intramuscular, and AD5 & PMED) elicited circulating neutralizing antibodies, the average titers were similar to each other and controls at time of challenge.

Circulating neutralizing antibody levels measured at week 22 reflected the influence of antigenic stimulation provided by RSV challenge; a pronounced increase in neutralizing antibody titer occurred between weeks 18 and 22 in previously unimmunized (naive) animals. Conversely, neutralizing antibody levels remained relatively unchanged or decreased in immunization groups. A neutralizing antibody titer decrease was particularly evident in AGMs vaccinated with adjuvanted proteins, suggesting that the viral challenge did not exert a discernible boosting effect on RSV-specific antibodies in these groups.

F and G protein binding IgG antibodies were detected in serum samples from immunized animals using ELISA. The magnitude and kinetics of those responses differed for the various immunization strategies (Figures 3 and 4). AGMs

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**Figure 3.** Endpoint anti-F immunoglobulin G (IgG) titers in serum samples from individual animals derived at week −1 (A), 2 (B), 10 (C), 18 (D), and 22 (E) of the experiment. F, Anti-F IgG avidities (represented as EC50 values for individual animals determined by ammonium thiocyanate assay) in serum samples derived at week 18. African green monkeys were immunized according to Table 1, prior to intranasal challenge at week 18 with 7.5 × 10^5 plaque-forming units of respiratory syncytial virus (RSV) M37. Bar denotes group median. Significant differences between treatment groups are denoted by encircled numbers referring to comparator groups. Significance was defined as P < .05 using nonparametric statistical testing (Kruskal–Wallis and Dunn test for multiple comparisons). Abbreviations: AD5, recombinant adenovirus; Alum, Alhydrogel “85”; CpG, immunostimulatory oligodeoxyribonucleotides; EC50, half maximal effective concentration; IgG, immunoglobulin G; IM, intramuscular; IN, intranasal; IMX, ISCOMATRIX adjuvant; PMED, particle-mediated epidermal delivery; RSV, respiratory syncytial virus, VSV, vesicular stomatitis virus.
immunized with adjuvanted proteins had higher titers \( (P < .05) \) of anti-F/G IgG in their serum as compared with unimmunized (naive) controls and monkeys previously infected with RSV (RSV intranasal) at time of challenge (week 18). Similarly, AGMs immunized with proteins formulated with CpG/Alum had circulating anti-F IgG titers above those detected in monkeys immunized using a combined viral vector-DNA approach (AD5 & PMED; \( P < .05 \)). Monkeys immunized using a combined intranasal/intramuscular immunization regimen (AD5 intranasal & proteins intramuscular) also had higher \( (P < .05) \) titers of anti-F/G IgG in their serum as compared with unimmunized (naive) controls at week 18. Although other immunization approaches (VSV & AD5 and AD5 & PMED) raised circulating anti-F/G IgG antibodies, the levels in serum derived at week 18 of the experiment were not different to other test group as adjudged by nonparametric statistical analysis. None of the immunization approaches efficiently induced high titers of nonneutralizing antibodies, as indicated by calculating the ratio of neutralizing to binding IgG titers in serum derived at week 18 (Supplementary Figure 2).

Ammonium thiocyanate ELISA analyses of antigen specific antibody avidities in serum derived at the time of challenge revealed that immunization with recombinant adenoviruses and PMED (AD5 & PMED) or VSV and AD5 (VSV & AD5) elicited circulating F-specific IgG antibodies of low avidity compared to vaccination with proteins formulated with CpG/Alum \( (P < .05) \); Figure 3). Monkeys immunized with proteins formulated with CpG/IMX had circulating F-specific IgG antibodies of higher avidity in comparison to AGMs immunized with recombinant adenoviruses and PMED (AD5 & PMED; \( P < .05 \)). Aside from
these differences, other treatment group comparisons did not indicate any differentiation for either F or G-specific IgG avidities, as adjudged by nonparametric analysis.

Anti-F IgG antibodies were detected in BAL derived at week 17 of the experiment (Supplementary Figure 1). AGMs immunized with adjuvanted proteins or intranasally delivered adenoviruses and intramuscularly injected adjuvanted proteins (AD5 intranasal & proteins intramuscular) had higher anti-F IgG titers in their BAL in comparison to unimmunized (naive) controls ($P < .05$). Four of six monkeys immunized intranasally with RSV (RSV intranasal) had detectable anti-F immunoglobulin A (IgA) in their BAL. However, no anti-G IgG or IgA was detectable in any of the BAL samples (results not shown), and viral neutralizing antibodies were only detected in BAL from a small proportion of vaccinees (Supplementary Figure 1C).

**T-Cell Response**

ELISpot analyses following restimulation of PBMCs derived at week 17 (Figure 5) with overlapping synthetic peptides indicated that immunization with CpG/Alum-adjuvanted proteins (proteins CpG/Alum) or heterologous immunization approach with intranasally delivered recombinant adenoviruses and intramuscular injection of CpG/Alum-adjuvanted proteins (AD5 intranasal & proteins intramuscular) elicited significant numbers of antigen specific IFN-γ-secreting T cells. The latter elicited significant numbers of IFN-γ-secreting T cells with reactivity toward both of the F protein peptide pools tested and G protein peptides. Three intramuscular immunizations with CpG/Alum-adjuvanted proteins tended to engender IFN-γ-secreting T cells with reactivity toward pooled peptides from the C-terminus half of the protein. None of the other immunization approaches (VSV & AD5, protein CpG/IMX, AD5 intranasal & proteins intramuscular, and AD5 & PMED) elicited statistically significant numbers of F/G-specific IFN-γ-secreting T cells in PBMCs derived at week 17.

**DISCUSSION**

The data in this report indicate that intranasal challenge of AGMs with RSV M37 has utility as a differentiating stage-gate for RSV vaccine candidates prior to further investment as development leads. Lack of clinical disease symptoms entails that, as is the case with rodent models of RSV infection, viral loads must be used as a surrogate protective marker. Vaccine candidate–mediated protection can be benchmarked against the profile of viral shedding in challenged AGMs previously infected intranasally with RSV M37, although it should be remembered that RSV infection does not confer the same level of immunity in humans [1]. The fact that several of the immunization approaches were poorly protective in AGMs, despite demonstrating efficacy in earlier rodent challenge experiments, highlights the complexities of translating RSV protection data generated in mice and cotton rats to higher species. Conversely, because the experimental immunization approaches were worked up in rodent models, intergroup comparisons must be performed cautiously as one or more of the vaccination

![Figure 5.](image-url)
procedures may not have been fully optimized for this AGM model.

Six AGMs were primed by intranasal instillation of admixed recombinant adenoviruses expressing F or G and then boosted by intramuscular injection of CpG/Alum-adjuvanted proteins. The animals then received a third immunization involving a second intranasal instillation of recombinant adenoviruses. Notably, this form of immunization generated measurable F and G specific T-cell responses and afforded a high level of protection against challenge as compared with the other vaccination approaches tested (Figure 1 and Supplementary Figure 3). Circulating neutralizing antibody levels were no greater than in any of the other immunization groups at time of challenge (Figure 2), making it difficult to attribute the comprehensive protection afforded by this immunization strategy solely to induction of a superior RSV-neutralizing serum antibody response. It might be anticipated that mucosal administration of F and G–expressing adenoviral vectors engenders localized humoral and cellular responses that efficiently counteract infection and/or viral replication in the respiratory mucosa. To this end, 3 of 6 AGMs immunized by intranasal instillation of admixed recombinant adenoviruses (AD5 intranasal & proteins intramuscular) had detectable levels of anti-F IgA in BAL samples derived at week 17 (Supplementary Figure 1), although anti-F IgA was also detected in some of the monkeys immunized parenterally with adjuvanted proteins.

Aluminium salt–adjuvanted RSV protein subunits have been tested extensively over the years, both preclinically and in seropositive humans [29, 31–35]. In light of its ability to potentiate antibody and cellular responses [36] and possibly booster reactivity in groups with reduced immune function (eg, the elderly [37]), we coadministered Cpg 24555 with F and G proteins coformulated with either Alhydrogel or ISCOMATRIX adjuvant. On the basis of literature precedence and our own murine studies, we expected the Cpg/ISCOMATRIX adjuvant system to efficiently induce antigen-specific T cells [16]. Although both adjuvanting strategies induced robust RSV neutralizing antibody responses, Cpg/Alum-adjuvanted proteins elicited F-specific T-cell responses and conferred significant protection of the nose and throat whereas proteins formulated with Cpg/ISCOMATRIX adjuvant were less efficacious in this regard (Figures 1, 2, and 5). These data may indicate a potential role for T-cell–mediated protection in this model. Inclusion of a palivizumab-treated group [11] would have been useful to provide information about the ability of neutralizing antibodies to protect the AGM respiratory tract without T-cell involvement.

**Supplementary Data**

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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