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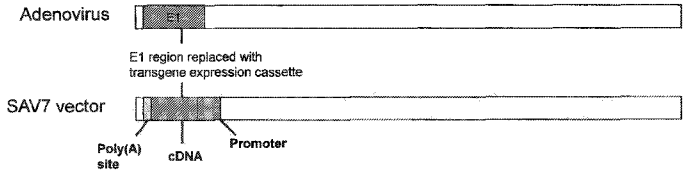

FIG. 1

(57) Abstract: The invention provides a replication-deficient monkey adenoviral vector, such as a serotype 7 simian adenoviral vector, which comprises at least one nucleic acid sequence encoding a respirator)' syncytial virus (RSV) antigen, such as an RSV F protein antigen. The invention also provides compositions and methods for treating or preventing RSV infection in a mammal by inducing an immune response against RSV using a simian adenoviral vector alone or with other vectors, including simian or human adenoviral vectors.

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RESPIRATORY SYNCYTIAL VIRUS (RSV) VACCINE

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 61/372,725, filed August 11, 2010, the content of which is incorporated by reference herein in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

This invention was made with Government support under Grant Number 1R43A1075686-01A1 awarded by the National Institute of Allergy and Infectious Diseases (NIAID). The Government has certain rights in this invention.

INCORPORATION-BY-REFERENCE OF MATERIAL SUBMITTED ELECTRONICALLY

Incorporated by reference in its entirety herein is a computer-readable nucleotide/amino acid sequence listing submitted concurrently herewith and identified as follows: One 270,937 Byte ASCII (Text) file named "Sequence_listing_ST25.TXT," created on August 5, 2011.

FIELD OF THE INVENTION

The invention relates to respiratory syncytial virus (RSV) and methods and compositions for treating and preventing RSV infection in mammals.

BACKGROUND OF THE INVENTION

Human respiratory syncytial virus (RSV) is the leading viral cause of lower respiratory illness and hospitalization in young children. The vast majority of children infected with RSV suffer from a mild upper respiratory tract infection; however, a small subset experience severe RSV-induced lower respiratory infection (LRI) and bronchiolitis that often requires hospitalization and can be life-

threatening (Collins et al., *Respiratory syncytial virus, In: Fields Virology*, Knipe and Howley, eds., Lippincott Williams & Wilcins, New York (1996), pp. 1313-1351). Since nearly every child eventually is infected with RSV, and significant LRI develops in 20-30% of RSV-infected children, RSV causes more than 130,000 pediatric hospitalizations annually in the United States (Shay et al., *JAMA*, *282*(15): 1440-1446 (1999), and World Health Organization, Initiative for Vaccine Research (IVR), Respiratory syncytial virus (RSV), http://www.who.int/vaccine_research/diseases/ari/en/index3.html (2007)).

Some risk factors for the development of severe RSV-induced illness have been clearly identified, including premature birth (Navas et al., *J. Pediatr.*, *121*(3): 348-54 (1992)), bronchopulmonary dysplasia (Groothuis et al., *Pediatrics*, *82*(2): 199-203 (1988)), congenital heart disease (MacDonald et al., *N. Engl. J. Med.*, *307*(7): 397-400 (1982)), and T cell immune deficiency (McIntosh et al., *J. Pediatr.*, 82(4): 578-90 (1973)). However, more than half of the children hospitalized with severe RSV-induced illness do not have an identified risk factor (Boyce et al., *J. Pediatr.*, *137*(6): 865-70 (2000)), which means that approximately 1-2% of otherwise healthy children without any identifiable risk factors suffer the potentially life-threatening consequences of RSV-induced illness (Collins et al., *supra*).

RSV-induced severe illness in children also has been correlated with the development of asthma (see, e.g., Sigurs et al., *Pediatrics*, *95*(4): 500-505 (1995); Welliver et al., *Pediatr. Pulmonol.*, *15*(1): 19-27 (1993); Cifuentes et al., *Pediatr. Pulmonol.*, *36*(4): 316-321 (2003); Schauer et al., *Eur. Respir. J.*, *20*(5): 1277-1283 (2002); Sigurs et al., *Am. J. Respir. Crit. Care Med.*, 161(5): 1501-1507 (2000); and Stein et al., *Lancet*, *354*(9178): 541-545 (1999)). The basis for this association is unknown, but may be due to underlying genetic factors, immune dysfunction, antigen-specific responses, or structural lesions caused by lung remodeling after severe RSV disease.

Although RSV infection is almost universal by age three, reinfection occurs throughout life because natural RSV infection does not provide complete immunity (Hall et al., *J. Infect. Dis.*, *163*(4): 693-698 (1991), and Muelenaer et al., *J. Infect. Dis.*, *164*(1): 15-21 (1991)). In the elderly, RSV is an important cause of

morbidity and mortality. In a retrospective cohort study, RSV was responsible for an annual average of 15 hospitalizations and 17 deaths per 1,000 nursing home residents, whereas influenza accounted for an average of 28 hospitalizations and 15 deaths in the same setting (Garofalo et al., *Pediatr. Allergy Immunol.*, *5*(2): 111-117 (1994)). Thus, RSV was isolated as frequently as influenza A in this population and was associated with comparable mortality as influenza A (Ellis et al., *J. Am. Geriatr. Soc.*, *51*(6): 761-72003; and Falsey et al., *J. Infect. Dis.*, *172*(2): 389-394 (1995)).

Currently there are no FDA-approved vaccines for the prevention of RSV infection or treatment of RSV-induced disease. The only FDA-approved medication for prophylaxis of RSV infection is SYNAGIS® (palivizumab) (MedImmune, Gaithersburg, MD), which is a humanized monoclonal antibody directed to an epitope in the A antigenic site of the RSV F protein administered to high-risk infants. Although SYNAGIS® represents a significant advance in the prevention of lower respiratory tract acute RSV disease and mitigation of lower respiratory tract infection, it has not been shown to be effective against RSV infection in the upper respiratory tract at permissible doses.

RSV vaccine development has suffered from a legacy of vaccine-enhanced disease in children after natural RSV infection (Kim et al., *Am. J. Epidemiol.*, *89*(4): 422-434 (1969); and Kapikian et al., *Am. J. Epidemiol.*, *89*(4): 405-421 (1969)). For example, in the early 1960s a formalin-inactivated alum-precipitated vaccine candidate (FI-RSV) was administered to RSV-naïve infants in the early 1960s, and although immunogenic, it did not protect the children against natural infection. In addition, vaccinees subsequently infected with RSV had increased hospitalization rates and more severe illness, including two deaths, relative to control children immunized with formalin-inactivated parainfluenza virus (Kapikian et al., *supra*, Chin et al., *Am. J. Epidemiol.*, *89*(4): 449-463 (1969); and Polack et al., *J. Exp. Med.*, *196*(6): 859-65 (2002)). Other approaches to RSV immunization have included live attenuated RSV, RSV subunit proteins, and parainfluenza virus chimeras. Live attenuated RSV vaccines have been tested in clinical trials of RSV-naïve infants, but have not been shown to achieve genetic stability of mutations, the optimal balance between attenuation for safety in infants,

or a protective immune response (Karron et al., *J. Infect. Dis.*, *191*(7): 1093-1104 (2005); and Bukreyev et al., *J. Virol.*, *79*(15): 9515-9526 (2005)). A live attenuated parainfluenza-RSV chimera vaccine containing the attachment (G) proteins of RSV types A and B has been administered intranasally and is expected to replicate safely in children (Tang et al., *J. Virol.*, *78*(20): 11198-11207 (2004); and Schmidt et al., *J. Virol.*, *76*(3): 1089-1099 (2002)). However, data from clinical testing is not yet available. Protein subunit vaccines based on RSV G and F proteins have been safely administered to adults and RSV-seropositive children, but are modestly immunogenic (Tristram et al., *Vaccine*, *12*(6): 551-556 (1994)). In this respect, purified subunit vaccines have not induced CD8+ T-cells and have been associated with IL-4 production, raising safety concerns for use in seronegative infants. Adjuvanting subunit protein vaccines with aluminum hydroxide, MPL, or a combination of MPL and QS21 did not prevent the IL-4 response (Murphy et al., *Vaccine*, *8*(5): 497-502 (1990); and Hancock et al., *J. Virol.*, *70*(11): 7783-7791 (1996)). Subunit vaccines also have been shown to induce IgE isotype antibody (Welliver et al., *J. Clin. Microbiol.*, *27*(2): 295-299 (1989)).

Thus, there remains a need for compositions and methods to effectively and safely prevent or treat RSV infection.

BRIEF SUMMARY OF THE INVENTION

The invention provides such compositions and methods for effectively and safely preventing or treating RSV infection in a mammal, preferably a human.

In one aspect, the invention provides a simian adenoviral vector (also referred to herein as a monkey adenoviral vector), preferably replication-deficient, comprising at least one nucleic acid sequence which encodes an RSV antigen. The RSV antigen in one embodiment is the RSV F protein antigen or a fragment thereof. In another embodiment, the simian adenoviral vector is a serotype 7 simian adenoviral vector. In some embodiments, the nucleic acid sequence is codon-optimized for expression in a mammal, preferably a human.

In some embodiments, the replication-deficient serotype 7 simian adenoviral vector comprises SEQ ID NO:9.

In another aspect, the invention provides a human adenoviral serotype 5 vector, preferably replication-deficient, comprising at least one nucleic acid sequence which encodes an RSV antigen. The RSV antigen in one embodiment is the RSV F protein antigen or a fragment thereof. In some embodiments, the replication-deficient human adenoviral serotype 5 vector comprises a sequence selected from the group consisting of SEQ ID NO:7 and SEQ ID NO:8.

In another aspect, a method is provided for inducing an immune response against RSV in a mammal. The method comprises administering to a mammal a composition comprising a replication-deficient, simian adenoviral vector comprising at least one nucleic acid sequence encoding an RSV antigen, which is preferably an RSV F protein antigen or fragment thereof, and a pharmaceutically acceptable carrier. In one embodiment, the simian adenoviral vector is of serotype 7. In some embodiments, the nucleic acid sequence is codon-optimized for expression in a mammal. In some embodiments, the nucleic acid sequence is expressed in the mammal to produce the RSV F protein and thereby induce a protective immune response against RSV in the mammal. In one embodiment, the mammal is a human. In some embodiments, the replication-deficient serotype 7 simian adenoviral vector administered comprises SEQ ID NO:9.

In some embodiments, the method further comprises administering a priming composition to the mammal prior to administering the simian adenoviral vector, while in other embodiments the method comprises administering a boosting composition to the mammal after administering the simian adenoviral vector. In some embodiments, the priming and boosting compositions comprise a plasmid or a viral vector comprising a nucleic acid sequence encoding an RSV antigen or a fragment thereof. The viral vector is preferably an adenoviral vector, which is preferably a simian adenoviral vector or a human adenoviral vector. The simian adenoviral vector is preferably of serotype 7, and the human adenoviral vector is preferably of serotype 5. In some embodiments, the RSV antigen is an RSV F protein antigen. In some embodiments, the replication-deficient serotype 7 simian adenoviral vector administered comprises SEQ ID NO:9. In some embodiments, the replication deficient human adenoviral serotype 5 vector comprises a sequence selected from the group consisting of SEQ ID NO:7 and SEQ ID NO:8.

In some embodiments, the human patient is younger than 3 years or older than 65 years.

In some embodiments, administration of the priming and boosting compositions is separated by about 4 weeks.

In some embodiments, the priming or boosting compositions comprise between about $1x10^9$ and $1x10^{12}$ particle units (pu) of the serotype 7 simian adenoviral vector and the serotype 5 human adenoviral vector.

In another aspect, the invention provides a method for treating or protecting against RSV infection in a human by inducing an immune response against RSV in the human, comprising administering to the human one or more compositions comprising a replication-deficient simian adenoviral vector and a pharmaceutically acceptable carrier, and optionally one or more compositions comprising a replication-deficient human adenoviral vector and a pharmaceutically acceptable carrier, wherein in some embodiments each adenoviral vector comprises at least one nucleic acid sequence encoding an RSV antigen. The RSV antigen is preferably an RSV F protein antigen or a fragment thereof, and the nucleic acid sequence is expressed in the human to produce the RSV antigen and thereby induce the immune response against RSV infection in the human. In some embodiments, the nucleic acid sequence encoding an RSV F protein antigen or fragment thereof is codon-optimized for expression in a mammal. In some embodiments, the replication-deficient simian adenoviral vector administered comprises SEQ ID NO:9. In some embodiments, the replication deficient human adenoviral vector comprises a sequence selected from the group consisting of SEQ ID NO:7 and SEQ ID NO:8.

In another aspect of the invention, a method is provided for substantially reducing the titer of RSV in the lungs of a mammal infected with RSV by inducing an immune response against RSV. The method comprises administering one or more compositions comprising a replication-deficient simian adenoviral vector and a pharmaceutically acceptable carrier, and optionally one or more compositions comprising a replication-deficient human adenoviral vector and a pharmaceutically acceptable carrier. In some embodiments, each adenoviral vector comprises at least one nucleic acid sequence encoding an RSV antigen or a fragment thereof,

preferably an RSV F protein antigen, which is expressed in the mammal to produce the RSV antigen and thereby induce an immune response against RSV that substantially reduces the titer of RSV in the lungs of the mammal. The mammal is preferably a human, and the nucleic acid sequence encoding an RSV antigen preferably is codon-optimized for expression in a mammal. In some embodiments, the replication deficient human adenoviral serotype 5 vector comprises a sequence selected from the group consisting of SEQ ID NO:7 and SEQ ID NO:8. In some embodiments, the replication-deficient simian adenoviral vector administered comprises SEQ ID NO:9.

In another aspect of the invention, vaccine compositions are provided for treating or protecting against RSV infection in a mammal, preferably a human. In one embodiment, a vaccine is provided for treating or protecting against RSV infection in a human by inducing an immune response against RSV in the human. In some embodiments, the vaccine comprises a replication-deficient serotype 7 simian adenoviral vector comprising at least one nucleic acid sequence encoding an RSV F protein antigen or a fragment thereof, and a pharmaceutically acceptable carrier. In some embodiments, the at least one nucleic acid sequence is codon-optimized for expression in a mammal and can be expressed in the mammal to produce the RSV F protein antigen and thereby induce an immune response against RSV in the mammal. In some embodiments, the replication-deficient serotype 7 simian adenoviral vector comprises SEQ ID NO:9.

In still another aspect of the invention, compositions are provided comprising a replication-deficient serotype 7 monkey adenoviral vector comprising the sequence of SEQ ID NO:12, as well as compositions comprising a replication-deficient human serotype 5 adenoviral vector comprising a sequence selected from the group consisting of SEQ ID NO:10 and SEQ ID NO:11. In some embodiments of the compositions, a nucleic acid sequence encoding a heterologous antigen is inserted within the vector sequences. Also provided are methods for inducing a protective immune response against RSV in a mammal, by administering these compositions.

The invention offers various benefits and advantages, including addressing the efficacy and safety concerns associated with prior RSV vaccines.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 depicts the adenovirus vector SV7-F, including the wild type simian adenovirus type 7 (SV7), the E1 deletion, and the structure of the expression cassette that includes the CMVtetO promoter ("Promoter"), RSV F_0 gene ("cDNA"), and the SV40 polyadenylation sequence ("Poly(A) site").

FIG. 2 is a flow chart depicting the construction of the plasmid containing the SAV7-F viral genome.

FIG. 3 depicts adenovirus vectors Ad5-F (Ad5.11D and modified Ad5-F, Ad5.4344), including the wild type adenovirus type 5, the E1-, E2B-, E3-, and E4-deleted adenovector backbone, and the GV11D adenovector backbone illustrating the deletions and the structure of sequences in place of the deleted sequences. Promoter = CMVTetO promoter; cDNA = RSV F0 transgene, Poly(A) site = SV40 polyadenylation sequence; muM polyA = transcription terminator and polyadenylation site; TIS1 = transcriptionally inert spacer 1; R = Rous Sarcoma Virus LTR.

FIG. 4 is a flow diagram depicting the construction of plasmid pAdE1(t.RSVF.0)E3(10)E4(TIS1) by homologous recombination (1 through 3) and rescue of Adt.RSVF0.11D. (1) and (2) illustration of homologous recombination between the Ad5 viral vector genome plasmid (1) with the shuttle plasmid (2) containing the CMVTetO.RSVF0 expression cassette. Schematics are not to scale.

FIG. 5 is a flow diagram depicting the construction of a modified Ad5-F vector (Ad4344.RSVF.0). Schematics are not to scale.

FIG. 6 is a photograph of a gel showing the expression of RSV F_0 from the adenovirus vector Ad5-F. RSV F_1 is present at ~50kDa. The protein samples (volumes of 10 ul down to 1 ul) were electrophoresed on a tris-glycine gel with 12% acrylimide and transferred to a PVDF membrane. The ECL plus western detection kit was used to visualize HRP using the Alpha Innotech with FluorChem software.

FIG. 7 is a photograph of a gel showing the expression of RSV F_0 from the adenovirus vector SAV7-F. RSV F_1 is present at ~50kDa. The protein samples

(volumes of 10 ul down to 1 ul) were electrophoresed on a tris-glycine gel with 12% acrylimide and transferred to a PVDF membrane. The ECL plus western detection kit was used to visualize HRP using the Alpha Innotech with FluorChem software.

FIG. 8 is a photograph showing RSV A2 plaques formed on a HEP2 monolayer and stained with Giemsa staining solution.

FIG. 9 is a diagram illustrating a protocol for immunizing mice against RSV using (i) a serotype 5 adenoviral vector encoding an RSF F_0 protein, and (ii) a formalin-inactivated alum-precipitated vaccine (F1-RSV).

FIG. 10 is a diagram illustrating a protocol for immunizing mice against RSV using human adenoviral vectors (serotypes 5 (Ad5.F.0) and 28 (Ad28.F.0)) and a simian adenoviral vector (serotype 7 (SV7.F.0)) encoding an F_0 protein.

FIG. 11 is a graph illustrating anti-RSV neutralizing antibody titers (EC_{50}) induced by a prime-boost immunization method which employs different combinations of Ad5, Ad28, and SV7 vectors encoding an RSV F_0 protein.

FIG. 12 is a diagram illustrating prime/boost RSV immunization regimens in mice which employ different combinations of adenoviral vectors based on human serotype 5 (rAd5) and simian serotype 7 (SAV-7) encoding an RSV F_0 protein.

FIGS. 13(A-C) show protection against RSV challenge mediated by SAV7 adenovirus vectors. FIG. 13(A) is a diagram illustrating an RSV immunization regimen in mice utilizing human (rAd5) and simian (SAV7) adenoviral vectors encoding the F protein alone, or the F protein and the M/M2 protein. FIG. 13(B) is a graph showing titers of RSV in lung tissue at 5 days post-challenge. FIG. 13(C) is a graph showing titers of RSV in nose tissue at 5 days post-challenge.

FIGS. 14(A-C) show protection against RSV challenge mediated by rAd28 adenovirus vectors. FIG. 14(A) is a diagram illustrating an RSV immunization regimen in mice utilizing human (rAd5 and rAd28) adenoviral vectors encoding the F protein alone, or the F protein and the M/M2 protein. FIG. 14(B) is a graph showing titers of RSV in lung tissue at 5 days post-challenge. FIG. 14(C) is a graph showing titers of RSV in nose tissue at 5 days post-challenge.

FIG. 15 is a graph showing TH2 cytokines in lung supernatants of vaccinated mice challenged with RSV. Control Ad5 = Ad5-mock (no transgene).

FIG. 16 is a diagram illustrating prime/boost RSV immunization regimens in mice which employ different combinations of adenoviral vectors based on human serotype 5 (rAd5), simian serotype 7 (SAd7), and simian serotype 11 (SAd11) encoding an RSV F_0 protein.

FIG. 17 is a graph showing TH2 cytokines in lung supernatants of vaccinated mice challenged with RSV. Control Ad5 = Ad5-mock (no transgene).

FIG. 18 is a diagram illustrating prime/boost RSV immunization regimes in cotton rats which employ different combinations of adenoviral vectors based on human serotype 5 (rAd5) and simian serotype 7 (SAd7), and FFB (storage buffer).

FIG. 19 is a graph showing the results of protective immunity against pulmonary RSV infection being induced by homologous and heterologous prime-boost strategies. RSV viral titers in the lungs were measured at day 5 post-challenge. Titers were determined by plaque assay and expressed as plaque forming units per gram of lung tissue. Assay limit of detection is 70 PFU / gram of lung. Data are represented as the mean ± SEM for viral titers in PFU/gram of lung for N = 5 cotton rats per prime group.

FIG. 20 is a graph showing the results of homologous and heterologous prime-boost regimens with rSAV7F.0/rAd5F.0 inducing neutralizing serum antibody. Neutralizing antibody titers were determined at 4 weeks after priming ("preboost") and before challenge at 8 weeks post-immunization ("prechallenge" = four weeks after boost, immediately before challenge). Titers were determined by plaque reduction neutralization assay and are represented as the mean (± SD) IC_{50} antibody titer for all 10 animals per prime group.

FIG. 21 is a graph showing that challenge with live RSV increases neutralizing serum antibody titers in all priming groups. Preboost and prechallenge data are reproduced from FIG. 20, with data added from the later time point of 10 days after RSV challenge ("Day 10 Post"). Antibody titers were measured by plaque reduction assay, and data are represented as the mean ± SD IC_{50} titers with N = 10 animals for the preboost and prechallenge endpoints and N = 5 animals for the day 10 post endpoint.

FIG. 22 is a graph showing the relationship of serum neutralizing activity with viral load. Each point on the chart is the viral titer plotted against serum neutralizing antibody titer of an individual animal. The lowest IC_{50} titer wherein the viral load was undetectable was 200. The threshold of protection is defined as the neutralizing antibody titer associated with a 100X or greater reduction in pulmonary RSV titers relative to levels found in FFB + FFB prime-boosted animals.

FIG. 23 is a graph showing that no homologous or heterologous combination of rSAV7F.0 and rAd5F.0 primes for immune responses resulting in recruitment of eosinophils to the BAL following challenge with live RSV, and potentially indicating the safety of these immunization regimens. Ten days after RSV challenge, cotton rats were euthanized and BAL was performed. The BAL cells were differentially stained, and eosinophil recruitment was evaluated. The percentage of eosinophils per total leukocytes were calculated and determined to be at or below the level of natural infection while significant numbers of eosinophils trafficked to the lung in FI-RSV-immunized animals after RSV challenge. Data are represented as the mean ± SD of the percentage of eosinophils in N=5 animals per priming group.

FIG. 24 is a graph showing that serum antibodies induced by Ad5-F neutralize an RSV B strain.

FIG. 25 is a diagram illustrating RSV immunization regimens in cotton rats for determining the relative immunogenicity of two adenovirus vaccine vectors, rAd5F.0 and modified Ad5-F (rAd4344F.0), for RSV.

FIG. 26 is a graph showing that immunization with rAd5F.0 or rAd4344F.0 vectors induced comparable levels of neutralizing serum antibodies. Titers of neutralizing antibodies in sera obtained at week 4 post-priming (day -1 before challenge) and at days 5 and 10 post-challenge were measured by PRNT. Data represent mean and standard deviation of IC_{50} values for N=15, 5, and 10 animals at day -1, day 5, and day 10 around challenge, respectively. * denotes titers statistically different from RSV-immunized animals at the same time point. # denotes titers statistically greater than titers from animals immunized with 10^7 PU

of same rAd vector at same time point. $p<0.05$ as determined by one-way ANOVA analyses (with Tukey's mean comparisons).

FIG. 27 is a graph showing that immunization with rAd5F.0 or rAd4344F.0 at both doses protected against RSV A2 infection. Cotton rats were challenged with live RSV 28 days after immunization, and RSV titers in the lung were measured five days after challenge. Data represent RSV titers as the number of PFU/gram lung with individual animals shown (◆), and the mean of each group shown (●). Limit of detection of the assay is 70 PFU/gram lung.

FIG. 28 is a graph showing that immunization with rAd5F.0 or rAd4344F.0 induced immune responses that do not result in immunopathology after RSV infection as evidenced by the lack of eosinophilia. Animals primed with rAd5F.0 or rAd4344F.0 had levels of eosinophils that were comparable to the level of RSV A2 primary infection. The FI-RSV control animals had eosinophilia as expected. Eosinophil cell counts were determined from BAL samples obtained 10 days post-RSV A2 challenge. Values plotted are mean percentage of total cells ± SD.

FIG. 29 is diagram illustrating RSV immunization regimens in cotton rats to determine protection against RSV A2 infection by immunization with a dose range of Ad5-F.

FIG. 30 is a graph showing viral load of RSV A2 in the lungs of cotton rats post-challenge. Immunization with Ad5-F at a dose of 1×10^7pu or greater reduced RSV A2 pulmonary replication to undetectable levels by day 5 post challenge. (n=5, mean +/- standard deviation)

FIG. 31 is a graph showing PRNT serum neutralizing antibody titers pre-challenge. Ad5-F was shown to induce serum IC_{50} titers against RSV A2 that increased as the immunization dose increased. IC_{50} is the inverse of the serum dilution yielding 50% inhibition of RSV infection. (n=10, mean +/- standard deviation).

FIG. 32 is a graph showing PRNT serum neutralizing antibody IC_{50} titers post-challenge. Serum neutralizing activity was boosted by natural infection with RSV A2 by day 5 post-challenge in animals immunized with Ad5-F. Pre-

challenge titers are the same as shown in FIG. 31, with n = 10; all other groups n = 5. Values shown are mean +/- standard deviation.

FIG. 33 is a graph showing the change in body weight of cotton rats after challenge with RSV A2. Values shown are mean percentage of pre-challenge animal weight +/- standard deviation.

FIG. 34 is a photograph showing cotton rat lymphocytes from a BAL sample of an FI-RSV immunized animal. BAL sample taken on day 10 post-challenge with RSV A2 was differentially stained with H&E. Differentiation between eosinophil and neutrophil was not possible.

FIG. 35 is a diagram illustrating an RSV immunization regimen in cotton rats for evaluating the ability of rAd5F.0 to induce neutralizing antibody.

FIG. 36 is a graph showing that high dose (1×10^9 pu) rAd5F.0 immunization elicited robust neutralizing antibody activity against RSV A2. Neutralizing antibody IC_{50} titers were determined by plaque reduction neutralization of serum samples. Data represent mean ± standard deviation IC_{50} of N = 6 to 10 animals per group at each time point.

FIG. 37 is a graph showing that high dose rAd5F.0 immunization elicited durable immune responses which completely protected against RSV challenge at 8 months post-immunization. RSV titers were measured at day 5 post-challenge by plaque assay. No detectable virus was found in RSV- or high dose rAd5F.0-primed cotton rats. Data are shown as box plots with mean titer denoted by the line, maximum and minimum titers denoted by X, and viral titers in individual animals shown by offset circles where N = 3 to 4 animals per group. The limit of detection for the assay is 70 PFU/gram lung.

FIG. 38 is a graph showing that low dose (1×10^7 pu) rAd5F.0 immunization elicited detectable neutralizing antibody activity against RSV A2. Neutralizing antibody IC_{50} titers were determined by plaque reduction neutralization assay of serum samples. Data represent mean ± standard deviation IC_{50} of N = 6 to 10 animals per group at each time point.

FIG. 39 is a graph showing that low dose rAd5F.0 immunization elicited durable immune responses which partially protected against RSV challenge. RSV titers were measured in lung homogenates at day 5 after challenge by plaque assay.

No detectable virus was found in 2 of 3 cotton rats immunized with low dose rAd5F.0 with the remaining animal having reduced viral titers relative to FI-RSV-primed cotton rats. Data are shown as box plots with mean titer denoted by the line, maximum and minimum titers denoted by X, and viral titers in individual animals shown by offset circles where N = 3 to 4 animals per group. The limit of detection for the assay is 70 PFU/gram lung.

FIG. 40 is a graph showing that RSV challenge boosts neutralizing serum antibody titers in all immunization groups. Sera were obtained at week 32 after priming (immediately before RSV challenge) and at day 10 after RSV challenge. Titers of neutralizing antibody were measured by plaque reduction neutralization assay. Data represent mean ± SD IC_{50} titers where N = 6 to 8 animals per group at week 32 and N=3 to 4 animals per group at day 10.

FIG. 41 is a graph showing that immunization with rAd5F.0 does not prime for immunopathologic immune responses. Ten days after RSV challenge BAL was performed and cells were differentially stained. Eosinophil recruitment was evaluated as the percentage of eosinophils within the number of total leukocytes in the BAL. Data represent the mean ± SD percentage of eosinophils where N = 3 to 4 cotton rats per group.

FIG. 42 is a graph showing the relationship of serum neutralizing activity with viral load. Each point on the chart is the viral titer plotted against serum neutralizing antibody titer of an individual animal. The lowest IC_{50} titer wherein the viral load was undetectable was 120. The threshold of protection is defined as the neutralizing antibody titer associated with a 100X or greater reduction in pulmonary RSV titers relative to levels found in FI-RSV-immunized animals.

FIG. 43 is a diagram illustrating RSV immunization regimens in cotton rats for low-dose immunization with SAV7 and SAV11 RSV F vectors.

FIG. 44 is a graph showing that immunization with low doses of SAV7F.0 and SAV11F.0 induced low levels of serum neutralizing activity, indistinguishable from mock immunization (FFB) except at the highest doses (*).

FIG. 45 is a graph showing that immunization with SAV7 and SAV11 at low doses induced partial protection.

FIG. 46 is a graph showing RSV pulmonary replication time course. All animals received 1 x 10^6 PFU of RSV A2.

FIG. 47 is a graph showing RSV pulmonary replication time course from published literature. All animals received RSV A2 dose of 1 X 10^4 PFU.

FIG. 48 is a diagram illustrating an RSV infection time course in cotton rats.

FIG. 49 is a diagram illustrating an RSV immunization regimen in cotton rats.

FIG. 50 is a graph showing that cotton rat weights are not a reliable indication of illness. (mean value ± StdD).

FIG. 51 is a graph showing that serum neutralizing activity was present pre-challenge for animals infected with RSV A2 and immunized with FI-RSV A2. The Hep 2 mock did not illicit any serum neutralizing activity. Each point represents an individual animal, with the mean value ± StdD also plotted.

FIG. 52 is a graph showing that serum neutralizing activity was increased by challenge with 1 X 10^7 pfu of RSV A2 by day 10 post challenge (mean value ± StdD).

FIG. 53 is a graph showing RSV concentration of lung homogenates. Sample 1 was spiked to a concentration of 1 X10^6 PFU/ml. Samples 2-5 were from animals infected with 1 x 10^6 PFU i.n. and harvested 4 days post-infection. Sample 6 was spiked to a concentration of 2 x 10^6 PFU/mL. Sample 7 was comprised of material taken from sample 6 then re-processed with the tissue dissociator.

FIG. 54 is a graph showing the level of pulmonary RSV of samples harvested 4 days post-infection with 1 X10^6 PFU of RSV i.n. Samples that were frozen prior to assay (2, 3) showed a three times reduction in pulmonary RSV compared to samples that were never frozen (4, 5).

FIG. 55 is a graph showing the titer of RSV in spiked lung homogenates plotted against the mathematically predicted titer.

FIG. 56 is a graph showing titration of an infectious dose of RSV and resultant number of plaques per well. Hep2 monolayers were infected with the indicated number of plaque forming units (pfu) and the number of plaques per well were counted. Optimum plaque number (60-80 per well; gray highlight) was

observed with input dose range of 25 to 35 pfu per well of RSV A2. Data shown are values from three replicate infections from independent dilutions and the average of the three replicates.

FIG. 57 is a graph showing an example of a linear curve of percentage of maximum plaque number.

FIGS. 58(A-C) are photographs BAL samples stained with modified Sirius Red and Gill's Hematoxylin. Eosinophils (white arrows) are identified by red stained granules in the cytoplasm, and multi-invaginated nucleus. Neutrophils (black arrows) are identified by pink stained granules in the cytoplasm, and nucleus with relatively fewer invaginations. (A) 600X magnification (oil immersion). (B and C) 400X magnification (oil immersion).

FIG. 59 is a graph showing neutralizing activity titer of serum samples harvested day 27 post-immunization. N=2 for 1:10, 1:20, and 1:40 dilutions. N=3 for the 1:100 dilution.

FIG. 60 is a graph showing eosinophils in BAL fluids expressed as a percentage of total cells (eosinophils, neutrophils, basophils, and monocytes). The broncho-alveolar lavage samples were collected day eight post-challenge. N=2 for 1:10, 1:20, and 1:40 dilutions. N=3 for the 1:100 dilution.

FIG. 61 is a graph showing that expression of RSV F_0 was cytotoxic, and that reducing transcription from the RSV F_0 transgene cassette in a M2A cell line resulted in increased cell viability.

FIG. 62 is a graph showing serum RSV neutralizing antibody titers at two weeks (week 5) and 4 weeks (week 7) post-boost immunization.

FIG. 63 is a graph showing body weight change post-challenge after immunization with various prime-boost combinations.

FIG. 64 is a graph showing titer of RSV in the lungs of mice at five days post-challenge.

Fig. 65 shows Tables 6 and 7. Table 6 shows representative plaque count with determination of mean count per dilution, and Table 7 shows an example of determination of percentage of maximum plaque count for graphing.

DETAILED DESCRIPTION

The present invention addresses the efficacy and safety concerns associated with prior RSV vaccines.

In some embodiments, the invention provides a simian adenoviral vector, which is preferably replication-deficient, comprising at least one nucleic acid sequence encoding an RSV antigen. The simian adenoviral vector may be administered as part of a composition, and also may be used in immunogenic methods for treating and preventing RSV infection. The simian adenoviral vector may be administered alone or with other vectors, including adenoviral vectors, preferably replication-deficient simian adenoviral vectors or replication-deficient human adenoviral vectors. In some embodiments, the simian adenoviral vector is a replication-deficient serotype 7 simian adenoviral vector. In some embodiments, the replication-deficient serotype 7 simian adenoviral vector comprises SEQ ID NO:9. In some embodiments, the simian adenoviral vector has at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% identity to the sequence of SEQ ID NO:9.

In some embodiments, the invention provides a human adenoviral serotype 5 vector, preferably replication-deficient, comprising at least one nucleic acid sequence which encodes an RSV antigen. The RSV antigen in one embodiment is the RSV F protein antigen or a fragment thereof. In some embodiments, the RSV antigen is codon-optimized. In some embodiments, the replication-deficient human adenoviral serotype 5 vector comprises a sequence selected from the group consisting of SEQ ID NO:7 and SEQ ID NO:8. In some embodiments, the human adenoviral vector has at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% identity to the sequences of SEQ ID NO:7 or SEQ ID NO:8.

In some embodiments, the invention also provides a replication-deficient serotype 7 simian adenoviral vector alone, that is, without limitation to a heterologous antigen-encoding nucleic acid sequence comprised therein. In some embodiments, the replication-deficient serotype 7 simian adenoviral vector comprises the nucleic acid sequence of SEQ ID NO:12. In some embodiments, the

invention also provides a replication-deficient human serotype 5 adenoviral vector alone, that is, without limitation to a heterologous antigen-encoding nucleic acid sequence comprised therein. In some embodiments, the replication-deficient human serotype 5 adenoviral vector comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:10 and SEQ ID NO:11.

The nucleic acid sequences of SEQ ID NO:10, SEQ ID NO:11, and SEQ ID NO:12 are of adenoviral vectors in which at least the essential E1 region has been deleted. See Examples 1 and 2. In some embodiments, a nucleic acid sequence encoding a heterologous antigen, preferably an RSV antigen, may be inserted into the vectors at the location of the E1 region deletion, using techniques known in the art. See also Examples 1 and 2. In some embodiments, the RSV antigen is an RSV F protein antigen, the nucleic acid sequence for which may be inserted into the vectors at the location of the E1 region deletion. For instance, in some embodiments of the simian and human adenoviral vectors of the invention, the nucleic acid sequence encoding an RSV F protein antigen was inserted between nucleotides 602 and 2328 of the vector sequence of SEQ ID NO:7, between nucleotides 602 and 2328 of the vector sequence of SEQ ID NO:8, and between nucleotides 662 and 2388 of the vector sequence of SEQ ID NO:9.

In some embodiments, the invention also provides a composition, or pharmaceutical composition, comprising one or both of a replication-deficient serotype 7 simian adenoviral vector and a replication-deficient human serotype 5 adenoviral vector, as described above, and in some embodiments a pharmaceutically acceptable carrier. In some embodiments is provided a composition, or pharmaceutical composition, comprising a replication-deficient serotype 7 simian adenoviral vector comprising the nucleic acid sequence of SEQ ID NO:12 and in some embodiments a pharmaceutically acceptable carrier. In some embodiments is provided a composition, or pharmaceutical composition, comprising a replication-deficient human serotype 5 adenoviral vector comprising a nucleic acid sequence selected from the group consisting of SEQ ID NO:10 and SEQ ID NO:11 and in some embodiments a pharmaceutically acceptable carrier.

In some embodiments, the invention provides a composition comprising a replication-deficient, E1 region-deleted, serotype 7 monkey adenoviral vector

comprising the sequence of SEQ ID NO:12. In other embodiments is provided a composition wherein a nucleic acid sequence encoding a heterologous antigen is inserted at the location of the E1 region deletion. In some embodiments, the invention provides a composition comprising a replication-deficient, E1 region-deleted, human serotype 5 adenoviral vector comprising a sequence selected from the group consisting of SEQ ID NO:10 and SEQ ID NO:11. In other embodiments is provided a composition wherein a nucleic acid sequence encoding a heterologous antigen is inserted at the location of the E1 region deletion.

In some embodiments, the invention also provides simian and human adenoviral vectors, as described above, and compositions comprising the same, wherein the simian adenoviral vector has at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% identity to the sequence of SEQ ID NO:12, and the human adenoviral vector has at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% identity to a sequence selected from the group consisting of SEQ ID NO:10 and SEQ ID NO:11.

Percentage of sequence identity refers to the degree of identity between any given query sequence and a subject sequence. A query nucleic acid or amino acid sequence is aligned to one or more subject nucleic acid or amino acid sequences using the computer program ClustalW (version 1.83, default parameters), which allows alignments of nucleic acid or protein sequences to be carried out across their entire length (global alignment). ClustalW calculates the best match between a query and one or more subject sequences, and aligns them so that identities, similarities and differences can be determined. Gaps of one or more residues can be inserted into a query sequence, a subject sequence, or both, to maximize sequence alignments. For fast pairwise alignment of nucleic acid sequences, the following default parameters are used: word size: 2; window size: 4; scoring method: percentage; number of top diagonals: 4; and gap penalty: 5. For multiple alignment of nucleic acid sequences, the following parameters are used: gap opening penalty: 10.0; gap extension penalty: 5.0; and weight transitions: yes. For fast pairwise alignment of protein sequences, the following parameters are used:

word size: 1; window size: 5; scoring method: percentage; number of top diagonals: 5; gap penalty; 3. For multiple alignment of protein sequences, the following parameters are used: weight matrix: blosum; gap opening penalty: 10.0; gap extension penalty: 0.05; hydrophilic gaps: on; hydrophilic residues: Gly, Pro, Ser, Asn, Asp, Gln, Glu, Arg, and Lys; residue-specific gap penalties: on. The output is a sequence alignment that reflects the relationship between sequences.

Adenoviruses belong to the family *Adenoviridae*, which is divided into five genera, *Mastadenovirus*, *Atadenovirus*, *Siadenovirus*, *Aviadenovirus*, and *Ichtadenovirus*. The adenoviruses in the genus *Mastadenovirus* infect only mammals and include the human, chimpanzee, and monkey adenoviruses. Adenoviruses isolated from humans and chimpanzees have been used extensively in the art as gene transfer vectors. Humans and chimpanzees are very closely related and are grouped together as hominids. In contrast, there is a significantly greater evolutionary distance between monkeys and hominids. In this respect, the monkeys diverged between 25 and 35 million years ago from the hominids, whereas the humans and chimpanzees diverged about 7 million years ago (Samonte et al., *Nature Reviews Genetics, 3*, 65-72 (2002)).

The term "simian," as used herein, refers to both new world and old world monkeys, and does not include any member of the family *Hominidae* (e.g., humans, chimpanzees, gorillas, and orangutans, which are also referred to as the "great apes"). The use of the term "simian" was discontinued in taxonomy because of the inaccurate grouping of a subset of great apes (all non-human Hominids) with monkeys. Classically, the virus name has reflected the first described host followed by a number, e.g., human adenovirus 1. For this reason, the adenoviral vectors in this application that were isolated from monkeys are referred to as monkey adenoviral vectors. Therefore, the simian adenoviral vector (or monkey adenoviral vector) of the invention is derived from an adenovirus isolated from a new world monkey or an old world monkey (collectively referred to herein as "monkeys"). New world monkeys include the families *Callitrichidae* (e.g., marmosets and tamarins), *Cebidae* (e.g., capuchins and squirrel monkeys), *Aotidae* (e.g., night or owl monkeys (douroucoulis)), *Pitheciidae* (e.g., titis, sakis and uakaris), and *Atelidae* (e.g., howler, spider, and woolly monkeys) (see, e.g.,

Hershkovitz (ed.), *Living New World Monkeys (Platyrrhini), Volume 1*, University of Chicago Press (1977)). Old world monkeys include animals in the family *Cercopithecinae*, such as, for example, macaques, baboons, and mangabeys (see, e.g., Whitehead, ed., *Old World Monkeys*, Cambridge University Press (2002))

Adenovirus serotypes are differentiated on the basis of neutralization assays. A serotype is defined as one which either exhibits no or limited cross-reaction with other types (see, Fauquet et al., (eds.), *Virus Taxonomy: The Eighth Report of the International Committee on Taxonomy of Viruses*, Academic Press, p. 216 (2005)). The serologically distinguishable serotypes (also referred to herein as adenovirus "types") are grouped into species. Classically, the virus name has reflected the first described host followed by a number, e.g., human adenovirus 1. For this reason, the adenoviral vectors in this application that were isolated from monkeys are referred to as monkey adenoviral vectors. The lack of cross neutralization combined with a calculated phylogenetic distance of more than 10% separates two virus isolates into different serotypes. In addition, species designation depends on other characteristics that differ between serotypes of adenovirus, including host range, DNA hybridization, RFLP analysis, percentage of GC in the genome, oncogenicity in rodents, growth characteristics, possibility of recombination, number of VA RNA genes, hemagglutination, genetic organization of the E3 region, and host range. Simian adenoviruses isolated from monkeys are more distant from both human adenoviruses and chimpanzee adenoviruses. The chimpanzee adenoviruses are closely related to common human adenoviruses of species B and E, so similar that the chimpanzee adenoviruses are grouped within the human species B and E. The limited phylogenic reconstructions for the simian adenoviruses reveal that the simian adenoviruses are quite distinct from the common chimpanzee and human adenoviruses (*Virus Taxonomy: VIIIth Report of the International Committee on Taxonomy of Viruses* (2005)). The phylogeny of adenoviruses that infect primates is disclosed in, e.g., Roy et al., *PLoS Pathog.*, *5*(7): e100050. doi:10.1371/journal.ppat.1000503 (2009).

Various origins, serotypes, or mixtures of serotypes can be used as the source of the viral genome for the simian (monkey) adenoviral vector (such as those described in, e.g., U.S. Patents 7,247,472 and 7,491,508). For instance, a

monkey adenovirus can be of serotype 1, 3, 6, 7, 11, 16, 18, 19, 20, 27, 33, 38, 39, 48, 49, 50, or any other monkey adenoviral serotype. A monkey adenovirus can be referred to by using any suitable abbreviation known in the art, such as, for example, SV, SAdV, or SAV. Preferably, the monkey adenoviral vector is a monkey adenoviral vector of serotype 3, 6, 7, 11, 16, 18, 19, 20, 27, 33, 38, or 39. More preferably, the monkey adenoviral vector is of serotype 7, 11, 16, 18, or 38. Most preferably, the monkey adenoviral vector is of serotype 7. These monkey adenoviruses, isolated from monkeys, have low sequence homology to human serotype 5 adenovirus, and are more closely related, though quite distinct, from the enteric F and G serotype adenoviruses. They contain two different fiber genes (long and short fibers) instead of one fiber gene, which suggests that they may target the gut mucosa, similar to gut-tropic human adenoviruses, where they are expected to stimulate mucosal immune responses. In addition, comparisons between viral hexon proteins suggest that monkey adenovirus serotypes 7, 11, 16, and 38 are distantly related to human adenoviruses, and are categorized more closely to gut-tropic adenoviruses (human Ad40, 41, and 52) than to other groups.

The simian and human adenoviral vectors used in accordance with embodiments of the invention comprise at least one nucleic acid that encodes an antigen. A "nucleic acid" is intended to encompass a polymer of DNA or RNA, i.e., a polynucleotide, which can be single-stranded or double-stranded and which can contain non-natural or altered nucleotides. Nucleic acids are typically linked via phosphate bonds to form nucleic acids or polynucleotides, though many other linkages are known in the art (e.g., phosphorothioates, boranophosphates, and the like). Any nucleic acid sequence that is inserted into the simian adenovirus genome also is referred to herein as a "heterologous" or "exogenous" nucleic acid sequence.

An "antigen" is a molecule that triggers an immune response in a mammal. An "immune response" can entail, for example, antibody production and/or the activation of immune effector cells. An antigen in the context of the invention can comprise any subunit, fragment, or epitope of any proteinaceous or non-proteinaceous (e.g., carbohydrate or lipid) molecule which provokes an immune response in a mammal. By "epitope" is meant a sequence of an antigen that is

recognized by an antibody or an antigen receptor. Epitopes also are referred to in the art as "antigenic determinants." The antigen can be a protein or peptide of viral, bacterial, parasitic, fungal, protozoan, prion, cellular, or extracellular origin, which provokes an immune response in a mammal, preferably leading to protective immunity. The antigen also can be a self-antigen, i.e., an autologous protein which the body has mistakenly identified as a foreign invader.

In some embodiments, the heterologous nucleic acid sequence preferably encodes an antigen of a pathogen. The pathogen can be a virus, such as respiratory syncytial virus (RSV), human immunodeficiency virus (HIV), foot-and-mouth disease (FMDV), herpes simplex virus (HSV), hepatitis C virus (HCV), ebola virus, or Marburg virus. The virus preferably is RSV. An RSV antigen in the context of the invention can comprise any proteinaceous RSV molecule or portion thereof that provokes an immune response in a mammal. An "RSV molecule" is a molecule that is a part of a respiratory syncytial virus, is encoded by a nucleic acid sequence of a respiratory syncytial virus, or is derived from or synthetically based upon any such molecule. Administration of an RSV antigen that provokes an immune response in accordance with the invention preferably leads to protective immunity against RSV. In this regard, an "immune response" to RSV is an immune response to any one or more RSV antigens.

The simian adenoviral vector of the invention can be replication-competent. For example, the simian adenoviral vector can have a mutation (e.g., a deletion, an insertion, or a substitution) in the adenoviral genome that does not inhibit viral replication in host cells. Preferably, however, the simian adenoviral vector is replication-deficient. By "replication-deficient" is meant that the simian adenoviral vector comprises an adenoviral genome that lacks at least one replication-essential gene function (i.e., such that the adenoviral vector does not replicate in typical host cells, especially those in a human patient that could be infected by the simian adenoviral vector in the course of the inventive method). A deficiency in a gene, gene function, or gene or genomic region, as used herein, is defined as a deletion of sufficient genetic material of the viral genome to impair or obliterate the function of the gene whose nucleic acid sequence was deleted in whole or in part. While deletion of genetic material is preferred, mutation of

genetic material by addition or substitution also is appropriate for disrupting gene function. Replication-essential gene functions are those gene functions that are required for replication (e.g., propagation) and are encoded by, for example, the adenoviral early regions (e.g., the E1, E2, and E4 regions), late regions (e.g., the L1-L5 regions), genes involved in viral packaging (e.g., the IVa2 gene), and virus-associated RNAs (e.g., VA-RNA1 and/or VA-RNA-2). More preferably, the replication-deficient simian adenoviral vector comprises an adenoviral genome deficient in at least one replication-essential gene function of one or more regions of the adenoviral genome. Preferably, the simian adenoviral vector is deficient in at least one gene function of the E1A region, the E1B region, or the E4 region of the adenoviral genome required for viral replication (denoted an E1-deficient or E4-deficient adenoviral vector). In addition to a deficiency in the E1 region, the simian adenoviral vector also can have a mutation in the major late promoter (MLP), as discussed in International Patent Application WO 00/00628. Most preferably, the simian adenoviral vector is deficient in at least one replication-essential gene function (desirably all replication-essential gene functions) of the E1 region and at least one gene function of the nonessential E3 region (e.g., an Xba I deletion of the E3 region) (denoted an E1/E3-deficient adenoviral vector). With respect to the E1 region, the simian adenoviral vector can be deficient in all or part of the E1A region and all or part of the E1B region. When the simian adenoviral vector is deficient in at least one replication-essential gene function in only one region of the adenoviral genome (e.g., an E1- or E1/E3-deficient adenoviral vector), the adenoviral vector is referred to as "singly replication-deficient." A particularly preferred singly replication-deficient adenoviral vector is, for example, a replication-deficient adenoviral vector requiring, at most, complementation of the E1A and E1B regions of the adenoviral genome, so as to propagate the adenoviral vector (e.g., to form adenoviral vector particles).

The simian adenoviral vector of the invention can be "multiply replication-deficient," meaning that the simian adenoviral vector is deficient in one or more replication-essential gene functions in each of two or more regions of the adenoviral genome, and requires complementation of those functions for replication.. For example, the aforementioned E1-deficient or E1/E3-deficient

simian adenoviral vector can be further deficient in at least one replication-essential gene function of the E4 region (denoted an E1/E4- or E1/E3/E4-deficient adenoviral vector), and/or the E2 region (denoted an E1/E2- or E1/E2/E3-deficient adenoviral vector), preferably the E2A region (denoted an E1/E2A- or E1/E2A/E3-deficient adenoviral vector). When the adenoviral vector is multiply replication-deficient, the deficiencies can be a combination of the nucleotide deletions discussed above with respect to each individual region. An adenoviral vector deleted of the entire E4 region can elicit a lower host immune response.

By removing all or part of, for example, the E1, E3, and E4 regions of the simian adenoviral genome, the resulting simian adenoviral vector is able to accept inserts of exogenous nucleic acid sequences while retaining the ability to be packaged into adenoviral capsids. The exogenous (or "heterologous") nucleic acid sequence can be positioned in the E1 region, the E3 region, or the E4 region of the adenoviral genome. Indeed, the nucleic acid sequence can be inserted anywhere in the simian adenoviral genome so long as the position does not prevent expression of the nucleic acid sequence or interfere with packaging of the simian adenoviral vector. The simian adenoviral vector also can comprise multiple (i.e., two or more) nucleic acid sequences encoding the same antigen. Alternatively, the adenoviral vector can comprise multiple nucleic acid sequences encoding two or more different antigens. Each nucleic acid sequence can be operably linked to the same promoter, or to different promoters depending on the expression profile desired by the practitioner, and can be inserted in the same region of the adenoviral genome (e.g., the E4 region) or in different regions of the adenoviral genome. For example, the simian adenoviral vector can comprise a nucleic acid sequence that encodes two or more different RSV antigens. Alternatively, the simian adenoviral vector can comprise two or more nucleic acid sequences that each encodes a different RSV antigen.

The simian adenoviral vector, when multiply replication-deficient, especially in replication-essential gene functions of the E1 and E4 regions, preferably includes a spacer sequence to provide for viral growth in a complementing cell line similar to that achieved by singly replication-deficient adenoviral vectors, particularly an E1-deficient adenoviral vector. The spacer

sequence can contain any nucleotide sequence or sequences which are of a desired length, such as sequences at least about 15 base pairs (e.g., between about 15 base pairs and about 12,000 base pairs), preferably about 100 base pairs to about 10,000 base pairs, more preferably about 500 base pairs to about 8,000 base pairs, even more preferably about 1,500 base pairs to about 6,000 base pairs, and most preferably about 2,000 to about 3,000 base pairs in length. The spacer element sequence can be coding or non-coding and native or non-native with respect to the adenoviral genome, but does not restore the replication-essential function to the deficient region. The spacer element preferably is located in the E4 region of the adenoviral genome. The use of a spacer in an adenoviral vector is described in U.S. Patent 5,851,806.

In some embodiments, the simian adenoviral vector requires complementation of replication-essential gene functions of the E1A, E1B, E2A, and/or E4 regions of the adenoviral genome for replication (i.e., propagation). In some embodiments, the simian adenoviral vector requires at most complementation of the E1A region, the E1B region, and/or the E4 region of the adenoviral genome for propagation. However, the adenoviral genome can be modified to disrupt one or more replication-essential gene functions as desired by the practitioner, so long as the simian adenoviral vector remains deficient and can be propagated using, for example, complementing cells and/or exogenous DNA (e.g., helper adenovirus) encoding the disrupted replication-essential gene functions. The simian adenoviral vector can be deficient in replication-essential gene functions of only the early regions of the adenoviral genome, only the late regions of the adenoviral genome, and both the early and late regions of the adenoviral genome. The simian adenoviral vector also can have essentially the entire adenoviral genome removed, in which case it is preferred that at least either the viral inverted terminal repeats (ITRs) and one or more promoters or the viral ITRs and a packaging signal are left intact (i.e., an adenoviral amplicon). In one embodiment, the simian adenoviral vector of the invention comprises an adenoviral genome that lacks native nucleic acid sequences which encode adenoviral proteins. Adenoviral genomic elements required for replication and packaging of the adenoviral genome into adenoviral capsid proteins can be retained. Minimal

adenoviral vectors lacking adenoviral protein coding sequences are termed "helper-dependent" adenoviral vectors, and often require complementation by helper adenovirus for efficient propagation. Examples of replication-deficient adenoviral vectors, including multiply replication-deficient adenoviral vectors, are disclosed in U.S. Patents 5,837,511; 5,851,806; 5,994,106; 6,127,175; 6,482,616; and 7,195,896, and International Patent Applications WO 94/28152, WO 95/02697, WO 95/16772, WO 95/34671, WO 96/22378, WO 97/12986, WO 97/21826, and WO 03/022311.

Complementing cell lines for producing the simian adenoviral vector include, but are not limited to, 293 cells (described in, e.g., Graham et al., *J. Gen. Virol.*, *36*: 59-72 (1977)), PER.C6 cells (described in, e.g., International Patent Application Publication WO 97/00326, and U.S. Patents 5,994,128 and 6,033,908), and 293-ORF6 cells (described in, e.g., International Patent Application Publication WO 95/34671 and Brough et al., *J. Virol.*, *71*: 9206-9213 (1997)). Additional complementing cells are described in, for example, U.S. Patents 6,677,156 and 6,682,929, and International Patent Application Publication WO 03/20879. Methods for propagating monkey adenovirus and monkey adenoviral vectors are described in International Patent Application Publication WO/2011/057248. In some instances, the cellular genome need not comprise nucleic acid sequences, the gene products of which complement for all of the deficiencies of a replication-deficient adenoviral vector. One or more replication-essential gene functions lacking in a replication-deficient adenoviral vector can be supplied by a helper virus, e.g., an adenoviral vector that supplies in *trans* one or more essential gene functions required for replication of the desired adenoviral vector. Helper virus is often engineered to prevent packaging of infectious helper virus. For example, one or more replication-essential gene functions of the E1 region of the adenoviral genome are provided by the complementing cell, while one or more replication-essential gene functions of the E4 region of the adenoviral genome are provided by a helper virus.

Ideally, the replication-deficient simian adenoviral vector is present in a composition, e.g., a pharmaceutical composition, substantially free of replication-competent adenovirus (RCA) contamination (e.g., the composition comprises less

than about 1% of replication-competent adenovirus on the basis of the total adenoviruses in the composition). Most desirably, the composition is RCA-free. Adenoviral vector compositions and stocks that are RCA-free are described in U.S. Patent 5,944,106, and International Patent Application WO 95/34671.

If the simian adenoviral vector is not replication-deficient, ideally the simian adenoviral vector is manipulated to limit replication of the vector to within a target tissue. For example, the simian adenoviral vector can be a conditionally-replicating adenoviral vector, which is engineered to replicate under conditions pre-determined by the practitioner. For example, replication-essential gene functions, e.g., gene functions encoded by the adenoviral early regions, can be operably linked to an inducible, repressible, or tissue-specific transcription control sequence, e.g., promoter. In this embodiment, replication requires the presence or absence of specific factors that interact with the transcription control sequence. In the treatment of viral infections, for example, it can be advantageous to control adenoviral vector replication in, for instance, lymph nodes, to obtain continual antigen production and control immune cell production. Conditionally-replicating adenoviral vectors are described further in U.S. Patent 5,998,205.

RSV is classified in the *Pneumovirus* genus of the *Paramyxoviridae* family (Collins et al., *supra*; Lamb & Kolakofsky, "Paramyxoviridae: the viruses and their replication," In: Knipe & Howley (eds.), *Fields Virology Vol. 1*, Lippincott, Williams & Wilkins, Philadelphia, pp. 1305-1340 (2001)). The RSV genome comprises a negative-sense polynucleotide molecule which, through complementary viral mRNAs, encodes eleven viral proteins: the nonstructural proteins NSl and NS2, N, P, matrix (M), small hydrophobic (SH), glycoprotein (G), fusion (F), M2(ORF1) (also known as M2-1), M2(ORF2) (also known as M2-2), and L (see, e.g., Mink et al., *Virology*, *185*: 615-624 (1991); Stec et al., *Virology*, *183*: 273-287 (1991); and Connors et al., *Virol.*, *208*: 478-484 (1995)). The nucleocapsid protein (N), phosphoprotein (P), and large polymerase protein (L) constitute the minimal components for viral RNA replication and transcription *in vitro* (Grosfield et al., *J. Virol.*, *69*: 5677-5686 (1995) ; Yu et al., *J. Virol.*, *69*: 2412-2419 (1995); U.S. Patents 6,264,957 and 6,790,449; and International Patent Application Publication WO 97/12032)). The N protein associates with the

genomic RNA to form the nucleocapsid, which serves as the template for RNA synthesis. The L protein is a multifunctional protein that contains RNA-dependent RNA polymerase catalytic motifs and also is likely responsible for capping and polyadenylation of viral mRNAs. However, the L protein alone is not sufficient for the polymerase function; the P protein is also required. The M2(ORF1) protein is a transcription antitermination factor required for processive RNA synthesis and transcription read-through at gene junctions (Collins et al., *supra*; Hardy et al., *J. Virol.*, *73*: 170-176 (1999); Hardy & Wertz, *J. Virol.*, *72*: 520-526 (1998)). M2 (ORF1) also is known as RNA polymerase elongation factor and is needed for the recovery of infectious RSV. The M2(ORF2) protein is involved in the switch between viral RNA transcription and replication (Bermingham & Collins, *Proc. Natl. Acad. Sci. USA*, *96*: 11259-11264 (1999) ; Jin et al., *J. Virol.*, *74*: 74-82 (2000)). The NSl and NS2 proteins have been shown to inhibit minigenome synthesis *in vitro* (Atreya et al., *J. Virol.*, *72*:1452-1461 (1998)).

The F (fusion) and G (attachment) glycoproteins are the two major protective antigens of RSV (Walsh et al., *J. Infect. Dis.*, *155*: 1198-1204 (1987)). The F protein is synthesized as a 68 kDa precursor molecule (F_0) which is proteolytically cleaved into disulfide-linked F_1 (about 48 kDa) and F_2 (about 20 kDa) polypeptide fragments (Walsh et al., *J. Virol.*, *47*: 171-177 (1983)). The G protein is heavily O-glycosylated giving rise to a glycoprotein of apparent molecular weight of about 90 kDa (Levine et al., *J. Gen. Virol.*, *69*: 2521-2524 (1987)). Two broad subtypes of RSV have been defined: RSV-A and RSV-B. The major antigenic differences between these subtypes are found in the G glycoprotein, while the F glycoprotein is more conserved (Wertz et al., *J. Virol.*, *61* (10): 3163-3166 (1987)).

In accordance with the invention, the RSV antigen can include all or part of, for example, the NS1 protein, the NS2 protein, the N protein, the P protein, the M protein, the SH protein, the G protein, the F protein, the M2-1 protein, the M2-2 protein, and the L protein. In some embodiments, the RSV antigen includes all or part of the F protein. In this respect, RSV infection of infants has been shown to induce antibodies to F and G, and F-specific responses are more consistently neutralizing (see, e.g., Welliver et al., *J. Clin. Microbiol.*, *27*(2): 295-299 (1989);

Ward et al., *J. Gen. Virol.*, *64* (Pt 9): 1867-76 (1983); Hendry et al., *J. Infect. Dis.*, *157*(4): 640-647 (1988); Wagner et al., *J. Clin. Microbiol.*, *24*(2): 304-306 (1986); Johnson et al., *J. Virol.*, *61*(10): 3163-3166 (1987); and Murphy et al., *J. Clin. Microbiol.*, *23*(6): 1009-1014 (1986)). An inverse correlation has been observed between the amount of neutralizing antibodies to F and G and the severity of illness (Fernald et al., *Pediatr. Res.*, *17*(9): 753-758 (1983)). Both immunization with the F protein and passive transfer of anti-F monoclonal antibodies have been shown to protect mice and cotton rats against RSV (see, e.g., Johnson et al., *supra*, Olmsted et al., *Proc. Natl. Acad. Sci. USA*, *83*(19): 7462-7466 (1986); Taylor et al., *Immunology*, *52*(1): 137-142 (1984); Walsh et al., J. Infect. Dis., 155(6): 1198-204 (1987); and Walsh et al., *Infect. Immun.*, *43*(2): 756-8 (1984)), and antibody induced by F is cross-protective against heterologous RSV strains (Johnson et al., *supra*). There is also evidence that antibodies to the F protein are sufficient to protect against disease in humans (see, e.g., Parnes et al., *Pediatr. Pulmonol.*, *35*(6): 484-489 (2003); and Sorrentino et al., *Pediatr. Infect. Dis. J.*, *19*(11): 1068-1071 (2000)).

In some embodiments, the antigen is a variant of a wild-type RSV polypeptide, or fragment thereof. In some embodiments, the variant has at least about 50%, at least about 60%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% identity to an amino acid sequence of an RSV polypeptide, or a fragment thereof. In some embodiments, the variants are those that vary from the reference or wild-type sequence by conservative amino acid substitutions, *i.e.*, those that substitute a residue with another of like characteristics. Typical substitutions are among Ala, Val, Leu and Ile; among Ser and Thr; among the acidic residues Asp and Glu; among Asn and Gln; and among the basic residues Lys and Arg, or aromatic residues Phe and Tyr. In some embodiments, the polypeptides are variants in which several, 5 to 10, 1 to 5, or 1 to 2 amino acids are substituted, deleted, or added in any combination.

In some embodiments, the sequence of the nucleic acid encoding the variant of the RSV polypeptide, or fragment thereof, has at least about 50%, at

least about 60%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% identity to SEQ ID NO:2 and SEQ ID NOS:4-6.

In some embodiments of the invention, the RSV antigen-encoding nucleic acid sequence comprises codons expressed more frequently in humans than in RSV. While the genetic code is generally universal across species, the choice among synonymous codons is often species-dependent. RSV replicates in the cytoplasm, and as a result the native mRNAs of RSV have cis signals that reduce the number of mRNAs that are transported from the nucleus. One of ordinary skill in the art would appreciate that, to achieve maximum protection against RSV infection the simian adenoviral vector must be capable of expressing high levels of RSV antigens in a mammalian, preferably a human, host. In this respect, the nucleic acid sequence preferably encodes the native amino acid sequence of an RSV antigen, but comprises codons that are expressed more frequently in mammals (e.g., humans) than in RSV. Changing all of the native RSV codons to those most frequently used in mammals will increase expression of the RSV antigen in the mammal (e.g., a human) (see, e.g., Kim et al., *Vaccine*, 28(22): 3801-3808 (2010),. Such modified nucleic acid sequences are commonly described in the art as "humanized," as "codon-optimized," or as utilizing "mammalian-preferred" or "human-preferred" codons.

In embodiments of the invention, an RSV nucleic acid sequence is said to be "codon-optimized" if at least about 60% (e.g., at least about 70%, at least about 80%, or at least about 90%) of the wild-type codons in the nucleic acid sequence are modified to encode mammalian-preferred codons. That is, an RSV nucleic acid sequence is codon-optimized if at least about 60% of the codons encoded therein are mammalian preferred codons. A preferred codon-optimized nucleic acid sequence encoding an RSV F protein comprises SEQ ID NO: 2. However, the invention is not limited to this exemplary sequence. Indeed, genetic sequences can vary between different strains, and this natural scope of allelic variation is included within the scope of the invention. Codon-optimized nucleic acid sequences encoding RSV antigens are disclosed in, for example, International Patent

Application Publication WO 2008/133663, which sequences are incorporated herein by reference.

In some embodiments, the codon-optimized nucleic acid sequence encoding an RSV F protein is generated manually. In some embodiments, the codon-optimized nucleic acid sequence is generated by computer software, where some or all of the codons of the native sequence are modified. Methods describing codon optimization have been published. See, e.g., Nakamura et. al., *Nucleic Acids Research* 1996, 24:214-215; WO98/34640). Another example is the Syngene method, which is a modification of the Calcgene method (Hale *et al. Protein Expression and Purification*, 12: 185-188 (1998)).

Additionally and alternatively, a codon-optimized nucleic acid sequence encoding an RSV antigen can be any sequence that hybridizes to the above-described sequences under at least moderate, preferably high, stringency conditions, such as those described in, e.g., Sambrook et al., *Molecular Cloning, a Laboratory Manual, 3rd edition*, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (2001). Determining the degree of homology can be accomplished using any suitable method (e.g., BLASTnr, provided by GenBank).

In some embodiments, the codon-optimized nucleic acid sequence encodes an amino acid sequence of an RSV F antigen that is a consensus sequence based on a comparison of a plurality of RSV F protein amino acid sequences.

In some embodiments, in addition to codon optimization, the nucleic acid sequence can be further modified, referred to collectively as "codon-modified." In some embodiments, the sequence can be further modified to enhance mRNA stability and increase expression. For example, in some embodiments, the GC content of the mRNA can be modified to improve stability. In some embodiments, the nucleic acid sequence can be modified to enhance ribosomal binding. In some embodiments, consensus and/or cryptic splice sites can be modified or removed. In some embodiments, modifications are made that affect repeat sequences and/or secondary structures in the mRNA in order to improve stability and/or translation. In some embodiments, the nucleic acid sequences are optimized and modified using various techniques or programs in the art, such as, for example, GeneOptimizer® software by GeneArt®. In some embodiments, the nucleic acids

can be optimized and modified using a combination of approaches, including manual approaches and computer program approaches.

In some embodiments, the codon-optimized nucleic acid sequence encoding the RSV F protein is selected from the group consisting of SEQ ID NO:2, SEQ ID NO: 4, SEQ ID NO:5 and SEQ ID NO: 6.

The simian adenoviral vectors of the invention also preferably comprise expression control sequences, such as promoters, enhancers, polyadenylation signals, transcription terminators, internal ribosome entry sites (IRES), and the like, that provide for the expression of the nucleic acid sequence in a host cell. Exemplary expression control sequences are known in the art and are described in, for example, Goeddel, *Gene Expression Technology: Methods in Enzymology*, Vol. 185, Academic Press, San Diego, Calif. (1990). Ideally, the RSV antigen-encoding nucleic acid sequence is operably linked to a promoter and a polyadenylation sequence. A large number of promoters, including constitutive, inducible, and repressible promoters, from a variety of different sources are well known in the art. Representative sources of promoters include for example, virus, mammal, insect, plant, yeast, and bacteria, and suitable promoters from these sources are readily available, or can be made synthetically, based on sequences publicly available, for example, from depositories such as the ATCC as well as other commercial or individual sources. Promoters can be unidirectional (i.e., initiate transcription in one direction) or bi-directional (i.e., initiate transcription in either a 3' or 5' direction). Non-limiting examples of promoters include, for example, the T7 bacterial expression system, pBAD (araA) bacterial expression system, the cytomegalovirus (CMV) promoter, the SV40 promoter, and the RSV promoter. Inducible promoters include, for example, the Tet system (U.S. Patents 5,464,758 and 5,814,618), the Ecdysone inducible system (No et al., *Proc. Natl. Acad. Sci.*, *93*: 3346-3351 (1996)), the T-REx™ system (Invitrogen, Carlsbad, CA), LACSWITCH™ System (Stratagene, San Diego, CA), and the Cre-ERT tamoxifen inducible recombinase system (Indra et al., *Nuc. Acid. Res.*, *27*: 4324-4327 (1999); *Nuc. Acid. Res.*, *28*: e99 (2000); U.S. Patent 7,112,715; and Kramer & Fussenegger, *Methods Mol. Biol.*, *308*: 123-144 (2005)).

A promoter can be selected by matching its particular pattern of activity with the desired pattern and level of expression of an antigen(s). For example, the simian adenoviral vector can comprise two or more nucleic acid sequences that encode different antigens and are operably linked to different promoters displaying distinct expression profiles. In this regard, a first promoter can be selected to mediate an initial peak of antigen production, thereby priming the immune system against an encoded antigen. A second promoter can be selected to drive production of the same or different antigen such that expression peaks several days after that of the first promoter, thereby "boosting" the immune system against the antigen. Alternatively, a hybrid promoter can be constructed which combines the desirable aspects of multiple promoters. For example, a CMV-Rous Sarcoma Virus hybrid promoter combining the CMV promoter's initial rush of activity with the Rous Sarcoma Virus promoter's high maintenance level of activity can be employed. In that antigens can be toxic to eukaryotic cells, it may be advantageous to modify the promoter to decrease activity in complementing cell lines used to propagate the simian adenoviral vector.

To optimize protein production, preferably the antigen-encoding nucleic acid sequence further comprises a polyadenylation site following the coding sequence. Any suitable polyadenylation sequence can be used, including a synthetic optimized sequence, as well as the polyadenylation sequence of BGH (Bovine Growth Hormone), polyoma virus, TK (Thymidine Kinase), EBV (Epstein Barr Virus), and the papillomaviruses, including human papillomaviruses and BPV (Bovine Papilloma Virus). A preferred polyadenylation sequence is the SV40 (Human Sarcoma Virus-40) polyadenylation sequence. Also, preferably all the proper transcription signals (and translation signals, where appropriate) are correctly arranged such that the nucleic acid sequence is properly expressed in the cells into which it is introduced. If desired, the nucleic acid sequence also can incorporate splice sites (i.e., splice acceptor and splice donor sites) to facilitate mRNA production.

If the RSV antigen-encoding nucleic acid sequence encodes a processed or secreted protein or peptide, or a protein that acts intracellularly, preferably the RSV antigen-encoding nucleic acid sequence further comprises the appropriate

sequences for processing, secretion, intracellular localization, and the like. The RSV antigen-encoding nucleic acid sequence can be operably linked to a signal sequence, which targets a protein to cellular machinery for secretion. Appropriate signal sequences include, but are not limited to, leader sequences for immunoglobulin heavy chains and cytokines (see, for example, Ladunga et al., *Current Opinions in Biotechnology, 11*: 13-18 (2000)). Other protein modifications can be required to secrete a protein from a host cell, which can be determined using routine laboratory techniques. Preparing expression constructs encoding antigens and signal sequences is further described in, for example, U.S. Patent 6,500,641. Methods of secreting non-secretable proteins are further described in, for example, U.S. Patent 6,472,176, and International Patent Application Publication WO 02/48377.

An RSV antigen encoded by the nucleic acid sequence of the simian adenoviral vector also can be modified to attach or incorporate the antigen on a host cell surface. In this respect, the antigen can comprise a membrane anchor, such as a gpi-anchor, for conjugation onto a cell surface. A transmembrane domain can be fused to the antigen to incorporate a terminus of the antigen protein into the cell membrane. Other strategies for displaying peptides on a cell surface are known in the art and are appropriate for use in the context of the invention.

The invention further provides a method of inducing an immune response against RSV in a mammal. In some embodiments, the method comprises administering to the mammal a composition comprising the aforementioned simian adenoviral vector and a pharmaceutically acceptable carrier, whereupon the nucleic acid sequence encoding the RSV antigen is expressed in the mammal to produce the RSV antigen and thereby induce an immune response against the pathogen. The simian adenoviral vector desirably is administered in a pharmaceutically acceptable (e.g., physiologically acceptable) composition, which comprises a carrier, preferably a physiologically (e.g., pharmaceutically) acceptable carrier, and the simian adenoviral vector. Any suitable carrier can be used within the context of the invention, and such carriers are well known in the art. The choice of carrier will be determined, in part, by the particular site to which the composition is to be administered and the particular method used to administer the composition.

Ideally, in the context of adenoviral vectors, the pharmaceutical composition preferably is free of replication-competent adenovirus. The pharmaceutical composition can optionally be sterile.

In accordance with the invention, the composition is administered to an animal, preferably a mammal, and most preferably a human, wherein the RSV antigen-encoding nucleic acid sequence is expressed to induce an immune response against RSV. The human may be in a population that has a high risk of acquiring RSV. Such high-risk populations include, for example, pregnant women, neonates, young infants, the immunocompromised, and the elderly. Most preferably, the human is younger than 3 years (e.g., about 2.5 years old, about 2 years old, about 1.5 years old, about 1 year old, about 9 months old, about 6 months old, about 3 months old, about 6 weeks old, about 4 weeks old, about 2 weeks old, about 1 week old, or about 1 day old) or older than 65 years (e.g., about 70 years old, about 75 years old, about 80 years old, about 85 years old, about 90 years old, about 95 years old, or older).

The immune response can be a humoral immune response, a cell-mediated immune response, or, desirably, a combination of humoral and cell-mediated immunity. Ideally, the immune response provides protection upon subsequent challenge with RSV. However, protective immunity is not required in the context of the invention. The inventive method also can be used for antibody production and harvesting.

In some embodiments, the effectiveness of an immune response against RSV can be determined by a neutralization assay. In some embodiments, the level of neutralizing activity in an immunized mammal corresponds to at least the level of neutralizing activity present in 25 μg/ml of SYNAGIS® (palivizumab). In some embodiments, the level of neutralizing activity in an immunized mammal corresponds to at least 40 μg/ml, at least 50 μg/ml, at least 100 μg/ml, at least 250 μg/ml, at least 500 μg/ml, or at least 1000 μg/ml of SYNAGIS®.

In some embodiments, the effectiveness of an immune response can be determined by detecting the presence of RSV in a biological sample from an immunized mammal after challenge or infection with RSV. In some embodiments, RSV can be detected and quantified in a biological sample by a plaque assay. In

some embodiments, viral titers of RSV in the lung can be measured post challenge with RSV to determine the effectiveness of the immune response. In some embodiments, viral titers are measured at 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days or 15 days post challenge or infection. In some embodiments, the viral titers are determined by a plaque assay as described in Example 5. In some embodiments, the effectiveness of the immune response is determined in a cotton rat animal model. In some embodiments, the RSV titers can be expressed as plaque forming units (pfu) per gram of lung tissue from the immunized mammal. In some embodiments, the titer of RSV in the lung of an immunized mammal following challenge with RSV is at or below the assay limit of detection. In some embodiments, the titer of RSV in the lung is 70 pfu or less/gram of lung tissue. In some embodiments, the titer of RSV in the lung is 100 pfu or less/gram of lung tissue. In some embodiments, the titer of RSV in the lung is 200 pfu or less/gram of lung tissue, 500 PFU or less/gram of lung tissue, 750 PFU or less/gram of lung tissue, 1000 PFU or less/gram of lung tissue, 5000 PFU or less/gram of lung tissue or 10,000 PFU or less/gram of lung tissue.

To enhance the immune response generated against an RSV antigen, the composition also can comprise an immune stimulator, or a nucleic acid sequence that encodes an immune stimulator. Immune stimulators also are referred to in the art as "adjuvants," and include, for example, cytokines, chemokines, or chaperones. Cytokines include, for example, Macrophage Colony Stimulating Factor (e.g., GM-CSF), Interferon Alpha (IFN-α), Interferon Beta (IFN-β), Interferon Gamma (IFN-γ), interleukins (IL-1, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, IL-15, IL-16, and IL-18), the TNF family of proteins, Intercellular Adhesion Molecule-1 (ICAM-1), Lymphocyte Function-Associated antigen-3 (LFA-3), B7-1, B7-2, FMS-related tyrosine kinase 3 ligand, (Flt3L), vasoactive intestinal peptide (VIP), and CD40 ligand. Chemokines include, for example, B Cell-Attracting chemokine-1 (BCA-1), Fractalkine, Melanoma Growth Stimulatory Activity protein (MGSA), Hemofiltrate CC chemokine 1 (HCC-1), Interleukin 8 (IL-8), Interferon-stimulated T-cell alpha chemoattractant (I-TAC), Lymphotactin, Monocyte Chemotactic Protein 1 (MCP-1), Monocyte Chemotactic Protein 3 (MCP-3), Monocyte Chemotactic Protein 4 (CP-4), Macrophage-Derived

Chemokine (MDC), a macrophage inflammatory protein (MIP), Platelet Factor 4 (PF4), RANTES, BRAK, eotaxin, exodus 1-3, and the like. Chaperones include, for example, the heat shock proteins Hsp170, Hsc70, and Hsp40.

Suitable formulations for the composition include aqueous and non-aqueous solutions, isotonic sterile solutions, which can contain anti-oxidants, buffers, and bacteriostats, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. The formulations can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials, and can be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, water, immediately prior to use. Extemporaneous solutions and suspensions can be prepared from sterile powders, granules, and tablets. In some embodiments, the carrier is a buffered saline solution. In some embodiments, the simian adenoviral vector is administered in a composition formulated to protect the simian adenoviral vector from damage prior to administration. For example, the composition can be formulated to reduce loss of the adenoviral vector on devices used to prepare, store, or administer the simian adenoviral vector, such as glassware, syringes, or needles. The composition can be formulated to decrease the light sensitivity and/or temperature sensitivity of the simian adenoviral vector. The composition thus may comprise a pharmaceutically acceptable liquid carrier, such as, for example, those described above, and a stabilizing agent selected from the group consisting of polysorbate 80, L-arginine, polyvinylpyrrolidone, trehalose, and combinations thereof. Use of such a composition will extend the shelf life of the vector, facilitate administration, and increase the efficiency of the inventive method. Formulations for adenoviral vector-containing compositions are further described in, for example, U.S. Patent 6,225,289, U.S. Patent 6,514,943, and International Patent Application Publication WO 00/34444.

The composition also can be formulated to enhance transduction efficiency. In addition, one of ordinary skill in the art will appreciate that the adenoviral vector can be present in a composition with other therapeutic or biologically-active agents. For example, factors that control inflammation, such as ibuprofen or steroids, can be part of the composition to reduce swelling and inflammation

associated with *in vivo* administration of the simian adenoviral vector. As discussed herein, immune system stimulators or adjuvants, e.g., interleukins, lipopolysaccharide, or double-stranded RNA, can be administered to enhance or modify any immune response to the RSV antigen. Antibiotics, i.e., microbicides and fungicides, can be present to treat existing infection and/or reduce the risk of future infection, such as infection associated with gene transfer procedures.

Any route of administration can be used to deliver the composition to the mammal. Indeed, although more than one route can be used to administer the composition, a particular route can provide a more immediate and more effective reaction than another route. In some embodiments, the composition is administered by intramuscular injection or intranasal administration. The composition also can be applied or instilled into body cavities, absorbed through the skin (e.g., via a transdermal patch), inhaled, ingested, topically applied to tissue, or administered parenterally by, for instance, intravenous, peritoneal, or intraarterial administration.

The composition can be administered in or on a device that allows controlled or sustained release, such as a sponge, biocompatible meshwork, mechanical reservoir, or mechanical implant. Implants (see, e.g., U.S. Patent 5,443,505), devices (see, e.g., U.S. Patent 4,863,457), such as an implantable device, e.g., a mechanical reservoir or an implant or a device comprised of a polymeric composition, are particularly useful for administration of the composition. The composition also can be administered in the form of sustained-release formulations (see, e.g., U.S. Patent 5,378,475) comprising, for example, gel foam, hyaluronic acid, gelatin, chondroitin sulfate, a polyphosphoester, such as bis-2-hydroxyethyl-terephthalate (BHET), and/or a polylactic-glycolic acid.

The dose of the composition administered to the mammal will depend on a number of factors, including the extent of any side-effects, the particular route of administration, and the like. The dose ideally comprises an "effective amount" of the simian adenoviral vector, i.e., a dose of simian adenoviral vector which provokes a desired immune response in the mammal. Desirably, the composition comprises a single dose of adenoviral vector comprising at least about $1x10^5$ particles (which also is referred to as particle units) of adenoviral vector. The dose

preferably is at least about $1x10^6$ particles (e.g., about $1x10^6$-$1x10^{12}$ particles), more preferably at least about $1x10^7$ particles, more preferably at least about $1x10^8$ particles (e.g., about $1x10^8$-$1x10^{11}$ particles or about $1x10^8$-$1x10^{12}$ particles), and most preferably at least about $1x10^9$ particles (e.g., about $1x10^9$-$1x10^{10}$ particles or about $1x10^9$-$1x10^{12}$ particles), or even at least about $1x10^{10}$ particles (e.g., about $1x10^{10}$-$1x10^{12}$ particles) of the adenoviral vector. The dose desirably comprises no more than about $1x10^{14}$ particles, preferably no more than about $1x10^{13}$ particles, even more preferably no more than about $1x10^{12}$ particles, even more preferably no more than about $1x10^{11}$ particles, and most preferably no more than about $1x10^{10}$ particles. In other words, a single dose of the simian adenoviral vector can comprise, for example, about $1x10^6$ particle units (pu), $2x10^6$ pu, $4x10^6$ pu, $1x10^7$ pu, $2x10^7$ pu, $4x10^7$ pu, $1x10^8$ pu, $2x10^8$ pu, $4x10^8$ pu, $1x10^9$ pu, $2x10^9$ pu, $4x10^9$ pu, $1x10^{10}$ pu, $2x10^{10}$ pu, $4x10^{10}$ pu, $1x10^{11}$ pu, $2x10^{11}$ pu, $4x10^{11}$ pu, $1x10^{12}$ pu, $2x10^{12}$ pu, or $4x10^{12}$ pu of the simian adenoviral vector.

Administration of the composition containing the simian adenoviral vector can be one or more components of a multistep regimen for inducing an immune response against RSV in a mammal, preferably a human. In this respect, the method further comprises administering to the mammal a boosting composition after administering the simian adenoviral vector to the mammal. In this embodiment, therefore, the immune response is "primed" upon administration of the composition containing the simian adenoviral vector, and is "boosted" upon administration of the boosting composition. Alternatively, the inventive method further comprises administering to the mammal a priming composition to the mammal prior to administering the simian adenoviral vector to the mammal. In this embodiment, therefore, the immune response is "primed" upon administration of the priming composition, and is "boosted" upon administration of the composition containing the simian adenoviral vector. Additional administrations of priming and boosting compositions are also possible.

Each of the priming composition and the boosting composition desirably is a gene transfer vector that comprises a nucleic acid sequence encoding an RSV antigen. Any gene transfer vector can be employed, including viral and non-viral gene transfer vectors. Examples of suitable viral gene transfer vectors include, but

are not limited to, retroviral vectors, adeno-associated virus vectors, vaccinia virus vectors, herpesvirus vectors, parainfluenza-RSV chimeric vectors (PIV-RSV), and adenoviral vectors. Examples of suitable non-viral vectors include, but are not limited to, plasmids, liposomes, and molecular conjugates (e.g., transferrin). Preferably, the priming composition or the boosting composition is a plasmid or an adenoviral vector. Alternatively, an immune response can be primed or boosted by administration of an RSV protein itself (e.g., an antigenic RSV protein) with or without a suitable adjuvant (e.g., alum, QS-21, insulin-derived adjuvant, etc.), a live-attenuated RSV particle, a virus-like particle, and the like. When the priming composition and/or the boosting composition is an adenoviral vector, it can be an adenoviral vector derived from any human or non-human animal as described herein. In some embodiments, the priming composition and/or the boosting composition comprises a human adenoviral vector (e.g., serotype 5, 28, or 35) or a simian adenoviral vector. For example, a priming composition containing a human serotype 5 adenoviral vector can be administered to a human, followed by administration of a boosting composition containing the simian adenoviral vector (e.g., a serotype 7 simian adenoviral vector). Alternatively, a priming composition containing the inventive simian adenoviral vector can be administered to a human, followed by administration of a boosting composition containing a human serotype 5 adenoviral vector. In another embodiment, a priming composition containing the simian adenoviral vector described herein (e.g. a serotype 7 simian adenoviral vector) can be administered to a human, followed by a second administration of the same composition. One of ordinary skill in the art will appreciate that any combination of human and/or simian adenoviral vectors encoding one or more RSV antigens can be employed as the priming or boosting composition in conjunction with a composition comprising the simian adenoviral vector of the present invention.

The gene transfer vector of the priming composition and the boosting composition preferably comprises at least one nucleic acid sequence encoding an RSV antigen. In some embodiments, the nucleic acid sequence encodes an F protein antigen or fragment thereof. The RSV antigen encoded by the nucleic acid sequence of the priming composition and/or the boosting composition can be the

same as the RSV antigen encoded by the simian adenoviral vector. Alternatively, the RSV antigen encoded by the nucleic acid sequence of the priming composition and/or the boosting composition can be different from the RSV antigen encoded by the simian adenoviral vector. In one embodiment, the gene transfer vector of the priming composition and/or the boosting composition comprises multiple (i.e., two or more) nucleic acid sequences encoding the same RSV antigen, as described herein. In another embodiment, the gene transfer vector of the priming composition and/or the boosting composition can comprise multiple nucleic acid sequences encoding two or more different antigens, as described herein.

Administration of the priming composition and the boosting composition can be separated by any suitable timeframe (e.g., at least about 1 week, 2 weeks, 4 weeks, 8 weeks, 12 weeks, 16 weeks, 24 weeks, 52 weeks, or a range defined by any two of the foregoing values). The boosting composition preferably is administered to a mammal (e.g., a human) at least about 2 weeks (e.g., 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 9 weeks, 10 weeks, 11 weeks, 12 weeks, 13 weeks, 14 weeks, 15 weeks, 16 weeks, 20 weeks, 24 weeks, 28 weeks, 35 weeks, 40 weeks, 50 weeks, 52 weeks, or a range defined by any two of the foregoing values) following administration of the priming composition. More than one dose of priming composition and/or boosting composition can be provided in any suitable timeframe. The dose of the priming composition and boosting composition administered to the mammal depends on a number of factors, including the extent of any side-effects, the particular route of administration, and the like.

In some embodiments, the priming composition comprises one or more replication-deficient adenoviral vectors of the invention. In some embodiments, the priming composition comprises one or more replication-deficient adenoviral vectors comprising a sequence selected from the group consisting of SEQ ID NO:7, SEQ ID NO:8 and SEQ ID NO:9. In some embodiments, the boosting composition comprises one or more replication-deficient adenoviral vectors of the invention. In some embodiments, the boosting composition comprises one or more replication-deficient adenoviral vectors comprising a sequence selected from the group consisting of SEQ ID NO:7, SEQ ID NO:8 and SEQ ID NO:9.

In some embodiments, the priming composition comprises a replication-deficient adenoviral vector comprising SEQ ID NO:9, and the boosting composition comprises one or more replication-deficient adenoviral vectors comprising a sequence selected from the group consisting of SEQ ID NO:7 and SEQ ID NO:8.

In some embodiments, the priming composition comprises one or more replication-deficient adenoviral vectors comprising a sequence selected from the group consisting of SEQ ID NO:7 and SEQ ID NO:8, and the boosting composition comprises a replication-deficient adenoviral vector comprising SEQ ID NO:9.

In other embodiments, the invention includes within its scope priming and boosting compositions comprising a replication-deficient serotype 7 simian adenoviral vector alone, that is, without limitation to a heterologous antigen-encoding nucleic acid sequence comprised therein. In some embodiments is provided a replication-deficient, E1 region-deleted serotype 7 simian adenoviral vector comprising the nucleic acid sequence of SEQ ID NO:12. In some embodiments, the invention also includes priming and boosting compositions comprising a replication-deficient human serotype 5 adenoviral vector alone, that is, without limitation to a heterologous antigen-encoding nucleic acid sequence comprised therein. In some embodiments is provided a replication-deficient, E1 region-deleted human serotype 5 adenoviral vector comprising a nucleic acid sequence selected from the group consisting of SEQ ID NO:10 and SEQ ID NO:11. In other embodiments, a nucleic acid sequence encoding a heterologous antigen is inserted at the location of the E1 region deletion.

In some embodiments, the priming composition comprises a replication-deficient, E1 region-deleted, simian adenoviral vector comprising the sequence of SEQ ID NO:12, and the boosting composition comprises a replication-deficient, E1 region-deleted, human adenoviral vector comprising a sequence selected from the group consisting of SEQ ID NO:10 and SEQ ID NO:11. In other embodiments, a nucleic acid sequence encoding a heterologous antigen is inserted at the location of the E1 region deletion.

In some embodiments, the priming composition comprises a replication-deficient, E1 region-deleted, human adenoviral vector comprising a sequence selected from the group consisting of SEQ ID NO:10 and SEQ ID NO:11, and the boosting composition comprises a replication-deficient, E1 region-deleted, simian adenoviral vector comprising the sequence of SEQ ID NO:12. In other embodiments, a nucleic acid sequence encoding a heterologous antigen is inserted at the location of the E1 region deletion.

Additional embodiments of the invention include the following:

1. A replication-deficient monkey adenoviral vector comprising at least one nucleic acid sequence which encodes a respiratory syncytial virus (RSV) F protein antigen or a fragment thereof, wherein the nucleic acid sequence is codon-optimized for expression in a mammal.

2. The monkey adenoviral vector of paragraph 1, wherein the vector is a serotype 7 monkey adenoviral vector.

3. The monkey adenoviral vector of any of paragraphs 1-2, wherein the vector further comprises one or more nucleic acid sequences encoding an RSV antigen other than the RSV F protein antigen.

4. The monkey adenoviral vector of any of paragraphs 1-3, wherein the mammal is a human.

5. A method for inducing a protective immune response against RSV in a mammal, which comprises administering to the mammal a composition comprising a replication-deficient serotype 7 monkey adenoviral vector comprising at least one nucleic acid sequence encoding an RSV F protein antigen or a fragment thereof, and a pharmaceutically acceptable carrier, wherein the nucleic acid sequence is codon-optimized for expression in a mammal and is expressed in the mammal to produce the RSV F protein antigen and thereby induce the immune response against RSV.

6. The method of paragraph 5, wherein the method further comprises administering a priming composition prior to administering the monkey adenoviral vector to the mammal.

7. The method of paragraph 6, wherein the priming composition comprises a plasmid or a viral vector comprising a nucleic acid sequence encoding an RSV antigen.

8. The method of paragraph 7, wherein the viral vector is an adenoviral vector.

9. The method of paragraph 8, wherein the adenoviral vector is a monkey adenoviral vector or a human adenoviral vector.

10. The method of paragraph 5, wherein the method further comprises administering a boosting composition after administering the monkey adenoviral vector to the mammal.

11. The method of paragraph 10, wherein the boosting composition comprises a plasmid or a viral vector comprising a nucleic acid sequence encoding an RSV antigen.

12. The method of paragraph 11, wherein the viral vector is an adenoviral vector.

13. The method of paragraph 12, wherein the adenoviral vector is a monkey adenoviral vector or a human adenoviral vector.

14. The method of paragraphs 9 or 13, wherein the nucleic acid sequence encoding an RSV antigen in the monkey adenoviral vector and human adenoviral vector encodes an RSV F protein antigen or a fragment thereof.

15. The method of paragraph 14, wherein the monkey adenoviral vector is of serotype 7.

16. The method of paragraph 15, wherein the human adenoviral vector is of serotype 5.

17. The method of paragraph 5, wherein the mammal is a human.

18. A method for inducing a protective immune response against RSV in a mammal, which comprises:

(a) administering to the mammal a priming composition comprising a replication-deficient monkey adenoviral vector or a replication-deficient human adenoviral vector, each comprising a nucleic acid sequence encoding an RSV F protein antigen or a fragment thereof, and a pharmaceutically acceptable carrier, and

(b) administering to the mammal a boosting composition comprising a replication-deficient monkey adenoviral vector or a replication-deficient human adenoviral vector, each also comprising a nucleic acid sequence encoding an RSV F protein antigen or a fragment thereof, and a pharmaceutically acceptable carrier,

wherein at least one of the priming or boosting compositions is a replication-deficient monkey adenoviral vector comprising a nucleic acid sequence encoding an RSV F protein antigen or fragment thereof which is codon-optimized for expression in a mammal, and whereby the nucleic acid sequences are expressed in the mammal to produce the RSV F protein antigen or fragment thereof and induce the immune response against RSV.

19. The method of paragraph 18, wherein the monkey adenoviral vector is of serotype 7.

20. The method of paragraph 19, wherein the human adenoviral vector is of serotype 5.

21. The method of paragraph 20, wherein the priming composition comprises a replication-deficient monkey adenoviral vector and the boosting composition comprises a replication-deficient human adenoviral vector.

22. The method of paragraph 20, wherein the priming composition comprises a replication-deficient human adenoviral vector and the boosting composition comprises a replication-deficient monkey adenoviral vector.

23. The method of paragraph 18, wherein the mammal is a human.

24. The method of paragraph 23, wherein the human is younger than 3 years or older than 65 years.

25. The method of paragraph 18, wherein administration of the priming composition and the boosting composition is separated by about 4 weeks.

26. The method of paragraph 19, wherein the priming or boosting compositions comprise between about $1x10^9$ and $1x10^{12}$ particle units (pu) of the serotype 7 monkey adenoviral vector.

27. The method of paragraph 20, wherein the priming or boosting compositions comprise between about $1x10^9$ and $1x10^{12}$ pu of the serotype 5 human adenoviral vector.

28. A method for treating or protecting against RSV infection in a human by inducing an immune response against RSV in the human, comprising administering to the human one or more compositions comprising a replication-deficient monkey adenoviral vector and a pharmaceutically acceptable carrier, and optionally one or more compositions comprising a replication-deficient human adenoviral vector and a pharmaceutically acceptable carrier, wherein each adenoviral vector comprises at least one nucleic acid sequence encoding an RSV F protein antigen or a fragment thereof which is expressed in the human to produce the RSV F protein antigen or fragment thereof and thereby induce the immune response against RSV infection in the human, wherein the nucleic acid sequence encoding an RSV F protein antigen or fragment thereof is codon-optimized for expression in a mammal.

29. The method of paragraph 28, wherein the at least one nucleic acid sequence encoding an RSV F protein antigen comprises SEQ ID NO:2.

30. The method of paragraph 28, wherein the monkey adenoviral vector is of serotype 7.

31. The method of paragraph 30, wherein the human adenoviral vector is of serotype 5.

32. An immunogenic vaccine composition for treating or protecting against RSV infection in a mammal by inducing an immune response against RSV in the mammal, wherein the vaccine comprises a replication-deficient serotype 7 monkey adenoviral vector comprising at least one nucleic acid sequence encoding an RSV F protein antigen or a fragment thereof, and a pharmaceutically acceptable carrier, wherein the at least one nucleic acid sequence is codon-optimized for expression in a mammal and can be expressed in the mammal to produce the RSV F protein antigen or fragment thereof and thereby induce the immune response against RSV in the mammal.

33. The vaccine composition of paragraph 32, wherein the mammal is a human.

34. A method for substantially reducing the titer of RSV in the lungs of a mammal infected with RSV by inducing an immune response against RSV, comprising administering to the mammal one or more compositions comprising a replication-deficient monkey adenoviral vector and a pharmaceutically acceptable carrier, and optionally one or more compositions comprising a replication-deficient human adenoviral vector and a pharmaceutically acceptable carrier, wherein each adenoviral vector comprises at least one nucleic acid sequence encoding an RSV antigen or a fragment thereof which is expressed in the mammal to produce the RSV antigen and thereby induce an immune response against RSV that substantially reduces the titer of RSV in the lungs of the mammal, wherein the

nucleic acid sequence encoding an RSV antigen or a fragment thereof is codon-optimized for expression in a mammal.

35. The method of paragraph 34, wherein the RSV antigen is an RSV F protein antigen.

36. The method of paragraph 35, wherein the mammal is a human.

37. The monkey adenoviral vector of any of paragraphs 1-4, wherein the monkey adenoviral vector comprises SEQ ID NO:9.

38. The method of any of paragraphs 5-17, wherein the replication-deficient serotype 7 monkey adenoviral vector comprises SEQ ID NO:9.

39. The method of any of paragraphs 7-16, wherein the viral vector is a replication-deficient human adenoviral vector comprising a sequence selected from the group consisting of SEQ ID NO:7 and SEQ ID NO:8.

40. The method of any of paragraphs 18-31, wherein the monkey adenoviral vector comprises SEQ ID NO:9 and the human adenoviral vector comprises a sequence selected from the group consisting of SEQ ID NO:7 and SEQ ID NO:8.

41. The immunogenic vaccine composition of paragraphs 32 or 33, wherein the replication-deficient serotype 7 monkey adenoviral vector comprises SEQ ID NO:9.

42. The method of any of paragraphs 34-36, wherein the replication-deficient monkey adenoviral vector comprises SEQ ID NO:9.

43. The method of any of paragraphs 34-36 or 42, wherein the replication-deficient human adenoviral vector comprises a sequence selected from the group consisting of SEQ ID NO:7 and SEQ ID NO:8.

44. The monkey adenoviral vector of any of paragraphs 1-4, wherein the nucleic acid sequence encoding the RSV F protein antigen or fragment thereof is a consensus sequence based on a comparison of a plurality of RSV F antigen sequences.

45. The monkey adenoviral vector of any of paragraphs 1-4 or 44, wherein the codon-optimized nucleic acid sequence has been further modified.

46. The monkey adenoviral vector of paragraph 45, wherein the further modification is selected from the group consisting of: modifying the GC content, enhancing ribosomal binding, modifying or removing consensus and/or cryptic splice sites, modifying repeat sequences, modifying the secondary structure in the mRNA in order to improve stability and/or translation, and combinations thereof.

47. The method of any of paragraphs 5-31, 34-36, 38-40, 42 or 43, wherein the immune response results in a level of neutralizing activity in the mammal which corresponds to a level of neutralizing activity of palivizumab selected from the group consisting of at least 40 μg/ml; at least 50 μg/ml; at least 100 μg/ml; at least 250 μg/ml; at least 500 μg/ml; and at least 1000 μg/ml.

48. The method of any of paragraphs 5-31, 34-36, 38-40, 42 or 43, wherein if the mammal is subsequently infected with RSV, the immune response results in a titer (of RSV per gram of lung tissue) selected from the group consisting of 70 PFU or less; 100 PFU or less; 200 PFU or less; 500 PFU or less; 750 PFU or less; and 1000 PFU or less.

49. The method of paragraphs 47 or 48, wherein the adenoviral vector is administered intramuscularly.

The simian adenoviral vector can be manipulated to alter the binding specificity or recognition of the adenovirus for a receptor on a potential host cell. For adenovirus, such manipulations can include deletion of regions of adenovirus coat proteins (e.g., fiber, penton, or hexon), insertions of various native or non-native ligands into portions of a coat protein, and the like. Manipulation of the coat protein can broaden the range of cells infected by the simian adenoviral vector or enable targeting of the simian adenoviral vector to a specific cell type.

Any suitable technique for altering native binding to a host cell, such as native binding of the fiber protein to its cellular receptor, can be employed. For example, differing fiber lengths can be exploited to ablate native binding to cells. This optionally can be accomplished via the addition of a binding sequence to the penton base or fiber knob. This addition of a binding sequence can be done either directly or indirectly via a bispecific or multispecific binding sequence. In an alternative embodiment, the adenoviral fiber protein can be modified to reduce the number of amino acids in the fiber shaft, thereby creating a "short-shafted" fiber (as described in, for example, U.S. Patent 5,962,311). Use of an adenovirus comprising a short-shafted adenoviral fiber gene reduces the level or efficiency of adenoviral fiber binding to its cell-surface receptor and increases adenoviral penton base binding to its cell-surface receptor, thereby increasing the specificity of binding of the adenovirus to a given cell. Alternatively, use of a simian adenoviral vector comprising a short-shafted fiber enables targeting of the simian adenoviral vector to a desired cell-surface receptor by the introduction of a nonnative amino acid sequence either into the penton base or the fiber knob.

In yet another embodiment, the nucleic acid residues encoding amino acid residues associated with native substrate binding can be changed, supplemented, or deleted (see, e.g., International Patent Application Publication WO 00/15823, Einfeld et al., *J. Virol.*, *75*(23): 11284-11291 (2001), and van Beusechem et al., *J. Virol.*, *76*(6): 2753-2762 (2002)) such that the simian adenoviral vector incorporating the mutated nucleic acid residues (or having the fiber protein encoded thereby) is less able to bind its native substrate.

Any suitable amino acid residue(s) of a fiber protein that mediates or assists in the interaction between the knob and the native cellular receptor can be mutated or removed, so long as the fiber protein is able to trimerize. Similarly, amino acids can be added to the fiber knob as long as the fiber protein retains the ability to trimerize. Suitable residues include amino acids within the exposed loops of the fiber knob domain, such as, for example, the AB loop, the DE loop, the FG loop, and the HI loop.

Any suitable amino acid residue(s) of a penton base protein that mediates or assists in the interaction between the penton base and integrins can be mutated or removed. Suitable residues include, for example, an RGD amino acid sequence motif located in the hypervariable region of the simian adenovirus penton base protein. The native integrin binding sites on the penton base protein also can be disrupted by modifying the nucleic acid sequence encoding the native RGD motif such that the native RGD amino acid sequence is conformationally inaccessible for binding to an integrin receptor, such as by inserting a DNA sequence into or adjacent to the nucleic acid sequence encoding the adenoviral penton base protein.

The simian adenoviral vector can comprise a fiber protein and a penton base protein that do not bind to their respective native cellular binding sites. Alternatively, the simian adenoviral vector comprises fiber protein and a penton base protein that bind to their respective native cellular binding sites, but with less affinity than the corresponding wild-type coat proteins. The simian adenoviral vector exhibits reduced binding to native cellular binding sites if a modified adenoviral fiber protein and penton base protein binds to their respective native cellular binding sites with at least about 5-fold, 10-fold, 20-fold, 30-fold, 50-fold, or 100-fold less affinity than a non-modified adenoviral fiber protein and penton base protein of the same serotype.

The simian adenoviral vector also can comprise a chimeric coat protein comprising a non-native amino acid sequence that binds a substrate (i.e., a ligand), such as a cellular receptor other than a native cellular receptor. The non-native amino acid sequence of the chimeric adenoviral coat protein allows the simian adenoviral vector comprising the chimeric coat protein to bind and, desirably, infect host cells not naturally infected by a corresponding adenovirus without the

non-native amino acid sequence (i.e., host cells not infected by the corresponding wild-type adenovirus), to bind to host cells naturally infected by the corresponding wild-type adenovirus with greater affinity than the corresponding adenovirus without the non-native amino acid sequence, or to bind to particular target cells with greater affinity than non-target cells. A "non-native" amino acid sequence can comprise an amino acid sequence not naturally present in the adenoviral coat protein or an amino acid sequence found in the adenoviral coat but located in a non-native position within the capsid. By "preferentially binds" is meant that the non-native amino acid sequence binds a receptor, such as, for instance, αvβ3 integrin, with at least about 3-fold greater affinity (e.g., at least about 5-fold, 10-fold, 15-fold, 20-fold, 25-fold, 35-fold, 45-fold, or 50-fold greater affinity) than the non-native ligand binds a different receptor, such as, for instance, αvβ1 integrin.

The simian adenoviral vector can comprise a chimeric coat protein comprising a non-native amino acid sequence that confers to the chimeric coat protein the ability to bind to an immune cell more efficiently than a wild-type adenoviral coat protein. In particular, the simian adenoviral vector can comprise a chimeric adenoviral fiber protein comprising a non-native amino acid sequence which facilitates uptake of the simian adenoviral vector by immune cells, preferably antigen presenting cells, such as dendritic cells, monocytes, and macrophages. In a preferred embodiment, the simian adenoviral vector comprises a chimeric fiber protein comprising an amino acid sequence (e.g., a non-native amino acid sequence) comprising an RGD motif, which increases transduction efficiency of the simian adenoviral vector into dendritic cells. The RGD-motif, or any non-native amino acid sequence, preferably is inserted into the adenoviral fiber knob region, ideally in an exposed loop of the adenoviral knob, such as the HI loop. A non-native amino acid sequence also can be appended to the C-terminus of the adenoviral fiber protein, optionally via a spacer sequence. The spacer sequence preferably comprises between one and two-hundred amino acids, and can (but need not) have an intended function.

In another embodiment, the simian adenoviral vector can comprise a chimeric virus coat protein that is not selective for a specific type of eukaryotic cell. The chimeric coat protein differs from a wild-type coat protein by an

insertion of a non-native amino acid sequence into or in place of an internal coat protein sequence, or attachment of a non-native amino acid sequence to the N- or C- terminus of the coat protein. For example, a ligand comprising about five to about nine lysine residues (preferably seven lysine residues) can be attached to the C-terminus of the adenoviral fiber protein via a non-functional spacer sequence. In this embodiment, the chimeric virus coat protein efficiently binds to a broader range of eukaryotic cells than a wild-type virus coat, such as described in U.S. Patent 6,465,253 and International Patent Application Publication WO 97/20051.

The ability of the simian adenoviral vector to recognize a potential host cell can be modulated without genetic manipulation of the coat protein, i.e., through use of a bi-specific molecule. For instance, complexing an adenovirus with a bispecific molecule comprising a penton base-binding domain and a domain that selectively binds a particular cell surface binding site enables the targeting of the simian adenoviral vector to a particular cell type. Likewise, an antigen can be conjugated to the surface of the adenoviral particle through non-genetic means.

A non-native amino acid sequence can be conjugated to any of the adenoviral coat proteins to form a chimeric adenoviral coat protein. Therefore, for example, a non-native amino acid sequence can be conjugated to, inserted into, or attached to a fiber protein, a penton base protein, a hexon protein, protein IX, VI, or IIIa, etc. Methods for employing such proteins are well known in the art (see, e.g., U.S. Patents 5,543,328; 5,559,099; 5,712,136; 5,731,190; 5,756,086; 5,770,442; 5,846,782; 5,962,311; 5,965,541; 5,846,782; 6,057,155; 6,127,525; 6,153,435; 6,329,190; 6,455,314; 6,465,253; 6,576,456; 6,649,407; 6,740,525; and 6,951,755, and International Patent Application Publications WO 96/07734, WO 96/26281, WO 97/20051, WO 98/07877, WO 98/07865, WO 98/40509, WO 98/54346, WO 00/15823, WO 01/58940, and WO 01/92549). The chimeric adenoviral coat protein can be generated using standard recombinant DNA techniques known in the art. Preferably, the nucleic acid sequence encoding the chimeric adenoviral coat protein is located within the adenoviral genome and is operably linked to a promoter that regulates expression of the coat protein in a wild-type adenovirus. Alternatively, the nucleic acid sequence encoding the chimeric adenoviral coat protein is located within the adenoviral genome and is

part of an expression cassette which comprises genetic elements required for efficient expression of the chimeric coat protein.

The coat protein portion of the chimeric adenovirus coat protein can be a full-length adenoviral coat protein to which the non-native amino acid sequence is appended, or it can be truncated, e.g., internally or at the C- and/or N- terminus. However modified (including the presence of the non-native amino acid), the chimeric coat protein preferably is able to incorporate into an adenoviral capsid. Where the non-native amino acid sequence is attached to the fiber protein, preferably it does not disturb the interaction between viral proteins or fiber monomers. Thus, the non-native amino acid sequence preferably is not itself an oligomerization domain, as such can adversely interact with the trimerization domain of the adenovirus fiber. Preferably the non-native amino acid sequence is added to the virion protein, and is incorporated in such a manner as to be readily exposed to a substrate, cell surface-receptor, or immune cell (e.g., at the N- or C-terminus of the adenoviral protein, attached to a residue facing a substrate, positioned on a peptide spacer, etc.) to maximally expose the non-native amino acid sequence. Ideally, the non-native amino acid sequence is incorporated into an adenoviral fiber protein at the C-terminus of the fiber protein (and attached via a spacer) or incorporated into an exposed loop (e.g., the HI loop) of the fiber to create a chimeric coat protein. Where the non-native amino acid sequence is attached to or replaces a portion of the penton base, preferably it is within the hypervariable regions to ensure that it contacts the substrate, cell surface receptor, or immune cell. Where the non-native amino acid sequence is attached to the hexon, preferably it is within a hypervariable region (Crawford-Miksza et al., *J. Virol.*, *70*(3): 1836-44 (1996)). Where the non-native amino acid is attached to or replaces a portion of pIX, preferably it is within the C-terminus of pIX. Use of a spacer sequence to extend the non-native amino acid sequence away from the surface of the adenoviral particle can be advantageous in that the non-native amino acid sequence can be more available for binding to a receptor, and any steric interactions between the non-native amino acid sequence and the adenoviral fiber monomers can be reduced.

In other embodiments (e.g., to facilitate purification or propagation within a specific engineered cell type), a non-native amino acid (e.g., ligand) can bind a compound other than a cell-surface protein. Thus, the ligand can bind blood- and/or lymph-borne proteins (e.g., albumin), synthetic peptide sequences such as polyamino acids (e.g., polylysine, polyhistidine, etc.), artificial peptide sequences (e.g., FLAG), and RGD peptide fragments (Pasqualini et al., *J. Cell. Biol.*, *130*: 1189 (1995)). A ligand can even bind non-peptide substrates, such as plastic (e.g., Adey et al., *Gene*, *156*: 27 (1995)), biotin (Saggio et al., *Biochem. J.*, *293*: 613 (1993)), a DNA sequence (Cheng et al., *Gene*, *171*: 1 (1996), and Krook et al., *Biochem. Biophys., Res. Commun.*, *204*: 849 (1994)), streptavidin (Geibel et al., *Biochemistry*, *34*: 15430 (1995), and Katz, *Biochemistry*, *34*: 15421 (1995)), nitrostreptavidin (Balass et al., *Anal. Biochem.*, *243*: 264 (1996)), heparin (Wickham et al., *Nature Biotechnol.*, *14*: 1570-73 (1996)), and other substrates.

Disruption of native binding of adenoviral coat proteins to a cell surface receptor can also render it less able to interact with the innate or acquired host immune system. Adenoviral vector administration induces inflammation and activates both innate and acquired immune mechanisms. Adenoviral vectors activate antigen-specific (e.g., T-cell dependent) immune responses, which limit the duration of transgene expression following an initial administration of the vector. In addition, exposure to adenoviral vectors stimulates production of neutralizing antibodies by B cells, which can preclude gene expression from subsequent doses of adenoviral vector (Wilson & Kay, *Nat. Med.*, *3*(9): 887-889 (1995)). Indeed, the effectiveness of repeated administration of the vector can be severely limited by host immunity. In addition to stimulation of humoral immunity, cell-mediated immune functions are responsible for clearance of the virus from the body. Rapid clearance of the virus is attributed to innate immune mechanisms (see, e.g., Worgall et al., *Human Gene Therapy*, *8*: 37-44 (1997)), and likely involves Kupffer cells found within the liver. Thus, by ablating native binding of an adenovirus fiber protein and penton base protein, immune system recognition of an adenoviral vector is diminished, thereby increasing vector tolerance by the host.

Modifications to adenovirus coat proteins are described in, for example, U.S. Patents 5,543,328; 5,559,099; 5,712,136; 5,731,190; 5,756,086; 5,770,442; 5,846,782; 5,871,727; 5,885,808; 5,922,315; 5,962,311; 5,965,541; 6,057,155; 6,127,525; 6,153,435; 6,329,190; 6,455,314; 6,465,253; 6,576,456; 6,649,407; 6,740,525; and 6,951,755; and International Patent Applications WO 96/07734, WO 96/26281, WO 97/20051, WO 98/07865, WO 98/07877, WO 98/40509, WO 98/54346, WO 00/15823, WO 01/58940, and WO 01/92549.

The following examples further illustrate the invention but, of course, should not be construed as in any way limiting its scope.

EXAMPLE 1

This example describes the construction of an E1-deleted SAV7-F vector.

Construction of SAV7-F

An adenovirus vector, SAV7d1t.RSVF0 (SAV7-F) [SEQ ID NO: 9], was constructed that expresses the Respiratory Syncytial Virus (RSV) F_0 glycoprotein (F) (FIG. 1). The vector was derived from the monkey adenovirus simian adenovirus 7 (SV7). SV7 was grown in a 293-ORF6 cell line, and viral DNA was purified with standard DNA extraction techniques. The SV7 genome was rescued into a plasmid backbone by homologous recombination between SV7 DNA and a small plasmid containing SV7 terminal sequences (approximately 300 base pairs, hereinafter bp) (FIG. 2, steps 1 – 3). The SAV7 genome was modified by deleting the essential E1 region for virus replication to render it replication-deficient (FIG. 2, steps 4 – 5). The E1 region deletion junction includes bp 400 to 2199 nucleotides of the wild type virus. In place of E1 sequences the Immediate Early high expression CMV enhancer / promoter with tetracycline operator sites (CMVtetO) RSVF0 expression cassette was introduced. The expression cassette, located at the E1 region deletion junction, is right-to-left with respect to the viral genome (FIG. 2, steps 6 – 7).

E1-deleted SAV7 vector is replication-deficient:

The host species for SV7 is the rhesus macaque; the virus was originally discovered in rhesus macaques and monkey cell lines used for isolation and propagation of the virus (*Am J Hyg.* 1958 Jul;68(1):31-44). Two monkey cell

lines, LLC-MK2 (rhesus macaque) and BSC-1 (African Green Monkey) were infected with either SV7 or E1-deleted SAV7 without a transgene at 100 pu/cell and the number of viral progeny determined at 72 and 96 hours post-infection. SV7 infection resulted in the production of infectious viral progeny, ranging from 2.5 x 10^8 to 4.9 x 10^8 infectious units per culture well. In contrast, no infectious viral progeny were detected from infection of either cell type with the E1-deleted SAV7 (Table 1). Therefore, E1-deleted SAV7 vectors are replication-deficient. E1-deleted SAV7 vectors also have a low risk for generation of replication competent adenovirus (RCA) during production.

Table 1. Generation of infectious viral progeny (ffu*).

	LLC-MK2		BSC-1	
Virus	72 hpi**	96 hpi	72 hpi	96 hpi
SAV7, no transgene	0	0	0	0
SV7, wild type	2.7E+07	4.9E+08	2.5E+08	3.7E+08

* - focus-forming units
** - hours post-infection

Overview of SAV7-F vector construction

The plasmid pACsAd7E1(d1t.RSVF0) was constructed consisting of the entire adenovirus vector genome with CMVtetO.RSVF0 expression cassette in the E1 region. Isolation of a single genetic clone of the final pACsAd7E1(d1t.RSVF0) plasmid genome was achieved by two sequential colony-growth steps in bacteria. This viral vector genome encoded by the plasmid was converted to a live viral vector upon introduction into mammalian cells that complement for adenovirus vector growth. Subsequent expansion via serial passaging was performed to generate adenovirus vector stocks. The key steps to construct the RSVF0 expressing adenovector plasmid are summarized below:

1) Construction of plasmids: Insertion of the RSV F_0 transgene in the E1 region of the SAV7 plasmid by homologous recombination.

- Linearization and transformation of pACsAd7E1(d1t.ef) and pAd3511CMVtetOf.RSVF0 in BJDE3 *E. coli* Rec+ strain to generate pACsAd7E1(d1t.RSVF.0) (FIG. 2, steps 6 – 7).

2) Clonal isolation and purification of pACsAd7E1(d1t.RSVF.0) : Two sequential bacterial transformations of Rec- DH10B with endotoxin-free plasmid DNA.

3) Rescue of the SAV7d1t.RSVF0 adenovector: (a) the pACsAd7E1(d1t.RSVF.0) plasmid was linearized with the restriction endonuclease Pac I to liberate the adenovector genome; and (b) adherent 293-ORF6 cells were transfected with the linearized plasmid, resulting in conversion of the DNA into the viral vector SAV7-F.

4) Expansion of the SAV7-F adenovector by serial passaging was conducted to generate high titer cell-virus lysate, the vector was purified from the lysates by density-equilibrium centrifugation in continuous cesium chloride gradients, and the purified vector was dialyzed into Final Formulation Buffer (FFB: 10 mM Tris pH 7.8, 75 mM NaCl, 5% Trehalose, 25 ppm Polysorbate 80, 1 mM $MgCl_2$).

pACsAd7E1(d1t.RSVF0) plasmid synthesis steps

The adenovirus vector plasmid pACsAd7E1(d1t.RSVF.0) was constructed by standard DNA subcloning methods and homologous recombination in E. coli. To make pACsAd7E1(d1t.RSVF.0) the following cloning and recombination steps were involved.

The rescue plasmid p.sAd7Rescue was generated by subcloning the PCR products of SV7 wild type sequence. ~300 bp of SV7 left end of the genome including the left ITR and ~300 bp of the right end including right ITR of SV7 wt sequence was PCR amplified and subcloned into low copy plasmid. psAd7Rescue plasmid was used as a recipient to rescue the SV7 wild type genome.

To make pACsAd7, the psAD7Rescue plasmid was linearized with BglII. SV7 wild type DNA and the linearized psAd7Rescue plasmid were recombined by co-transformation of Rec+ *E. coli.*

pACsAd7, linearized, and pKSsAd7E1d400-2199t.ef.sv were recombined by cotransformation of *E. coli.* The resultant recombinant plasmid, pACsAd7E1(d1t.ef), consisted of the SAV7 genome with the E1 region deleted and the CMVtetO EGFP expression cassette inside the E1 deletion.

pACsAd7E1(d1t.ef) was linearized with SwaI restriction enzyme and dephosphorylated. The RSV F0 expression cassette CMVtetOf.RSVF.0 was

introduced by homologous recombination by cotransformation of *E. coli* with the linearized and dephosphorylated plasmid and the plasmid pAd3511CMVtetOf.RSVF.0. The resultant plasmid pACsAd7E1(d1t.RSVF.0) contained the full SAV7-F genome.

Rescue of SAV7-F vector

To construct the adenoviral vector SAV7-F, the plasmid pACsAd7E1(d1t.RSVF.0) was digested with restriction endonuclease Pac I and transfected into 293-ORF6 cells with Polyfect reagent (Qiagen). The adenovector-cell lysate from the transfected cells was serially passaged until full cytopathic effect was observed. The identity and integrity of SAV7-F was confirmed by PCR analysis.

SAV7-F purified stocks

Adenovector SAV7-F was produced in 293-ORF6 cells using cell-virus lysate from the transfection-serial passaging to construct SAV7-F. 293-ORF6 cells, 1.17 x 10^9 cells total, were infected at a multiplicity of infection of 0.4 ffu/cell. 100 mM of $ZnCl_2$ was added to the inoculum to give a final concentration of 35 µM of $ZnCl_2$. Twenty-four hours post- infection the infected cells were fed with an equal volume of medium (containing a final concentration of 4 mM of L-Glutamine) and split into 4 x 500 mL aliquots. The infected cells were harvested 72 hours post-infection, collected into sterile 500 mL centrifuge tubes, concentrated by centrifugation, and resuspended in Benzonase Buffer (25 mM Tris, 10 mM NaCl, 5 mM $MgCl_2$, 0.0025% Tween 80 buffer, pH 8.0). Purification was initiated by freeze-thaw lysis of the resuspended cells three times. Benzonase® was added at 1 unit/2 x 10^4 cells and 10% stock Triton X-100 was added to give a final concentration of 0.1% Triton X. The lysate was incubated at 37°C for up to 3.0 hours. The vector was purified from the lysate by two cesium chloride isopycnic gradients and one cesium chloride discontinuous gradient (22,500 rpm at 2-8°C). The vector bands were pulled from the gradient via needle and syringe, pooled, and dialyzed against 4 changes of ~1.5 L of FFB. The dialyzed vector was filtered through a 0.2µm filter (Pall Cat# 4192) and stored at ≤

-60° C. The identity and integrity of SAV7-F were confirmed by PCR analysis and F expression confirmed by western blot assay.

EXAMPLE 2

This example describes the construction of Ad5-F vectors.

Construction of Ad5-F

Two Ad5-F vectors, Adt.RSVF0.11D (SEQ ID NO:7) and Ad4344RSVF.0 (SEQ ID NO:8), were constructed that express the RSV F_0 glycoprotein (F) (FIG. 3). The adenovectors consist of the *human adenovirus C*, serotype 5 (Ad5) genome with deletions in the E1, E3, and E4 regions or in the E1, E2B, E3, and E4 regions, and are replication-deficient due to the deletion of the essential functions provided by E1 and E4. These multiply deleted vectors are called "GV11" and are efficiently propagated in an E1 and E4 complementing cell, 293-ORF6. In place of E1 or E1 and E2B sequences the CMVtetO RSVF0 expression cassette was introduced (FIG. 3). The expression cassette, located at the E1 region deletion junction or E1-E2B deletion junctions, is right-to-left with respect to the viral genome. In place of the E4 sequences are either a spacer element (TIS1), or the genes encoding pIX and the E2B product, IVa2.

Region deletions: Adt.RSVF0.11D has an E1 region deletion of bases 356 through 3510. A unique feature of Ad4344RSVF.0 is the extension of the E1 region deletion through base 4340. This deletion provides an Ad5 vector that does not have significant homology to Ad5 DNA sequences in the complementing cell line on the right side of the E1 region because the portion of Ad5 DNA in 293 cells and their derivatives only extends to base 4343. This extended deletion removes the pIX open reading frame and a portion of the RNA processing elements for the IVa2 gene, i.e., E2B. Therefore, the genes encoding the pIX promoter, pIX open reading frame, IVa2 genomic coding sequence, and the genomic transcription termination and polyadenylation sites for both genes were placed into the E4 region. The expression of IVa2 is controlled by the Rous Sarcoma Virus Long Terminal Repeat (LTR) promoter. Additionally, a transcription terminator and polyadenylation site, muM, was inserted adjacent to Ad5 base 4341, to provide proper RNA processing of precursor RNAs, such as E2B, E2A, and E4 genes,

transcribed from right-to-left off the adenovirus genome during viral vector propagation. The E3 region is not essential for the growth of Ad5 in tissue culture and the deletion spans the bases 28593 through 30471. The E4 region deletion (inclusive of 32827 to 35563) removes all the E4 open reading frames, therefore eliminating essential elements for Ad5 replication. In addition to these major deletions, there is a two base deletion, relative to the Ad5 wild-type sequence, in the VA RNA I region (10594 and 10595). This two base deletion, derived from the Ad5 mutant dl324, interrupts a minor alternative transcription start site for VA RNA I (Ginsberg, H.S., The Adenoviruses. Plenum Press, New York, 1984).

These multiple-deletion adenovector backbones have been termed GV11. The GV11 adenovector reduces the risk of replication competent adenovirus (RCA) generation during production. Since two regions essential for adenovirus replication have been deleted and those regions are non-contiguous, the generation of an RCA would require two independent recombination events in a single adenovector genome. The rate of RCA generation is expected to be extremely rare, as two events would be required and any recombination event in E4 would be of the infrequent non-homologous type. The RCA rate in a first-generation Ad5 system, which measures one homologous recombination event, was recently reported to be 5.6×10^{-10} to 6.7×10^{-11} RCA per infectious unit (Duigou, G.J. & Young, C.S. 2005. *J Virol* 79, 5437-5444). Thus, the frequency of two events would be $<10^{-20}$ RCA per particle unit. In addition, the extension of the deletion of E1 through part of E2B (base 4340) removes a significant portion of the homology between the Ad5 vector and the portion of Ad5 DNA in 293-based cell lines, as described above. This will further decrease the efficiency of recombination between the Ad5 vector and the Ad5 DNA in the cell.

Construction of Adt.RSVF0.11D PI

The plasmid pAdE1(t.RSVF.0)E3(10)E4(TIS1) was constructed that encodes the entire Adt.RSVF0.11D adenovirus vector genome with CMVtetO.RSVF0 expression cassette in E1 region (FIG. 4). Isolation of a single genetic clone of the final vector genome was achieved by two sequential colony-growth steps in bacteria. This viral vector genome encoded by the plasmid was

converted to a live viral vector upon introduction into mammalian cells that complement for adenovirus vector growth. Subsequent expansion via serial passaging was performed to generate adenovirus vector stocks. The key steps for constructing the RSV F0 expressing adenovector plasmid (as depicted in FIG. 4) are summarized below:

1) Construction of plasmids: (a) insertion of the RSV F0 gene into an adenovector expression shuttle plasmid by standard restriction enzyme subcloning; and (b) insertion of CMVTetO.RSVF0 gene into a full length adenovector genome plasmid by homologous recombination in *E.coli* to generate the RSV F0-expressing adenovector genome in plasmid form (pAdE1(t.RSVF.0)E3(10)E4(TIS1)).

2) Limiting dilution bacterial transformation with pAdE1(t.RSVF.0)E3(10)E4(TIS1).

3) Construction of the Adt.RSVF0.11D adenovector: (a) plasmid pAdE1(t.RSVF.0)E3(10)E4(TIS1) is linearized with the restriction endonuclease Pac I to liberate the adenovector genome; and (b) adherent 293-ORF6 cells are transfected with the linearized plasmid, resulting in conversion of the DNA into the viral vector Adt.RSVF0.11D.

4) Expansion of the Adt.RSVF0.11D adenovector by serial passaging to generate high titer cell-virus lysate, the vector purified from the lysates by density-equilibrium centrifugation in cesium chloride gradients, and the purified vector dialyzed into Final Formulation Buffer (FFB: 10 mM Tris pH 7.8, 75 mM NaCl, 5% Trehalose, 25 ppm Polysorbate 80, 1 mM $MgCl_2$).

Rescue of Adt.RSVF0.11D

To construct the adenoviral vector Adt.RSVF0.11D, the plasmid pAdE1(t.RSVF.0).E3(10)E4(TIS1) was digested with the restriction endonuclease Pac I and transfected into 293-ORF6 cells with the Polyfect reagent (Qiagen). The adenovector-cell lysate from the transfected cells was serially passaged until full cytopathic effect was observed. The identity and integrity of Adt.RSVF0.11D in the cell-virus lysates were confirmed by PCR analysis and F expression was confirmed by western blot assay.

Adt.RSVF0.11D purified stocks

Adenovector Adt.RSVF0.11D was produced in 293-ORF6 cells using cell-virus lysate from the transfection-serial passaging to construct Adt.RSVF0.11D. 293-ORF6 cells, 7.5 x 10^8 cells total, were infected with 20 FFU per cell. 100 mM of $ZnCl_2$ was added to the inoculum to give a final concentration of 35 μM of $ZnCl_2$. The infected cells were harvested 72 hours post infection, and collected into sterile conical tubes, concentrated by centrifugation, resuspended in 25 mM Tris, 10 mM NaCl, 5 mM $MgCl_2$, 0.0025% Tween 80 buffer, pH 8.0. Purification was initiated by freeze thawing the resuspended cells three times. Benzonase® was added at 1 unit/2e4 cells and the lysate was incubated at 37°C for 2.5 hours. Cesium chloride isopycnic gradient centrifugation was performed overnight 3 times at 22,500 rpm at 2-8°C. The vector bands were pulled from the gradient via needle and syringe, pooled, and dialyzed against 4 changes of 1.4 L of Final Formulation Buffer (FFB: 10 mM Tris pH 7.8, 75 mM NaCl, 5% Trehalose, 25 ppm Polysorbate 80, 1 mM $MgCl_2$:Lonza catalog number 04-1056Q). The dialyzed vector was filtered using a 0.2 um filter (Pall Cat# 4192) and stored at ≤ -60° C. The identity and integrity of Adt.RSVF0.11D were confirmed by PCR analysis and F expression confirmed by western blot assay.

Construction of Ad4344.RSVF.0 vector

The plasmid pAdE1(4344t.RSVF0)E3(10)E4(R.IVa2s.PIX2) was constructed that encoded the entire Ad4344.RSVF.0 adenovirus vector genome with CMVtetO.RSVF0 expression cassette in the E1 region (FIG. 5). Isolation of a single genetic clone of the pAdE1(4344t.RSVF0)E3(10)E4(R.IVa2s.PIX2) plasmid was achieved by two sequential colony-growth steps in bacteria. This viral vector genome encoded by the plasmid was converted to a live viral vector upon introduction into mammalian cells that complement for adenovirus vector growth. Subsequent expansion via serial passaging was performed to generate adenovirus vector stocks. The key steps for constructing the RSV F0 expressing adenovector plasmid (as depicted in FIG. 5) are summarized below:

1) Construction of plasmids: (a) insertion of the RSVF0 gene into an adenovector expression shuttle plasmid by standard restriction enzyme subcloning; and (b)

insertion of CMVTetO.RSVF0 gene into a full length adenovector genome plasmid by homologous recombination in *E. coli* to generate the RSV F0-expressing adenovector genome in plasmid form; (c) insertion of R.IVa2 and pIX genes into the E4 region of a full length adenovector genome plasmid by homologous recombination in *E. coli* to generate pAdE1(4344t.RSVF0)E3(10)E4(R.IVa2s.PIX2) adenovector genome in plasmid form.

2) Rescue of the Ad4344.RSVF.0 adenovector: (a) the pAdE1(4344t.RSVFO)E3(10)E4(R.IVa2s.PIX2) plasmid was linearized with the restriction endonuclease Pac I to liberate the adenovector genome; and (b) adherent 293-ORF6 cells are transfected with the linearized plasmid, resulting in conversion of the DNA into the viral vector Ad4344.RSVF.0.

3) Expansion of the Ad4344.RSVF.0 adenovector by serial passaging to generate high titer cell-virus lysate, the vector purified from the lysates by density-equilibrium centrifugation in cesium chloride gradients, and the purified vector dialyzed into Final Formulation Buffer (FFB: 10 mM Tris pH 7.8, 75 mM NaCl, 5% Trehalose, 25 ppm Polysorbate 80, 1 mM $MgCl_2$).

Rescue of Ad4344.RSVF.0 vector

To construct the adenoviral vector Ad4344.RSVF.0, the plasmid pAdE1(4344t.RSVFO)E3(10)E4(R.IVa2s.PIX2) was digested with the restriction endonuclease Pac I and transfected into 293-ORF6 cells with Polyfect reagent (Qiagen). The adenovector-cell lysate from the transfected cells was serially passaged until full cytopathic effect was observed. The identity and integrity of SAV7-F was confirmed by PCR analysis.

Ad4344.RSVF.0 purified stocks

Adenovector Ad4344.RSVF.0 was produced in 293-ORF6 cells using cell-virus lysate from the transfection-serial passaging of Ad4344.RSVF.0. 293-ORF6 cells, 4.75 $x10^8$ cells total, were infected at a multiplicity of infection of 1.0 FFU per cell. 100 mM of $ZnCl_2$ was added to the inoculum to give a final concentration of 35 μM of $ZnCl_2$. The infected cells were harvested 72 hours post infection, and

collected into sterile conical tubes, concentrated by centrifugation, resuspended in 25 mM Tris, 10 mM NaCl, 5 mM $MgCl_2$, 0.0025% Tween 80 buffer, pH 8.0. Purification was initiated by freeze thawing the resuspended cells three times. Benzonase® was added at 1 unit/2e4 cells and the lysate was incubated at 37°C for 2.5 hours. Cesium chloride isopycnic gradient centrifugation was performed overnight 3 times at 22,500 rpm at 2-8°C. The vector bands were pulled from the gradient via needle and syringe, pooled, and dialyzed against 4 changes of 1.4 L of FFB. The dialyzed vector was filtered through a 0.2 µm filter (Pall Cat# 4192) and stored at ≤ -60° C. The identity and integrity of Ad4344.RSVF.0 were confirmed by PCR analysis and F expression was confirmed by western blot assay.

EXAMPLE 3

This example describes modes for expressing the RSV F gene sequence.

Control of F0 gene expression by a constitutive expression cassette

The Immediate Early high expression CMV enhancer / promoter with tetracycline operator sites (CMVtetO) controls the initiation of transcription. Within this sequence is the viral enhancer, CAAT box, TATA box, two copies of the 20 nucleotide tetracycline operator sequence (tetO) from transposon Tn10, and the CMV transcription start site. The tetO sites are inactive in mammalian cells since tetracycline-based gene expression regulation is specific for a prokaryotic system (reviewed in Blau HM, Rossi FM. Tet B or not tet B: advances in tetracycline-inducible gene expression. *Proc Natl Acad Sci U S A.* 1999 Feb 2;96(3):797-9.). The tetO sites allow for repressed transgene expression when the viral vector is propagated in a cell line with the tetracycline repressor. The CMV promoter expresses in a wide variety of mammalian cells immediately upon introduction into cells. The RSV F0 transgene was introduced between the CMVtetO promoter and SV40 early polyA. To further optimize the expression of the transgene from the CMVtetO promoter an artificial intron was created by placing a splice donor and a splice acceptor sequence upstream of the RSV F0 initiation codon.

The RSV F_0 gene sequence and expression in vitro

An amino acid sequence for the F protein was chosen based on a consensus sequence of RSV, strain A, F glycoprotein sequences in GenBank (NCBI). The DNA sequence to encode the consensus F sequence was chemically synthesized (GeneArt) and tested for expression. The first consensus amino acid sequence generated (SEQ ID NO: 3) did not express an F protein that was detectable by western blot analysis (data not shown). A second F amino acid sequence was generated (SEQ ID NO: 1) that (1) removed a misfolding of the protein by proline – proline at 101 – 102; wherein pro102 was changed to alanine and (2) used alternative amino acid residues with equal appearance in the consensus: isoleucine 379 to valine and methionine 447 to valine. The expression of this F protein was confirmed by western blot analysis (FIGS. 6 and 7). The F amino acid sequence (SEQ ID NO: 1) is encoded by the DNA sequence in SEQ ID NO: 2.

RSV F_0 Protein Expression Assay

The F glycoprotein is generated as a precursor, F_0 of ~70 kDa that is processed to F_1 (~48 kDa) and F_2 (~28 kDa). To determine expression of the F protein, 293 cells were infected at 500 pu / cell with Ad5-F or SAV7-F. Infected cells were harvested 24 hours post-infection (hpi), as follows. The culture medium was removed, cell monolayers were washed with DPBS and lysed using 500 ul of HEPES based-buffer, then transferred to eppindorph tubes and pelleted at 1000g for 10 minutes. Supernatants containing protein products were collected and stored at -80°C. Protein products were then separated by SDS-PAGE using a Tris-acetate gel containing 7% acrylimide for 60 minutes at 150 V. The X-cell Sure Lock system (Invitrogen) was used to transfer proteins to a PVDF membrane (pore size 0.2um, 60 minutes at 30V). The membrane was blocked overnight at 4°C in 5% nonfat milk/ TBST. The membrane was probed with the primary antibody goat α-RSV pAb7133P (Maine Biotechnology) and the secondary antibody rabbit α-goat HRP Ap106P (Millipore, Invitrogen). All antibodies were diluted to 1:250 and incubated for 60 min. at room temperature. ECL Plus detection reagents were applied and the membrane was exposed using the Alpha Innotech with FluorChem

software. The F_1 protein was clearly present (FIGS. 6 and 7), thus showing that the adenovirus vectors expressed the F protein antigen.

EXAMPLE 4

This example describes a 293-ORF6 cell line that reduces transgene expression during vector construction and growth, and thus is able to reduce RSV F protein cytotoxicity.

The F protein has been demonstrated to kill tumor cells when expressed intra-tumorally (Hoffmann D, Grunwald T, Bayer W, Wildner O. Immune-mediated anti-neoplastic effect of intratumoral RSV envelope glycoprotein expression is related to apoptotic death of tumor cells but not to the size of syncytia. World J Gastroenterol. 2008 Mar 28;14(12):1842-50. PubMed PMID: 18350621) and has been shown to induce apoptosis by a p53-dependent pathway (Eckardt-Michel J, Lorek M, Baxmann D, Grunwald T, Keil GM, Zimmer G. The fusion protein of respiratory syncytial virus triggers p53-dependent apoptosis. J Virol. 2008 Apr;82(7):3236-49. Epub 2008 Jan 23. PubMed PMID: 18216092). The F protein can also induce cell-cell fusion (Hoffmann D, Grunwald T, Bayer W, Wildner O. Immune-mediated anti-neoplastic effect of intratumoral RSV envelope glycoprotein expression is related to apoptotic death of tumor cells but not to the size of syncytia. World J Gastroenterol. 2008 Mar 28;14(12):1842-50. PubMed PMID: 18350621).

There can be potential negative effects of high-level expression of the F protein as a transgene product from the adenovirus vector during growth. Therefore, the F protein open reading frame was engineered into an expression cassette with elements allowing repression of F protein expression. The adenovirus vector with such an expression cassette can then be propagated on a cell line that constitutively expresses a repressor protein, the result being reduced expression of the transgene. A genetically stable 293-ORF6-cell line that constitutively expresses tetracycline repressor (TetR) has been constructed and named M2A. The M2A cell line is very efficient at reducing transgene expression during vector construction and growth. TetR binds to a defined DNA sequence, the Tet operator (TetO). When the TetO sequences are placed between the TATAA box of the

CMV promoter and site of transcription initiation they have no effect on the activity of the promoter in the absence of TetR. In the presence of TetR, expression directed by the CMV promoter with TetO (CMVTetO) is reduced. This provides a system that inhibits transgene expression during productive virus replication and provides high CMV promoter activity during delivery of the transgene to the target cells (conditions in the absence of TetR).

As a measure of the potential cytotoxicity of high-level expression of the F antigen during adenovector growth, the complementing cell lines 293-ORF6 and M2A were infected with an adenovirus vector expressing green fluorescent protein (Adgfp) or an RSV-F adenovirus vector (AdF), with a multiplicity of infection of 10 FFU/cell. At 24, 48 and 72 hours post-infection the viable cells were counted by trypan blue exclusion using a Beckman Coulter ViCell XR automated cell counting device. There was no difference in cell viability at the three time points within vector groups; therefore the data from the three time points were combined. The viability of 293-ORF6 cells was lower upon infection with AdF than when infected with Adgfp, demonstrating a cytoxicity of the RSV F protein (FIG. 61). In contrast, there was no difference in M2A viability. Therefore, infection of packaging cells by an adenovirus vector expressing high levels of the RSV F protein causes F protein-specific reduction in cell viability and reducing expression by the transcription control system described here prevents the loss of cell viability.

EXAMPLE 5

This example describes a protocol for determining the active titer of RSV using a plaque based assay. This assay may be used to determine the titer of RSV stocks and of RSV in animal tissues. (See also Kim et al., *Vaccine* 28(22): 3801-3808 (2010), also describing protocols for measuring RSV titer, including in the lungs and bronchoalveolar lavage (BAL) fluid)

1) Seed (2) 12-well plates with Hep2 cells 2.5 x 10^5 to 3.0 x 10^5 cells per well 36 hours prior to infection and incubate at 37°C and 5% CO_2.

2) Grow Hep-2 cells to 80% confluence in 12-well tissue culture plates.

3) Quick-thaw (15-25s.) a vial of RSV (or animal tissue sample, if frozen) in a 37°C water bath and put it on ice.

4) Make serial 10-fold dilutions from 1:1 to 1:100,000,000 in EMEM+10%FBS.

5) Aspirate media from Hep-2 monolayer, and add 100ul of undiluted virus and each dilution of virus in triplicate (i.e. one column of 3 wells per dilution of virus).

6) Infect at RT for 1 hour with constant rocking. (Do not place the plates in moving air.)

7) Aspirate the inoculums.

8) Overlay cells with 1 ml EMEM+10%FCS containing 0.75% methylcellulose. (Autoclave 3.75 g methylcellulose in 500 ml bottle with a stir bar. Add 500ml EMEM+10%FBS and stir at RT until methylcellulose is in solution, about 2-3 days. Add antibiotic/antimycotic solution to 1% final concentration).

9) Incubate plates 4-5 days at 37°C until plaques are observed (FIG. 8).

10) Do not aspirate.

11) Fix and stain each monolayer with hematoxylin (20min.) wash, then stain with eosin (5min.). (or use Geimsa stain as indicated below):

a. Add 2 ml 10% formalin to wells containing overlay medium for 1 hour.

b. Wash in tap water.

c. Add enough Giemsa stain solution to cover each well (~300 ul.).

d. Let stand for 45 min.

e. Wash in tap water and air-dry overnight.

f. Count plaques under a dissecting microscope by touching the plate with a pen at the plaque location.

12) Calculate the titer.

a. Use the count with a minimum of 10 plaques.

b. Average the 3 repetitions.

c. Divide the plaque count by the quotient of the dilution and the volume used for infection.

For example, for a count of 10 plaques in the -6 dilution with a 100ul inoculum volume, the calculation would be.

$$10 / (.000001*.1) = 1 \times 10^8 \text{ pfu/ml}$$

In one plaque assay, 19, 16, and 11 plaques were counted in the three repetitions of the RSV A2 stock diluted to 1×10^8 pfu/ml. This equates to a titer of 1.5×10^8 pfu/mL.

EXAMPLE 6

This example describes that *in vivo* protection against RSV infection can be provided by adenoviral vectors which express the RSV F protein.

Vaccine vectors based on multiple serotypes of adenovirus including a codon-modified consensus sequence of RSV F (SEQ ID NO: 2) were generated. Multiple F constructs were evaluated to determine if the F_1 protein alone, cytoplasmic tail truncations, or deletion of the fusion peptide would improve immunogenicity. None of these constructs were as stable or immunogenic as the original full length consensus F construct, so it was selected for further testing.

The adenovirus vectors based on serotypes human Ad5, human Ad28, simian adenovirus 7 (SV7), SV11, and SV38 were engineered essentially as described in the Examples above (see also Kahl et al., *Vaccine*, *28*: 5691-5702 (2010); and Lemiale et al., *Vaccine*, *25*: 2074-2084 (2007)) to constitutively express the RSV F_0 protein from the CMV-driven expression cassette located in the E1 region. Expression of the RSV F_0 protein was confirmed by Western blot analysis. HEK-293 cells were infected with the F_0 adenovectors and protein extracts were generated from the cells at 24 hours post infection (hpi). The full-length fusion protein, F_0 (70kDa) was expressed, however, the protein is rapidly processed into two parts via furin cleavage into F_1 (~48kDa) and F_2 (~28kDa) subunits. The protein extracts were subjected to SDS-PAGE, transferred to a blotting membrane, and probed with primary polyclonal antibody Goat anti-RSV pAb7133P (Maine Biotech, Portland, ME), and secondary antibody rabbit anti-goat HRP Ap106P (Invitrogen, Carlsbad, CA). The F_1 form was detected as a band at ~50kDa, and the F_2 form may have been detected as a band at ~25 kDa, while

unprocessed F_0 was not detected. The antibodies did not react with cellular protein from 293 cells or with Ad5 viral proteins.

Mice (CB6F1/J H-2d/b) were immunized at week 0 and challenged with RSV according to the protocol illustrated in FIG. 9. A single administration of Ad5.F.0 induced RSV neutralizing antibody. However, the single administration did not fully prevent weight loss, which is a measure of illness. The vaccination regimen reduced replication of RSV in the lung to below detectable levels and reduced RSV replication in the nose. Cellular immune responses were measured to determine whether administration of the vaccine vectors primed animals for an immunopathological response to RSV challenge and/or antigen-specific T-cell responses. Animals immunized with Ad5.F.0 did not have eosinophilia in their lungs following RSV challenge. Indeed, there were essentially no measurable eosinophils in the bronchoalveolar lavage (BAL) fluid, as with the mock immunized animals. In contrast, more than 40% of the total cells were eosinophils in BAL samples from animals receiving a formalin-inactivated alum-precipitated vaccine (FI-RSV). Thus, administration of FI-RSV immunopotentiated mice for a pathological response to RSV. The helper cell/cytotoxic cell phenotypes of lymphoctyes in the BAL samples were determined. The T-cell responses induced by Ad5.F.0 were biased toward CD8+, in contrast to FI-RSV immunization, which potentiated a CD4+ T-cell response. Additionally, functional assessment of the CD8+ T-cell populations in the spleens of mice immunized by Ad5.F.0 demonstrated that F protein-specific CD8+ T-cell responses were induced by immunization, whereas mock and FI-RSV vaccination did not induce F antigen-specific CD8+ T-cells. Thus, rAd5 delivery of the F_0 protein induces a robust CD8+ T cell response.

Further understanding of the potential for vaccine enhanced disease can come from analysis of lung cytokines. Cytokine levels in lung supernatant at 5 days post-challenge were measured by ELISA (Table 2). The cytokines IL-4, IL-5, and IL-13 are markers for CD4+ T-cells of the T_H2 type, which are associated with RSV vaccine-enhanced disease. The magnitude of these cytokines in the lungs of animals undergoing a primary RSV infection indicates a level that is below that associated with enhanced disease. In contrast, animals immunized with

FI-RSV demonstrate the markedly increased magnitude of these cytokines upon RSV challenge associated with vaccine-enhanced disease. The relative levels of these three cytokines measured in the Ad5-mock (no transgene) and FI-RSV immunized animals were consistent with expectations (FIG. 15). The cytokine levels in the lungs of animals immunized with Ad5-F and challenged with RSV were the same or lower than in the animals immunized with Ad5-mock. Therefore, immunization with Ad5 did not immunopotentiate a TH2 T-cell response, consistent with an absence of vaccine-enhanced disease.

Table 2. ELISA kits from R & D Systems.

Cytokine	ELISA kit Cat#
IL-4	DY404
IL-5	DY405
IL-13	DY413

The recombinant adenovirus vectors were evaluated alone and in prime-boost combinations for induction of RSV neutralizing antibody in CB6F1/J H-2d/b mice according to the protocol illustrated in FIG. 10. All vectors were immunogenic, and the SV7 vector appeared to be particularly potent when used as the boost (FIG. 11). The most potent combinations of vectors tested were Ad28 followed by SV7, and Ad5 followed by SV7 (FIG. 11). Both of these regimens approached the level of neutralizing activity present in 1000 μg/ml of SYNAGIS®, which is more than twenty-times higher than the level needed for protection by SYNAGIS® (Johnson et al., *Journal of Infectious Diseases, 176*: 1215–24 (1997)).

Additional testing of prime-boost regimens, such as those illustrated in FIG. 12, demonstrated the protection provided by the vaccine regimens. At two and four weeks post-boost immunization, the RSV neutralizing activity in serum approached the level of neutralizing activity present in 1000 μg/ml of SYNAGIS® (FIG. 62) . This level of neutralizing activity in vitro was associated with protection against illness, as measured by weight loss (FIG. 63). All prime-boost regimens prevented weight loss post-RSV challenge. In agreement with the serum

neutralizing titers and protection against weight loss, replication of RSV in the lung was reduced to undetectable levels (FIG. 64).

The results of this example demonstrate that RSV F protein delivered by an adenoviral vector based on human serotype 5 is immunogenic, protective, and does not potentiate for vaccine-enhanced immunopathology, and that prime-boost immunization methods utilizing simian and human adenoviral vectors can effectively prevent severe RSV-induced illness in mice.

EXAMPLE 7

This example demonstrates that adenoviral vectors based on human serotype 28 are not fully protective, while a simian adenoviral vector serotype 7 can induce protective immunity against RSV.

Adenovirus vectors of different serotypes expressing the chimeric protein M/M2 were constructed essentially as described in the Examples above. The expression of M/M2 was verified by western blot analysis. HEK-293 cells were infected with the M/M2 adenoviral vectors and protein extracts were generated from the cells at 24 hpi. The formula molecular weight of the M/M2 protein was 51.3 kDa. The protein extracts were subjected to SDS-PAGE, transferred to a blotting membrane, and probed with primary polyclonal antibody Goat anti-RSV pAb7133P (Maine Biotech, Portland, ME), and secondary antibody rabbit anti-goat HRP Ap106P (Invitrogen, Carlsbad, CA). The M/M2 protein was detected as a band at ~52 kDa. The antibodies did not react with cellular protein from HEK-293 cells or with Ad5 viral proteins.

The adenoviral vectors were evaluated for potency using the F gene alone and F + M/M2 in single administration vaccination protocols, as illustrated in FIGS. 13(A) and 14(A), to determine whether induction of additional CD4+ and CD8+ T cell responses would enhance the F-induced protective response. The viral clearance in the lungs and noses of animals immunized with SAV7-F was comparable to Ad5 and reached below the limit of quantitation. (FIGS. 13(B) and 13(C)) In contrast, immunization with rAd28-F did not fully protect the animals. The addition of the M/M2 antigen to the immunization with rAd28-F improved viral clearance in lungs and noses post challenge. (FIGS. 14(B) and 14(C))

Further understanding of the potential for vaccine enhanced disease can come from analysis of lung cytokines. Although an absence of TH2 cytokines was observed in animals immunized with Ad5-F (see Example 6), it was unknown whether SAV7 would also not immunopotentiate animals for enhanced disease. Cytokine levels in lung supernatant at 5 days post-challenge were measured by ELISA (Table 3). The cytokines IL-4, IL-5, and IL-13 are markers for CD4+ T-cells of the T_H2 type, which are associated with RSV vaccine-enhanced disease. The magnitude of these cytokines in the lungs of animals undergoing a primary RSV infection indicates a level that is below that associated with enhanced disease. In contrast, animals immunized with FI-RSV demonstrate the markedly increased magnitude of these cytokines upon RSV challenge associated with vaccine-enhanced disease. The cytokine levels in the lungs of animals immunized with SAV7-F and challenged with RSV were the same or lower than in the animals immunized with Ad5-mock (FIG. 17). Therefore, immunization with SAV7, as with Ad5, did not immunopotentiate a TH2 T-cell response, consistent with an absence of vaccine-enhanced disease.

Table 3. ELISA kits from R & D Systems.

Cytokine	ELISA kit Cat#
IL-4	DY404
IL-5	DY405
IL-13	DY413

The results of this example demonstrate that rAd5 and SAV7 vaccine vectors induce protective immunity against RSV, and that not all rAd vectors induce protective immunity against RSV, as illustrated by the lack of protection following rAd28-RSV immunization. This example also shows that animals vaccinated with SAV7 vaccine vectors are not immunopotentiated for vaccine-enhanced disease.

EXAMPLE 8

This example describes methods for inducing an immune response against RSV in a mammal by a heterologous prime-boost regimen utilizing human and simian adenoviral vectors.

The adenoviral vectors based on human serotype 5 (Ad5), simian serotype 7 (SAd7), and simian serotype 11 (SAd11) expressing the RSV F_0 protein described in Example 5 were administered to CB6F1/J H-2d/b mice in various prime-boost combinations, as illustrated in FIG. 16. Specifically, mice were administered a priming composition containing $1x10^9$ pu of rAd5-F, SAd7-F or SAd11-F at day 0 via i.m. injection. Mice were then boosted at 4 weeks and 8 weeks following administration of the priming composition with an adenoviral vector that was in some cases different from the adenoviral vector included in the priming composition. At 26, 54, and 82 weeks post-injection of the priming composition, RSV neutralizing activity in serum was measured.

The most potent combinations of vectors tested were SAd7-F followed by rAd5-F, and rAd5-F followed by rAd5-F. Both of these regimens approached the level of neutralizing activity present in 1000 μg/ml of SYNAGIS®.

The results of this example demonstrate that a heterologous prime-boost immunization regimen using a simian adenoviral vector based on serotype 7 and a human adenoviral vector based on serotype 5 induces anti-RSV neutralizing antibodies in mice.

EXAMPLE 9

This example demonstrates that the adenoviral vectors encoding an RSV F protein can induce protective immunity against RSV infection in cotton rats.

The cotton rat has been used as a model for human respiratory virus diseases (see, e.g., Eichelberger, *Viral Immunol.*, *20*(2): 243-249 (2007); Niewiesk, *Curr. Top. Microbiol. Immunol.*, *330*: 89-110 (2009); Niewiesk et al., *Lab Anim.*, *36*(4): 357-372 (2002); and Murphy et al., *Virus Res.*, *11*(1): 1-15 (1988)). Escalating doses of rAd5-F ($1x10^7$ pu to $1x10^{10}$ pu) were administered to cotton rats via i.m. injection. Animals were challenged with 10^6 pu of RSV intranasally at 4 days after adenoviral vector injection. At 5 and 10 days post-challenge, animals

were evaluated for RSV replication in the lung, serum neutralizing antibodies, eosinophilia in bronchoalveolar lavage (BAL) fluid, and lung histology.

Immunization with the Ad5 vectors induced protective immunity against RSV, and the vector dose correlated with the observed serum neutralizing antibody titer. In addition, all neutralizing antibody titers were higher than the protection threshold in a murine model. The observed neutralizing antibody response persisted to at least three months post-immunization. Recovery of RSV from the lungs was reduced to undetectable levels by vaccination with the Ad5 vectors.

An RSV vaccine optimally will need to protect against both strains of RSV, known as RSV A and RSV B. There are many isolates of RSV A and RSV B strains. The adenoviral vectors described herein express an F protein with an amino acid sequence most similar to RSV A strain isolates. The cotton rat sera generated by immunization with Ad5-F was tested for neutralizing activity against a B strain isolate, WV/14617/85 (WV) (ATCC cat# VR-1400). There was strong neutralizing activity against RSV WV (FIG. 24). In addition, as with the RSV A strain isolate A2 (ATCC cat# VR-1540), the vector dose correlated with the observed serum neutralizing antibody titer.

The results of this example demonstrate the efficacy of recombinant adenoviral vectors for RSV vaccination in a second rodent model and demonstrate the breadth of the immune response to divergent types of RSV.

EXAMPLE 10

This example compares neutralizing antibody responses and protection against challenge in cotton rats immunized with heterologous and homologous prime – boost regimens.

Various heterologous and homologous combinations of rSAV7F.0 and rAd5F.0 were tested, and each induced serum neutralizing antibody (FIG. 20) at levels sufficient to fully protect against challenge with live RSV, with no virus detected in the lungs of rSAV7F.0- and rAd5F.0-immunized mice five days post-challenge (FIG. 19). Serum neutralizing antibody responses having IC_{50} values of 200 and higher were associated with protection (data not shown).

Cotton rats (10 animals per group, 6 weeks old) were immunized with one of three adenovirus-based prime-boost regimens (FIG. 18), with the priming immunization administered at week 0, followed by boost at week 4. The two regimens of homologous prime-boost tested were 1) rAd5F.0 at $1x10^8$ PU per dose and 2) rSAV7F.0 at $1x10^9$ PU per dose. To differentiate between the effects of a boost immunization and continued development of the immune response from the prime, control groups of cotton rats were primed with either rAd5F.0 or rSAVF.0 at week 0 and then boosted with FFB at week 4. The heterologous prime-boost regimen consisted of priming with rSAV7F.0 ($1x10^9$pu) with a subsequent rAd5F.0 ($1x10^8$ PU) boost. A positive control for protective RSV immunity consisted of animals immunized intranasally (i.n.) with $1x10^6$ PFU live RSV A2 strain. As a control for induction of immunopathology, a group of cotton rats was immunized with FI-RSV (1:100) intramuscularly at week 0. Negative control animals were injected i.m. with FFB at week 0 and at the time of boost (week 4). Serum samples were collected at week 4 (pre-boost) and at week 8 (pre-challenge). All animals were challenged i.n. with $1x10^6$ PFU live RSV A2 at week 8. One subset of animals was euthanized at day 5 post-challenge for analyses of RSV titers in the lung. The remaining cotton rats in each group were euthanized at day 10 post-challenge. Experimental endpoints at day 10 included serum for antibody measurements, BAL for evaluation of eosinophilia, followed by removal of lungs for quantitation of RSV titers.

Although all regimens were protective, enhanced immunogenicity of heterologous rSAV7F.0 priming followed by rAd5F.0 boost was observed. This was demonstrated by 1) induction of significantly higher levels of neutralizing antibody by rSAV7F.0 priming as compared to rAd5F.0 priming ($p<0.0001$) and 2) by the ability of rAd5F.0 to significantly boost antibody levels relative to both pre-boost levels ($p<0.0001$) and to rSAV7F.0-primed, FFB-boosted antibody responses ($p=0.007$). The combination of rSAV7F.0 prime and rAd5F.0 boost generated the highest levels of pre-challenge neutralizing antibody which were further increased following live RSV A2 challenge (FIGS. 20-21), resulting in neutralizing antibody levels more than 2-fold greater than primary RSV infection and re-infection. The 4-week interval between prime and boost may have been too short for an optimal

boost of neutralizing antibody titer. Importantly, no homologous or heterologous combination of rSAV7F.0 and rAd5F.0 immunogens predisposed for eosinophil recruitment to the BAL following RSV challenge (FIG. 23), demonstrating the safety and lack of Th2-associated immunopathology of this vaccine regimen. These data demonstrate efficacy of the rSAV7F.0/rAd5F.0 heterologous prime-boost strategy for increasing the titer of neutralizing antibody.

As stated above, all the regimens provided complete protection against pulmonary infection with $1x10^6$ PFU live RSV A2 as evidenced by undetectable RSV titers in the lung at day 5 post-challenge (FIG. 19). Complete protection was also seen in control animals immunized with RSV A2 while partial protection was observed in 3 of 5 FI-RSV-immunized animals with the remaining 2 cotton rats having no detectable virus. Expected viral titers, averaging $2x10^5$ PFU/gram of lung, were observed in the mock-immunized FFB control animals.

The heterologous rSAV7F.0 prime / rAdF.0 boost produced a robust pre-challenge neutralizing antibody group mean titer of 2413 (FIG. 20), a titer which was significantly greater than the titer in all other prime-challenge combinations ($p<0.0001$). Additionally, while there were no significant increases observed in the level of neutralizing antibody titers in animals boosted with the homologous adenovirus vector or with FFB, boosting rSAVF.0-primed cotton rats with rAd5F.0 did result in a significant increase in neutralizing antibody titers relative to both pre-boost antibody levels of the same animals ($p<0.0001$) and to pre-challenge titers in rSAVF.0-primed animals boosted with FFB ($p=0.007$).

A robust increase in levels of neutralizing antibody titers in the serum was observed in all prime-boost regimens at day 10 after challenge with RSV A2 (FIG. 21). However, the titers in all other prime-boost immunization groups remained markedly below the neutralizing titers observed in the rSAV7F.0 prime / rAd5F.0 boost group.

As assessed by quantitation of viral titers in the lung (FIG. 19) and neutralizing antibody in the serum (FIGS. 20 and 21), complete protection from RSV A2 challenge was observed in immunized animals with IC_{50} neutralizing antibody titers greater than or equal to 200. (FIG. 22)

Cells collected by BAL were differentially stained, then examined for the presence of eosinophils. As stated above, no eosinophilia was observed in cotton rats immunized with any combination of the rSAV7F.0 or rAd5F.0 vectors (FIG. 23). These data demonstrate the inability of these vaccine regimens to induce strong T_H2 T-cell responses and serve as an important indication of the potential safety of this viral vector vaccine technology.

EXAMPLE 11

This example compares the relative immunogenicity of the standard rAd5 vector, Adt.RSVF0.11D (rAd5F.0), to a modified rAd5 vector that has a larger deletion of the E1 region, Ad4344RSVF.0.11D (rAd4344F.0).

Immunization with either 1 x 10^7 or 1 x 10^9 PU of rAd5F.0 or rAd4344F.0 induced significant levels of neutralizing serum antibody prior to RSV challenge with marked increases in antibody levels occurring after RSV challenge. Antibody responses induced by rAd5F.0 and rAd4344F.0 immunization were comparable at each dose, and neutralizing antibody titers were significantly greater in animals immunized with 1 x 10^9 PU at all time points measured. Immunization with rAd5F.0 and rAd4344F.0 fully protected against RSV challenge. There was no enhancement of lung eosinophilia in rAd5F.0- and rAd4344F.0-immunized animals following RSV challenge. Thus, the immunogenicity and safety of the modified rAd4344F.0 vector is equivalent to the rAd5F.0 vector.

Cotton rats were immunized IM with 1x10^7 PU, 1x10^9 PU of the adenovirus vectors, rAd4344F.0 and rAd5F.0 or experimental controls (FIG. 25). Serum was collected one day before challenge for measurement of neutralizing antibody titers. The animals were intra-nasally challenged on day 28 post-immunization with 1x10^6 PFU RSV A2. Serum, BAL fluid, and lungs were collected (FIG. 25).

Blood was obtained four weeks after immunization, day -1 before challenge, from all animals and at the day 5 and day 10 post-challenge time points. Neutralizing serum antibody titers (IC_{50}) were determined in all samples by PRNT assay. Immunization with live RSV and each dose of rAd5F.0 and rAd4344F.0 induced significant antibody production relative to FFB-immunized cotton rats (FIG. 26). Animals immunized with FI-RSV had low neutralizing antibody titers;

of the 15 animals, 7 had titers below the assay limit of 32, the IC_{50} of the 8 animals with measureable titers was 69 ± 20 (mean ± SD). Immunization with live RSV i.n. resulted in significantly higher IC_{50} titers relative to the titers of all the other groups prior to challenge. Importantly, when the IC_{50} titers of animals immunized with the two adenovirus vectors were compared, there was no significant difference in IC_{50} titers elicited by the same doses of each adenovirus vector. When the effect of the 100-fold difference in immunization dose was analyzed, animals immunized with $1x10^9$ PU of either rAd5F.0 or rAd4344F.0 had significantly greater neutralizing antibody titers than cotton rats immunized with $1x10^7$ of the same virus. At day 5 post-challenge, animals immunized with $1x10^9$ PU of either rAd5F.0 and rAd4344F.0 had significantly higher antibody titers than did animals immunized by primary RSV infection, although titers were similar in these 3 groups at day 10 post-challenge (FIG. 26). Taken together, these results demonstrate that the two rAd5 vector constructs induced comparable neutralizing serum antibody titers with similar dose response patterns.

RSV titers were measured in the lungs five days after challenge. All immunized animals had significantly lower RSV titers relative to FFB-immunized cotton rats (FIG. 27). All animals immunized with live RSV, rAd5F.0, or rAd4344F.0 and 2 of 5 FI-RSV-immunized animals had no detectable RSV in the lungs (FIG. 27).

As an indication of vaccine safety, eosinophil recruitment to the BAL was examined ten days after RSV challenge. As expected, significant eosinophilia was observed in FI-RSV-immunized cotton rats (FIG. 28). However, mock immunization with FFB or immunization with live RSV, rAd5F.0, or rAd4344F.0 did not prime for immune responses resulting in immunopathology following RSV challenge (FIG. 28).

The results of this example demonstrate that the increased deletion in the Ad5 E1 region did not alter the immunogenicity or safety potential of the rAd5F.0 vector design. The modified rAd4344F.0 construct was shown to be comparably immunogenic, inducing neutralizing antibody titers comparable to rAd5F.0 and immune responses which fully protected against live RSV challenge.

EXAMPLE 12

This example determines protection against RSV challenge by vaccination of cotton rats with a dose range of Ad5-F.

Protection against RSV A2 infection conferred by immunization with Adt.RSVF.O.11D (Ad5-F), an adenovirus serotype 5 vector that expresses a codon-optimized, full length RSV glycoprotein F_0, was determined. Immune responses induced to the RSV F_0 antigen and protection against challenge as a function of Ad5-F dose were determined by serum neutralizing activity and RSV viral load in the lung. Additionally, body weight loss was evaluated as an indicator of illness in the cotton rat RSV challenge model.

The immunization, challenge, and tissue collection schedule is shown as a timeline in FIG. 29. Animals were immunized with RSV A2, FI-RSV, four doses of Ad5-F, or buffer for mock-immunization. Blood for serum was collected on day 27 post-immunization, the animals were challenged with RSV A2 on day 28, and tissues were collected at days 5 and 10 post-challenge. Body weights were obtained daily from the day of challenge through the day of harvest.

Protection against RSV challenge

All animals immunized with 1 x 10^7pu to 1 x 10^{10}pu of AdF.0 were protected against challenge with RSV A2. Immunization with Ad5-F reduced RSV A2 pulmonary replication to undetectable levels by day 5 post challenge (FIG. 30). The FFB and FI-RSV immunized control animals were not protected from intranasal challenge with 1 x 10^6pfu of RSV A2. High titers of RSV A2 (1 x 10^7pfu) were measured in the lungs of cotton rats on day 5 post challenge, and were cleared by day 10. The animals that were initially infected intranasally with 1 x 10^7pfu of RSV A2 were protected from RSV A2 challenge in that pulmonary replication in these animals was reduced to undetectable levels.

RSV neutralizing antibody titers

Systemic neutralizing antibody titers were determined from PRNTs of cotton rat serum samples. Immunization with increasing doses of Ad5-F induced increasing serum neutralizing activity (FIG. 31). Every 10 fold increase in immunization dose corresponded to a 2 fold increase in pre-challenge neutralizing activity on day 27 post-immunization ranging from an IC_{50} mean titer of 214

induced by 1 x 10^7pu to an IC_{50} mean titer of 1662 induced by 1 x 10^{10} pu. Individual animals immunized with Ad5-F in the doses of 1 x 10^9 pu and 1 x 10^{10} pu had IC_{50} titers of up to 2100 which was greater than the titer of all but three of the RSV immunized animals. The pre-challenge mean IC_{50} titer induced by natural infection with 1 x 10^7 pfu of RSV A2, being 1544, was comparable to and within the standard deviation of Ad5-F at 1 X 10^{10} pu. The FI-RSV and FFB immunized control animals had minimal pre-challenge neutralizing activity probably due to background neutralization or virocidal activity.

Post-challenge serum neutralizing activity showed a boost in PRNT titers of all groups by day 10 post-challenge (FIG. 32). Notably, animals immunized with Ad5-F had an increase in serum neutralization at day 5 post-challenge, although less than two-fold on average. In contrast, animals immunized with FI-RSV, RSV, or mock had no increase in post-challenge IC50 by day 5.

Body weight change as an indicator of RSV illness:

No significant weight loss was observed in any immunization groups following RSV A2 challenge dose of 1 X 10^7pfu (FIG. 33). Therefore, weight loss is not a reliable indicator of illness in the cotton rat model.

Determination of eosinophilia:

Eosinophilia determination was not possible due to the lack of differential staining of cotton rat eosinophils, basophils, and neutrophils (FIG. 34). The hematoxylin + eosin staining protocol for mouse lymphocyte differential staining was not appropriate for cotton rat cells.

EXAMPLE 13

This example determines the durability of RSV neutralizing antibody titer and protection against challenge induced by Ad5F.0. In particular, this example evaluates the ability of two different doses of rAd5 expressing RSV F to induce neutralizing antibody in cotton rats, examines the durability of those responses, determines the ability of those responses to protect against RSV challenge, and evaluates the extent of eosinophilia after RSV challenge as a measure of immunopathology (FIG. 35).

Cotton rats immunized with a single i.m. injection of rAd5F.0 at a high dose, 1 x 10^9 particle units (pu), generated robust and durable titers of neutralizing serum antibodies which were comparable to neutralizing titers induced by primary RSV infection (FIG. 36). Six months or longer after immunization neutralizing serum antibody titers in high dose rAd5F.0- and live RSV-immunized animals were similar. Though antibody titers were decreasing, all animals in both groups were protected against RSV challenge as assessed by RSV titers in the lung five days post-challenge (FIG. 37).

In contrast, cotton rats immunized with a single i.m. injection of rAd5F.0 at a low dose, 1 x 10^7 pu, produced neutralizing serum antibodies at titers lower than live RSV infection, but much greater than those induced by FI-RSV-immunization (FIG. 38). Antibody responses in low dose rAd5F.0-immunized animals exhibited similar kinetics as live RSV infection with peak titers observed at week 4 post-immunization and waning to a response plateau between 12 and 16 weeks after priming. However, the titer of responses in the low dose rAd5F.0-immunized animals appeared to stabilize at the threshold level of protection. When challenged with RSV, 2 of the 3 cotton rats were fully protected, while the remaining 1 animal was partially protected with a 79% reduction in RSV titers at day 5 post-challenge (FIG. 39). Comparing the RSV neutralizing titers immediately prior to challenge with the lung viral load of individual animals demonstrated a threshold titer for protection of 120 (FIG. 42).

Analyses of animals ten days after RSV challenge demonstrated increased titers of neutralizing serum antibody in all groups relative to titers at week 32 post-immunization and immediately prior to challenge (FIG. 40). Furthermore, a lack of enhanced pulmonary eosinophilia in bronchoalveolar lavage samples suggested minimal risk of immunopathology with of rAd5F.0 following subsequent RSV infection (FIG. 41).

Two doses (1 x 10^7 pu or 1 x 10^9 pu) of rAdF.0 were tested by a single intramuscular immunization. A positive control set of animals was infected intranasally with 1 x 10^6 PFU live RSV A2, and a negative control group was immunized with FI-RSV (A2 strain) diluted 1:40. Blood was taken for serum at intervals as indicated in FIG. 35, and titers (IC_{50}) of neutralizing serum antibody

determined by PRNT. All animals were challenged with 1 x 10^6 PFU live RSV A2 with two post-challenge endpoints of five animals/group/endpoint, for a total of 40 animals in the study. The cotton rats were challenged with RSV at week 32 after immunization as animals were beginning to die due to causes unrelated to the study.

Cotton rats were primed by primary RSV infection or with FI-RSV, low dose (1 X 10^7 particle units) rAd5F.0, or high dose (1 X 10^9 particle units) rAd5F.0, and sera were collected post-immunization (FIG. 35). High dose rAdF.0 immunization elicited robust and durable titers of neutralizing serum antibody comparable to levels induced by primary RSV infection at week 4 after priming while FI-RSV priming resulted in poor induction of neutralizing serum antibody (FIG. 36). While neutralization titers in RSV-infected control animals began to decrease from week 4 to week 8, reaching a plateau around week 12, the antibody titers in cotton rats immunized with high dose rAd5F.0 continued to increase and peaked by week 8 at levels 3 times greater than those induced by RSV infection. Although titers began to wane, antibody levels induced by high dose rAd5F.0 immunization remained well above titers in RSV-infected animals until week 24 (6 months), then similar titers were seen in both groups (FIG. 36). At week 32 post-immunization, both high dose rAd5F.0 and RSV priming groups were completely protected against RSV challenge as demonstrated by undetectable viral titers five days post-challenge (FIG. 37). Thus, high dose rAd5F.0 immunization induced levels of neutralizing serum antibody comparable to primary RSV infection with comparable durability and which are sufficient to fully protect cotton rats against RSV challenge.

Cotton rats immunized with low dose rAd5F.0 generated neutralizing antibody responses with kinetics similar to those induced by primary RSV infection (FIG. 38). The titers induced by rAd5F.0 were markedly lower than in RSV-infected cotton rats, but above FI-RSV-induced titers, throughout the course of this study. By week 16 post-immunization, the mean low dose rAdF.0-induced IC_{50} titer was 129 and remained constant through 32 weeks (FIG. 38). At day 5 post-RSV challenge 2 of 3 cotton rats were fully protected, having no detectable virus while 1 animal was partially protected with RSV titer of 86,824 PFU/gram

lung as compared to a mean titer of 411,790 PFU/gram lung in the FI-RSV-immunized RSV-challenged cotton rats (FIG. 39). Protection against RSV challenge correlated with titers of neutralizing antibodies in individual rAd5F.0-immunized animals (Table 4). Therefore, while capable of inducing moderate levels of neutralizing serum antibody, neutralizing antibody titers in cotton rats immunized with low dose rAd5F.0 reached a lower peak response and were maintained at a lower titer over the course of this study in comparison to titers cotton rats immunized with RSV or with high dose rAd5F.0.

Table 4. Paired comparison of neutralizing antibody IC50 and RSV titers in cotton rat sera 28 days after immunization with 1×10^7 pu of rAd5F.0.

Animal	IC_{50}	RSV titer (PFU/gram lung)
rAd5F.0-primed animal 1	239.8	Not detectable
rAd5F.0-primed animal 2	140.9	Not detectable
rAd5F.0-primed animal 3	85.4	86,824

After challenge, neutralizing antibody responses increased 2.7-, 5.5-, 3.9-, and 3.1-fold in cotton rats immunized with RSV, FI-RSV, low dose rAd5F.0, and high dose rAd5F.0, respectively (FIG. 40). The presence and specificity of B cell memory responses in all groups was thus confirmed by these increased titers in neutralizing serum antibody titers ten days after RSV challenge.

Immunization with either low or high dose rAd5F.0 induced RSV-specific memory responses which did not result in vaccine-enhanced disease after RSV challenge. This was evidenced by the presence of minimal eosinophils in bronchoalveolar lavage samples from rAd5F.0-immunized animals ten days post-challenge (FIG. 41). The lack of eosinophilia provides an indication of safety for both high and low dose rAd5F.0 immunogens.

The threshold for protection in this experiment was a neutralizing serum antibody titer with an IC_{50} titer $\geq$ 120, measured immediately prior to challenge (FIG. 42). The threshold level of protection is defined by at least a 100-fold reduction in RSV pulmonary replication relative to the negative control group (FI-RSV-immunized animals here). In this study complete protection occurred with greater than 1000-fold reduction in RSV titers five days after RSV challenge in all

animals immunized with high dose rAd5F.0 and in 2 of 3 animals immunized with low dose rAd5F.0. This threshold of protection correlated with neutralizing serum antibody titers with $IC_{50} \geq 120$ as measured by PRNT. The low dose rAd5F.0-immunized animal not fully protected had an IC_{50} of 89 at week 32 post-immunization. While not completely protected, the titers of neutralizing antibody were sufficient for partial protection with a 79% reduction in RSV titers at day 5 post-challenge (86,824 PFU/gram lung as compared to a mean titer of 411,790 PFU/gram lung in FI-RSV-primed animals).

EXAMPLE 14

This example determines thresholds for protection conveyed by low dose immunization with SAV7F.0 and SAV11F.0 in the cotton rat model.

Animals were immunized with RSV A2 (1×10^6 pfu) intranasally (i.n.), mock-immunized with FFB by intramuscular injection (i.m.), or immunized i.m. with SAV7F.0 or SAV11F.0 with 1×10^3 pu, 1×10^4 pu, or 1×10^5 pu. The animals were challenged with 1×10^6 pfu RSV i.n. and tissues harvested at 5 days post-challenge (FIG. 43).

Immunization with low doses of SAV7F.0 (SAV7d1t.RSVF.0) and SAV11F.0 (SAV11d3t.RSVF.0) induced minimal levels of serum neutralizing activity (FIG. 44). The PRNT titers were comparable between the adenovirus vectors and doses. However, there was a clear difference in protection against RSV replication (FIG. 45). Protection induced by SAV7F.0 was correlated with dose, with increasing partial protection from $1x10^3$ pu dose to $1x10^4$ pu dose. At the $1x10^5$ pu dose four out of five animals were fully protected. In contrast, the protection afforded by SAV11F.0 was less predictable by dose, with no protection at $1x10^3$ pu dose and incomplete protection at $1x10^4$ pu and $1x10^5$ pu doses.

This example shows that SAV7 induced a superior protective response than SAV11.

EXAMPLE 15

This example determines the replication kinetics of RSV (RSV A2) in the cotton rat model. The titer of RSV across the time points of the experiment (1)

confirmed the infectivity of the RSV stock and (2) confirmed the appropriate time post-infection for assessing maximal reduction in RSV replication in RSV challenge experiments.

Female cotton rats (*Sigmodon hispidus*) were inoculated intranasally with 1 X 10^6 PFU of RSV A2 in 100 ul of EMEM. RSV A2 lot number 1005 with a titer of 2.2 X 10^7 PFU/mL was used. Each time point group was three animals each. Upon harvest lungs were flash frozen and stored at -80° C until assayed for RSV titer. The titer of RSV in the lungs of the cotton rats was determined by plaque assay on Hep2 cells (passage nineteen).

Published literature (Prince et al. *Amer J Path* 93:771 (1978)) provided a timeframe for capturing the rise and fall of RSV titer in cotton rat lungs following infection by the intranasal route. The study was comprised of seven time points across a two week range (FIG. 48). The dose of RSV, 1 x 10^6 pfu, was matched to the dose of RSV for challenging cotton rats in vaccine-mediated protection experiments.

The time-course of RSV replication in the lung of the cotton rats (FIG. 46) matched the previously published studies (FIG. 47). The infection by RSV in pulmonary tissue was characterized by the detection of RSV at 6 x 10^4 PFU/gram of lung tissue on the first day post–infection (dpi) and the viral load increased to 1 x 10^6 PFU / gram lung tissue at 3 dpi. RSV titers decreased rapidly and no RSV was detected at 6 dpi (limit of detection 70 PFU / gram lung tissue).

EXAMPLE 16

This example enables the use of the RSV challenge model in cotton rats by establishing methods and procedures for the administration of viruses, evaluating illness, and measuring serum neutralizing antibody and RSV titers in lung tissue. Additionally, stocks of immunogens used as controls in other studies were tested.

Animals were immunized, blood collected 27 days later for serum neutralization assays, and challenged on day 28 post-immunization with RSV A2. Groups of 5 animals per immunization were sacrificed on days 4, 7, and 10 post-challenge and tissues were harvested for viral, pathological, and immunological analyses (FIG. 49).

Implementation of methods and procedures: Cotton rat handling procedures were sufficient to allow for the administration of immunogens and RSV challenge virus, obtaining blood for serum, BAL for eosinophilia determination, and determining body weights. The administration of isoflourane anesthesia was sufficient to immobilize the animals for intranasal administration; however, one anesthesia-related death was observed. RSV neutralizing activity of serum samples were obtained, while RSV lung titers and eosinophilia were not.

Evaluation of illness: There were no visible signs of animal illness, such as ruffled fur or reduced activity, following administration of the immunogens or RSV challenge. Body weights of the cotton rats were tracked daily following RSV challenge. Body weight averages did not differ at any time point between any groups, thus, weight loss in not a measurement of RSV-induced illness in cotton rats (FIG. 50).

Serum neutralizing antibody titers: The collection of serum and measurement of the neutralizing activity by Plaque Reduction Neutralization Test (PRNT) provided consistent and expected relative values. Animals immunized at day 0 with RSV or FI-RSV had day 27 serum neutralization EC_{50} titers to RSV, whereas animals immunized with Hep2 cell lysate did not, based on a serum titration cut-off of 1:10 (FIG 51). All animals had RSV neutralizing titers at day 10 post-challenge, consistent with the intranasal administration of RSV to all animals (FIG. 52). The relative titers pre-challenge and post-challenge were consistent, namely, RSV > FI-RSV > Hep2 lysate.

Animal procedures were performed as described in Example 21. Serum neutralization was determined as described in Example 18, with the exception that the serum dilutions ranged from 1:10 to 1:20480. Pulmonary RSV was determined as described in Example 16.

Weight loss associated with RSV infection in the mouse model has traditionally been a reliable indication of illness (J. Med. Virol. 1988. 26:153-162; J. Virol. 2011. 85:5782-5793). Mouse models of RSV infection can show up to 20% weight loss by day six post-infection. Post-challenge weight loss tracking of cotton rats showed there was no significant weight loss (FIG. 50).

Serum neutralizing activity was quantified, both pre- and post-challenge. The average EC_{50} titer for RSV infected animals was 661 +/- 247, in comparison, the average titer for the animals receiving FI-RSV was 33 +/- 22 (FIG. 51). The Hep2 mock infected animals had no detectable serum neutralizing activity. The titers of RSV infected animals ranged from 200 to 1000. By contrast, the FI-RSV infected animals had titers ranging from undetectable to 80. The serum neutralizing activity of all animals was boosted significantly by challenge with 1 X 10^7 PFU of RSV A2. The average serum neutralization of RSV A2 infected and FI-RSV immunized animals increased post challenge by five-fold and 30-fold, respectively (FIG. 52). Previously uninfected animals, having been treated with Hep2 lysate (mock), showed an increase in serum neutralizing activity to an average EC_{50} of 200.

Titers of RSV in the lung of cotton rats were not obtained; there were no plaques observed in the assay. This may have been due to carbon dioxide vapor leaking into the vials with the harvested lungs. The pH of the medium in the vial had a slightly yellow tint upon thawing the vial, indicating the medium had become more acidic. It was observed that the electrical tape used to prevent carbon dioxide vapor leakage did not maintain its integrity during quick freeze.

Definitive data on eosinophilia was not obtained. The H&E staining procedure commonly used to differentiate eosinophils in murine BAL samples was not sufficient to differentiate cotton rat eosinophils from neutrophils. The other procedures (BAL collection, generation of microscope slides with appropriate cell density, cell fixation) appear to be sufficient.

EXAMPLE 17

This example a plaque-forming unit assay was developed to determine the RSV viral titer in cotton rat lung homogenates. The effect of sample processing on the RSV A2 viral titer of pulmonary tissue was also assessed.

Eight week old cotton rats were immunized intra-nasally with 1 X10^6 PFU of RSV A2 and lungs were harvested four days post-infection, predicted from published literature to be the peak viral load. The RSV titers in fresh (never frozen, processed and assayed as quickly as was possible) and frozen homogenates

were determined. The effect of homogenization was determined by comparing the titers of lung homogenates spiked with RSV before or after processing with the GentleMACS tissue dissociator. The effect of lung homogenate on RSV titer was determined by comparing the titers of RSV samples mixed with either buffer or with non-infected lung homogenate.

Animal infection and tissue harvest: Five female cotton rats eight weeks of age were obtained from HSD. Rat number 1 was mock infected intra-nasally with 100 ul EMEM+10%FBS. Rats numbered 2-5 were infected with 1 $X10^6$ PFU of RSV A2 (LN:1005, titer = 3 $X10^7$PFU/ml) in 100 uL of EMEM+10%FBS. The cotton rats were harvested 4 days post infection at peak viral titer. Lungs were harvested into 5 mL dram vials containing 2 mL of EMEM+10%FBS. The mass of each lung was determined differentially by weighing each vial without and with the lung, the difference of which being equal to the lung mass. The cap of each vial was sealed using electrical tape to minimize the transference of CO_2 to the contents of the vial. Samples were either immediately flash-frozen using an ethanol+ dry ice bath or placed on wet ice until assayed approximately one hour later.

Cells for titer assay: Hep2 cells passage number 20 were seeded into 12 well tissue culture plates at a density of 2.5 $X10^5$ cells per well in 1 mL of EMEM+10%FBS, 36 hours prior to infection with experimental samples.

Tissue processing: Fresh or thawed lungs from infected or uninfected animals were homogenized using the GentleMACS tissue dissociator set to Mlung_02.01, then clarified by low-speed centrifugation at 4° C. Frozen lungs were thawed in a 37° C water bath.

Spiking of tissue samples with RSV: (1) To determine the effect of lung homogenate on RSV titer, the frozen lung from the naïve animal (rat number 1) was thawed, processed, and then spiked with RSV A2 (LN:1005) to a final predicted titer of 1 $x10^6$ PFU / mL. (2) To determine the effect of the GentleMACS tissue dissociator on RSV titer, one fresh lung from a naïve animal was processed as above and spiked to a predicted titer of 2 x 10^6 PFU / mL. An aliquot was then processed a second time on the GentleMACS dissociator. The spiked sample that was processed one time was also used to determine the effect of lung homogenate on RSV titer.

Lung homogenate dilution range for assay: The resulting supernatants were serially diluted 10-fold from 1:10 to $1:10^7$. Triplicate infections of the Hep2 cells were performed with 100 ul of each dilution.

RSV titer (PFU per mL for spiked samples, PFU per gram of lung tissue for infected cotton rat lung samples) was determined by plaque assay on Hep2 cells as in Example 5.

The titer of RSV in lung homogenates was consistent with the expected titer based on an RSV infection dose of 1×10^6 PFU intra-nasally and a day 4 post-infection time-point (*Amer J Path* 93:771). The highest titers were obtained in lung homogenates of fresh lungs. Uninfected lung homogenate did not affect titer measurements when mixed with RSV. In contrast, processing of the lungs to generate the homogenate (freezing and thawing the lungs, homogenizing the lungs) reduced the titer of RSV. For practical considerations, lungs were frozen and subsequently processed with the GentleMACS tissue dissociator, although both procedures result in reduction of RSV titer.

The titer of RSV in lung homogenates that were flash-frozen, thawed, and processed with the GentleMACS dissociator was 5.6×10^5 PFU / gram of lung tissue (average of samples 2 and 3 in FIG. 54). The presence of cotton rat lung tissue did not affect the RSV titer in the two homogenates spiked with RSV after all tissue processing because the measured titers matched the predicted titers (FIG. 53, samples 1 and 6, expected titers of 1×10^6 and 2×10^6 PFU / mL, respectively). Processing lung tissue with the GentleMACS tissue dissociator had the effect of lowering the viral titer of RSV spiked lung homogenates by approximately one-half (FIG. 53, samples 6 and 7). However, flash freezing and subsequent thaw of lungs appeared to reduce titers by a factor of three (FIG. 53, average of frozen lungs = 1.62×10^5 PFU / gram, compared to average of fresh 5.83×10^5 PFU / gram; FIG. 54, frozen lungs = 0.56×10^6 PFU / gram, compared to fresh lungs = 1.71×10^6 PFU / gram).

EXAMPLE 18

This example determines the lower limit of detection of the established RSV lung titer assay used for the quantification of WT RSV strain A2 in cotton rat lung tissue.

Cotton rat lung homogenate comprised of naïve cotton rat lungs was spiked with different concentrations of live RSV A2 and assayed for RSV concentration (PFU / mL). The measured RSV titers of the spiked lung homogenates were compared to the mathematically predicted titers based on dilution.

The RSV stock had a titer of 2.23×10^8 PFU/ml. The stock was diluted to 1×10^8 PFU/ml using serum free EMEM (448ul LN2005 B2, QS to 1ml). The diluted RSV stock was titrated in parallel with the spiked lung homogenates. The resulting titer was 1.53×10^8 PFU/ml.

Harvested lungs were placed into 2 mL of EMEM+10%FBS and flash frozen using an ethanol / dry ice bath. Upon thawing the lungs were homogenized as described in Example 16. Lung homogenates were spiked with RSV to concentrations (PFU / mL) ranging from 1.0×10^7 to 1.0×10^2 at 10-fold increments, as well as 30, 10, and 3 PFU / mL. One replicate of the spiked lung homogenate was prepared and that single replicate was assayed in triplicate.

Cotton rat tissues were harvested as described in Example 22. The RSV lung titer assay was performed as described in Example 17.

The lower limit of detection for this RSV lung titer assay was determined to be 70.4 PFU / gram of lung tissue, corresponding to an RSV plaque-forming unit quantitation limit of 5 PFU / mL. Specifically, the assay detected a titer of 10 PFU / mL at a spike concentration of 5 PFU / mL (FIG. 55) and zero plaques in the lung homogenate without RSV spike (data not shown). Thus, the assay can detect a titer to 5 PFU / mL, corresponding to 70.4 PFU / gram of lung tissue (with lung weight average of 0.284 grams added to 2 mL of buffer upon harvest). There was no noticeable inhibitory effect of cotton rat lung homogenate on the replication of RSV A2 at spike concentrations of 1,530 PFU / mL and below. The measured titers of RSV in samples ranging from 15,300 PFU / mL to 1.5×10^7 PFU / mL varied from 2-fold to 7-fold lower than expected. There was no association between expected titer and the magnitude of the difference in the measured titer.

Therefore, it is likely that variability arising from the single replicate of spiked lung homogenate explains the differences in measured and expected titers.

EXAMPLE 19

This assay determines assay conditions for a plaque reduction neutralization test (PRNT) that will provide quantitation of neutralizing activity of serum or other fluids against RSV strain A2.

Conditions for optimal detection of RSV neutralizing activity were determined. The passage number and density of the Hep2 cells plated were critical parameters. Optimal plaque formation occurred when Hep2 cells at passage 30 or less were seeded at 2.5×10^5 cells per well (Table 5). An RSV concentration of 30pfu/well yielded 60 to 80 plaques per well, the optimum range for accurate plaque counting (FIG. 56). Wright-Geimsa staining solution was easier to use than hematoxylin and eosin staining. A base protocol and example calculations are shown below. IC_{50} (half-maximal inhibitory concentration) is the inverse of the dilution giving a 50% reduction in the number of plaques per well. IC_{50} also is equivalent to EC_{50} (half-maximal effective concentration).

The assay is conducted generally by titrating the number of Hep2 cells to produce an 80% confluent monolayer 36 hours after seeding on a 12-well tissue culture plate, and titrating the concentration of RSV A2 for infection to produce the optimal number of RSV plaques for counting in each well of a 12-well tissue culture plate.

Table 5. Titration of seeding density for Hep2 cells in 12 well tissue culture plate and confluency of the monolayer 36 hours post-seed.

Cell seeded per well	Confluency (percent of dish covered by cells)
1.0×10^5	60
1.5×10^5	70
2.0×10^5	75
2.5×10^5	80
3.0×10^5	90
3.5×10^5	>99

The RSV neutralization assay is conducted as follows:

1) Prepare methylcellulose overlay (10% EMEM containing 0.75% methylcellulose (3.75 g per 500 mL)). Weigh methylcellulose into storage bottle, autoclave, then add EMEM+10%FBS. Place on stir plate for 3 days. Store at 4° C. Add 1% antibiotic / antimycotic solution immediately prior to use.
2) Plate Hep2 cells, passage 30 or lower, in 12-well plates and let grow to 80% confluence. This should occur by 36 hours post-seed.
3) When cells are 80% confluent, this is day 0 of assay. On this day, make 2-fold serial dilutions of serum samples from 1:32 to 1:65536, diluting serum into 10% EMEM, to a final volume of 200 μL in U-bottom microtiter plates. Perform dilutions in triplicate. Include a negative (PBS) control and a positive control serum.
4) Place methylcellulose overlay solution on stir plate at RT.
5) Quick-thaw the RSV neutralization stock and dilute in 10% EMEM, using a dilution that produces 60-80 plaques per well (approximately 30 pfu/well). (With RSV LN1005 at 3 x 10^7 pfu /mL serial dilute 1:10, 1:10, 1:10, 1:10). Add 200 μL of diluted RSV to each well of diluted serum.
6) Incubate at 37° C for 1 hr.
7) Aspirate media from wells of Hep2 cells. Add 100 μL of diluted serum-RSV mixture to triplicate wells.
8) Incubate at RT for 1 hr. with constant rocking.
9) Aspirate inoculum then overlay with 1mL methylcellulose overlay solution.
10) Incubate at 37° C for 4-5 days until plaque formation.
11) Do not aspirate. Fix by adding 10% formalin for 1 hour.
12) Aspirate then wash using tap water.
13) Aspirate, wash, and add 300 uL Wright-Geimsa staining solution and allow stain to adhere for 1 hour.
14) Wash with tap water.
15) Air-dry and count plaques under the dissecting microscope.
16) Count plaques and determine the mean of three reps (FIG. 65 - Table 6).
17) Determine the percentage of maximum plaque count for each dilution (FIG. 65 - Table 7).

18) Graph the mean plaque numbers as a percentage of maximum plaque number on the Y axis, with the serum dilution on the X axis in log10 scale producing a standard curve (FIG. 57).

19) Remove any low or high plateaus.

20) Determine the IC_{50} titer by solving the equation that defines the curve using 0.5 as the Y value (See equation below).

An example of the determination of IC_{50} from the linear curve in FIG. 57 is as follows:

Y= % neutralization (IC); X= titer

Solve for Y;

Y=EXP((X+0.733)/0.2151)

If IC =50;

Y=EXP((.5+0.733)/0.2151)

Y=308.65

EXAMPLE 20

This example describes procedures for improving the differentiation of cotton rat eosinophils by modifying a published staining protocol (Llewellyn BD. An improved Sirius red method for amyloid. J Med Lab Technol.1970 Jul;27(3):308-9). The optimal staining method is characterized by color differences in the eosinophilic granules of lymphocytes. The published staining method lacks difference in color as the primary indicator for differentiation between an eosinophil and a neutrophil in the cotton rat model. This results in morphology being the primary determination of an eosinophil, not differences in color produced by differential staining.

This modified Sirius Red / Gills Hematoxylin staining method provided better differentiation of eosinophils from other lymphocytes in the BAL samples of cotton rats. The improvement in differential staining simplifies the counting of eosinophils by reducing the reliance on morphological differences between eosinophils and neutrophils. With this modified method, eosinophilic granules in

eosinophils stain bright red, and eosinophilic granules in neutrophils stain a muted pink color.

Experimental eosinophilia was induced in cotton rats via single immunization with FI-RSV LN:1007 diluted 1:20 in EMEM+10%FBS. Animals were challenged 28 days later with 1 x 10^6 pfu of RSV A2, intranasally. BAL fluids were collected at 14 days post-challenge. Microscope slides of BAL samples were prepared and stained according to the method below or by the published staining protocol described above.

Modified Sirius Red / Gills Hematoxylin staining method to differentiate cotton rat eosinophils

1) Obtain BAL samples from wet ice and wash twice by centrifugation at 4°C and 2000 RPM in eppendorph 5415R microfuge centrifuge with rotor type 10/06 FL020 FA-45-24-11. Resuspend cells in 200uL D-PBS.

2) Prepare microslides using the Cytospin 4 at 300 RPM for 10 min.

3) Fix cells overnight using Millonig formalin solution.

4) Rinse slides three times in absolute ethanol for 10 minutes each.

5) Bring slides to water using:

   a) 95% ethanol for 15 min.

   b) 80% ethanol for 15 min.

   c) 70% ethanol for 15 min.

6) Progressively stain the nuclei via immersion in Gill's Hematoxylin Solution for 1 min.

7) Wash 3 times in tap water.

8) Wash 1 time in 60% ethanol for 5 min.

9) Progressively stain eosinophilic granules via immersion in Alkaline Sirius Red Solution overnight.

10) Wash 3 times in tap water.

11) Dehydrate samples using:

a) 70% ethanol for 15 min.

b) 80% ethanol for 15 min.

c) 95% ethanol for 15 min.

d) Pure ethanol for 15 min.

12) Air dry samples for a minimum of 2 hours.

13) Coverslip.

14) Air dry samples overnight.

Microscopy

1) Observe slides under oil immersion at 400X or 600X magnification.

2) Record the number of eosinophils and the total number of lymphocytes for a minimum of 10 non-overlapping fields and a minimum of 300 total lymphocytes.

BAL samples were collected as described in Example 22.

The modified Sirius Red method readily distinguishes between an eosinophil and a neutrophil using eosinophilic color differences to distinguish an eosinophil from a neutrophil (FIG. 58) These two lymphocyte types are the most difficult to distinguish as they both have eosinophilic granules and a similar nuclear morphology, bi-, and tri- lobed for the eosinophil and multi-lobed for the neutrophil. The modified Sirius Red method has specific eosinophilic granule presentation and distinct nuclear presentation making it relatively easy to distinguish between the two morphologies.

EXAMPLE 21

RSV vaccine safety may be determined by modeling vaccine-enhanced immunopathology. This example determines the dilution of Formalin-inactivated RSV (FI-RSV) LN1007 that will produce a key marker for enhanced disease, lung eosinophilia, in cotton rat models of vaccination. Following intramuscular vaccination with a dilution range of FI-RSV the cotton rats were challenged with live RSV intranasally and the percentage of eosinophils in bronchoalveolar lavage (BAL) samples determined. The FI-RSV dilution determined herein as optimal for

producing eosinophilia may then be utilized as a safety control for evaluating RSV vaccines.

A single immunization with FI-RSV was administered to male cotton rats six weeks of age (n = 3 per group). The FI-RSV immunizations were intramuscular injections of dilutions 1:10, 1:20, 1:40, and 1:100. Blood was collected by retro-orbital bleed at 27 days post-immunization for serum neutralizing activity and animals were challenged with $1X10^6$ pfu, of RSV A2, intranasally, on day 28 post-immunization. BAL fluids were collected at sacrifice, 8 days post-challenge. Three animals, one each in the 1:10, 1:20, and 1:40 FI-RSV immunization groups, were found dead at different times, cause unknown, prior to challenge with RSV A2. Animal procedures were performed as described in Example 22. BAL samples were stained as described in Example 20. Serum neutralization was determined as described in Example 19.

The stock of FI-RSV used caused enhanced immunopathology, as represented by relative cell counts of eosinophils in the BAL samples. A key result was the induction of serum neutralizing antibody at sub-protective titer. Animals immunized with the 1:100 dilution of the FI-RSV had a serum neutralizing antibody titer of 40 prior to challenge (FIG. 59) and had the highest eosinophilia response, 49% (FIG. 60).

The serum neutralizing antibody titer at 27 days post-immunization showed a dose-dependent increase from FI-RSV dilutions 1:10 through 1:40 (FIG. 59). The magnitudes of the responses were in the protective range, except for the lowest response in the 1:10 dilution group. In contrast, the neutralizing antibody titer was lower at the 1:100 dilution and clearly below protective levels.

The percentage of eosinophils in BAL fluid from animals immunized with FI-RSV dilutions 1:10 through 1:40 were low (less than 10%) and did not change across the dilution range (FIG. 60). However, the BAL fluids from the 1:100 dilution immunizations contained high percentages of eosinophils (mean = 49%), indicative of vaccine-enhanced immunopathology.

EXAMPLE 22

This example describes the procedures for harvesting rodent tissues for RSV virological and immunological analyses.

Retro-orbital sinus bleed of mouse and cotton rat

The following method is intended for survival blood collection from mice and cotton rats. It is imperative that the animal be properly restrained. If the animal is allowed to move its head, severe injury to the eye or surrounding tissues could occur. The retro-orbital sinus is the site located behind the eye at the medial or lateral canthus. This venous sinus is located just underneath the conjunctiva membrane. Restrain the animal by the scruff method. A topical ophthalmic anesthetic must be used prior to performing this procedure. Wait 5 - 10 seconds after the anesthetic is applied before attempting this procedure. Gently blot away excess anesthetic with a clean gauze pad, being careful not to scratch the cornea. With a gentle rotating motion, insert the heparin coated Thiele tube through the sinus membrane. Continue rotating the tube at the back of the orbit until blood flows. Collect the appropriate volume of blood.

Intra-cardiac blood collection

Cardiac bleeding is performed by cardiac puncture under anesthesia. After a rodent is anesthetized, it is placed on its back, exposing the chest and abdomen. The thumb and forefinger are placed on either side of the sternum, and the chest is palpated to locate the heartbeat. A needle is inserted into the left side of the chest in the direction of the strongest heartbeat, and the plunger is gently pulled back. If no blood enters the needle, the needle is gently withdrawn and reinserted. When blood collection is complete, the syringe is gently withdrawn, the needle removed from the syringe, and the blood is emptied into a collection tube. The rodent is then euthanized.

Lung harvest for histology

Euthanize the animal. With ethanol, wet the animal down to minimize contamination of tissues with fur. With forceps, grab hold of the skin anteriorly to the urethral opening. Using scissors, cut along the ventral midline from the sternum to the chin, being careful to only cut the skin and not the muscle wall underneath. Lift the sternum with your forceps, puncture the diaphragm and cut

through each side of the sternum up through the cervical girdle. Be careful to keep the points of your scissors pointed upward in order to prevent any damage to the thoracic organs underneath. Extract the desired lung from the animal. Inflate the lung with 10% formalin using a 30 gauge needle. Place lung into 2ml of 10% formalin.

Lung harvest for RSV titer

Euthanize the animal. With ethanol, wet the animal down to minimize contamination of tissues with fur. With forceps, grab hold of the skin anteriorly to the urethral opening. Using scissors, cut along the ventral midline from the sternum to the chin, being careful to only cut the skin and not the muscle wall underneath. Lift the sternum with the forceps, puncture the diaphragm and cut through each side of the sternum up through the cervical girdle. Be careful to keep the points of the scissors pointed upward in order to prevent any damage to the thoracic organs underneath. Extract the desired lung from the animal. Place lung into pre-weighed dram vial containing 2ml of EMEM+10%FBS. Weigh for determination of lung mass. Seal lid using electrical tape. Quickly freeze the tissue using a cold bath containing ethanol and dry ice. Transfer to storage at -80^{0}C.

Bronchoalveolar lavage

Euthanize the animal. With ethanol, wet the animal down to minimize contamination with fur. Using scissors make a small incision in the animal skin at the neck. Dissect tissue from neck to expose trachea. Load a 3 cc syringe with 1 mL sterile D-PBS. Place syringe in end of 18 gauge catheter needle and insert into trachea toward the lungs. Carefully inject D-PBS into the animals' lungs then massage the lungs. Aspirate saline by pulling barrel of syringe. Inject recovered lavage fluid into 2 ml eppendorf-style tube on wet ice. Store in labeled vials indicating (animal ID #, experiment #, date).

Harvest of nasal wash

Euthanize the animal. With ethanol, wet the animal down to minimize contamination of tissues with fur. Using scissors, cut along the ventral midline from the sternum to the chin. Dissect tissue from neck to expose trachea. Load a 3 cc syringe with 500 uL sterile D-PBS. Place syringe in end of 18 gauge catheter

needle and insert into trachea toward the nose. Inject D-PBS into the animals' nasal passages taking care to have the fluid exiting the nose drip into eppendorf-style tube on wet ice.

Harvest of noses for titer

Euthanize the animal. With ethanol, wet the animal down to minimize contamination of tissues with fur. Using scissors remove the nose and cut along the snout. Pull back skin to reveal skull. Remove the incisors. Remove the upper jaw and nasal cavity by cutting at an angle from the upper jaw towards the frontal sinus below each eye. Place the excised material into a 15 mL conical containing 2 mL of EMEM+10%FBS. Seal lid with electrical tape. Quickly freeze the tissue using a cold bath containing ethanol and dry ice. Transfer to storage at -80^0 C until assayed.

Weighing of rodents for health monitoring

Tare balance with a clean cage. Add the animal to the cage. Record the mass of the animal. Return the animal to the original cage. Obtain a new cage for each treatment group to avoid cross contamination.

Serum harvest

Whole blood in labeled centrifuge tubes will stand at room temperature for at least 20 minutes and not more than two hours. The tubes are placed in a centrifuge and spun for 10 minutes at 6000 RPM using an eppendorf 5415R centrifuge with rotor type 10/06 FL020 FA-45-24-11. The tubes are removed gently to avoid remixing of the serum and blood solids. The serum is removed via micropipette and placed in a fresh labeled collection tube. A new micropipette tip is used for each serum sample collected. The used micropipette tips and residual blood is disposed of in a Sharp-safe container.

Survival, morbidity, and moribundity monitoring

Animals will be monitored daily for clinical signs of morbidity and moribundity. At the onset of morbidity the frequency of monitoring by personnel experienced in recognizing signs of morbidity will be increased from daily to twice daily to include holidays and weekends. Cotton rats will be weighed daily without being placed under anesthesia. A clinical illness grade will be assigned by a blinded examiner using a scale derived by assigning numbers to a set of clinical

features seen in cotton rats with different degrees of illness: 0 - healthy; 1 - barely ruffled; 2 - ruffled, but active; 3 - ruffled and inactive; 4 - ruffled, inactive, hunched, and gaunt; 5 - dead. Photographs of cotton rats to document clinical illness will be obtained at appropriate intervals. Any animal classified as being degree 4 will be euthanized.

Cotton rat monitoring for fighting

Cotton rats must be carefully monitored and evaluated for fighting. Should any evidence of blood be found in or on the transport container or alopecia be noted, cotton rats will be immediately separated and singly housed. Any wounds will be clipped and topical antibiotic applied as appropriate. As a prophylactic measure, all cotton rats will be singly housed on arrival.

All references, including publications, patent applications, and patents, cited herein are hereby incorporated by reference to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein.

The use of the terms "a" and "an" and "the" and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms "comprising," "having," "including," and "containing" are to be construed as open-ended terms (i.e., meaning "including, but not limited to,") unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be

construed as indicating any non-claimed element as essential to the practice of the invention.

Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

WHAT IS CLAIMED IS:

1. A composition comprising a replication-deficient, E1 region-deleted, serotype 7 monkey adenoviral vector comprising the sequence of SEQ ID NO:12.

2. The composition of claim 1, wherein a nucleic acid sequence encoding a heterologous antigen is inserted at the location of the E1 region deletion.

3. A composition comprising a replication-deficient, E1 region-deleted, human serotype 5 adenoviral vector comprising a sequence selected from the group consisting of SEQ ID NO:10 and SEQ ID NO:11.

4. The composition of claim 3, wherein a nucleic acid sequence encoding a heterologous antigen is inserted at the location of the E1 region deletion.

5. A method for inducing a protective immune response against RSV in a mammal, which comprises administering to the mammal priming and boosting compositions comprising a replication-deficient, E1 region-deleted, monkey adenoviral vector comprising the sequence of SEQ ID NO:12 or a replication-deficient, E1 region-deleted, human adenoviral vector comprising a sequence selected from the group consisting of SEQ ID NO: 10 and SEQ ID NO:11.

6. The method of claim 5, wherein a nucleic acid sequence encoding a heterologous antigen is inserted at the location of the E1 region deletion.

7. The method of claim 6, wherein the priming composition comprises the monkey adenoviral vector comprising the sequence of SEQ ID NO:12, and the boosting composition comprises the human adenoviral vector comprising a sequence selected from the group consisting of SEQ ID NO:10 and SEQ ID NO:11.

8. The method of claim 6, wherein the priming composition comprises the human adenoviral vector comprising a sequence selected from the group consisting of SEQ ID NO:10 and SEQ ID NO:11, and the boosting composition comprises the monkey adenoviral vector comprising the sequence of SEQ ID NO:12.

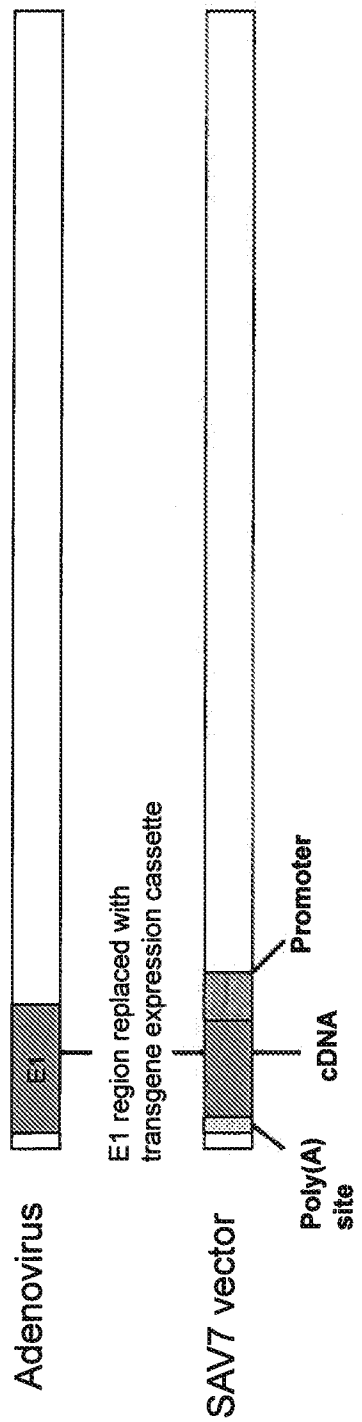
9. The composition of claims 2 or 4, wherein the nucleic acid sequence encoding a heterologous antigen is codon-optimized.

10. The method of any one of claims 6-8, wherein the nucleic acid sequence encoding a heterologous antigen is codon-optimized.

11. A composition comprising a replication-deficient, E1 region-deleted, serotype 7 monkey adenoviral vector comprising a sequence having at least about 80% identity to the sequence of SEQ ID NO:12.

12. A composition comprising a replication-deficient, E1 region-deleted, human serotype 5 adenoviral vector comprising a sequence having at least about 80% identity to a sequence selected from the group consisting of SEQ ID NO:10 and SEQ ID NO:11.

FIG. 1


Adenovirus
E1
E1 region replaced with
transgene expression cassette
SAV7 vector
Promoter
cDNA
Poly(A)
site

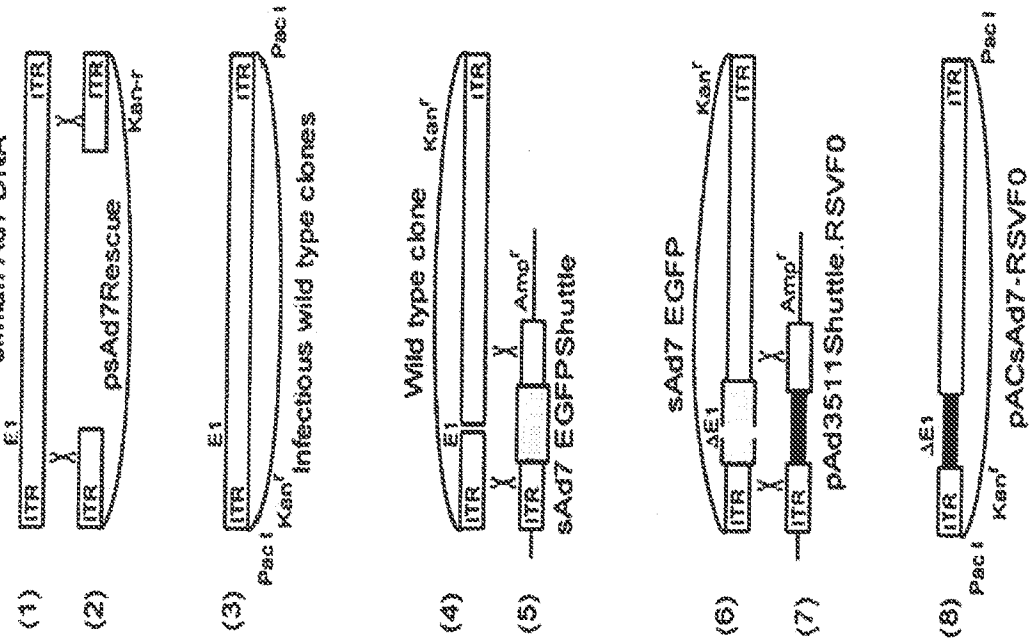

simian Ad7 DNA
E1
ITR
ITR
(1)
psAd7Rescue
ITR
ITR
Kan-r
(2)
Infectious wild type clones
E1
ITR
ITR
Pac I
Pac I
Kan^r
(3)
Wild type clone
Kan^r
E1
ITR
ITR
(4)
sAd7 EGFPShuttle
Amp^r
ITR
ITR
(5)
sAd7 EGFP
Kan^r
ΔE1
ITR
ITR
(6)
pAd35.11Shuttle.RSVF0
Amp^r
ITR
ITR
(7)
pACsAd7-RSVF0
ΔE1
ITR
ITR
Pac I
Kan^r
Pac I
(8)

FIG. 2

FIG. 3

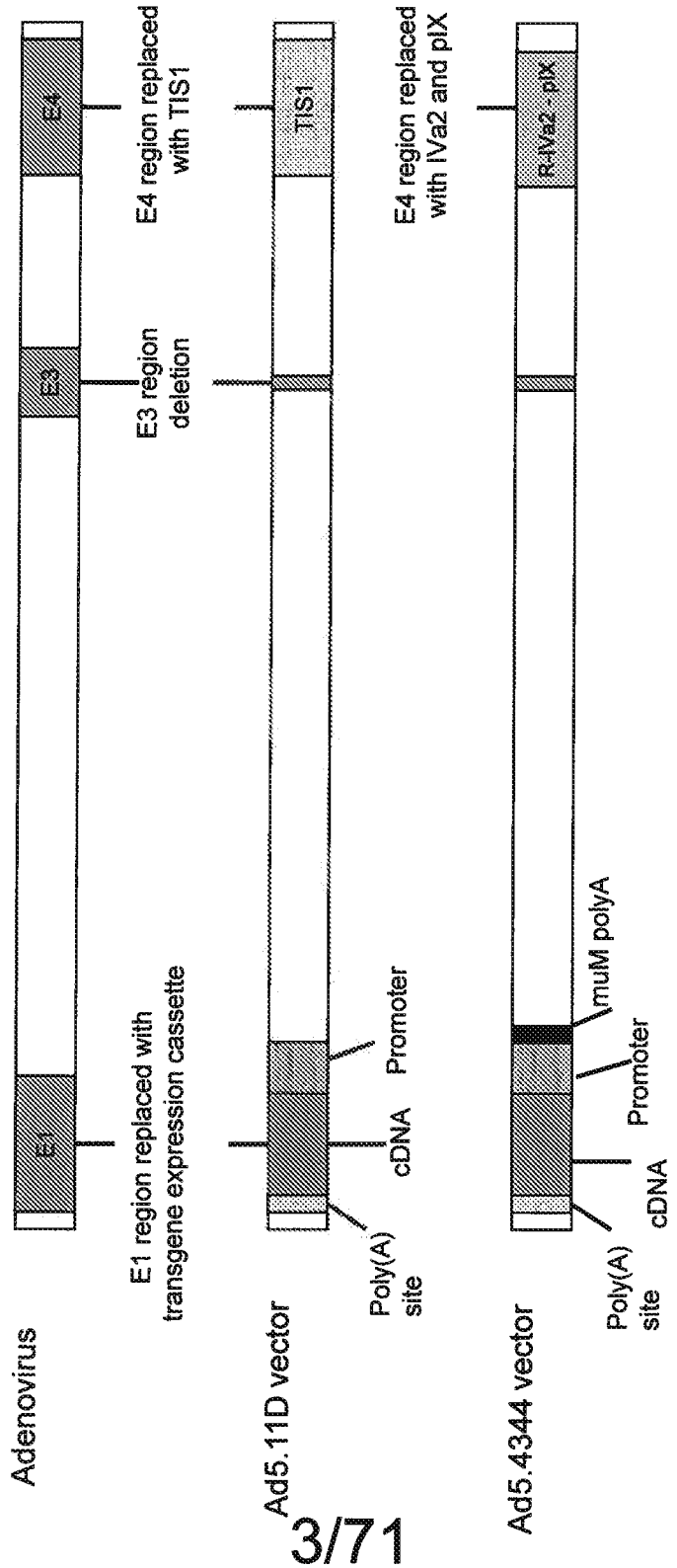
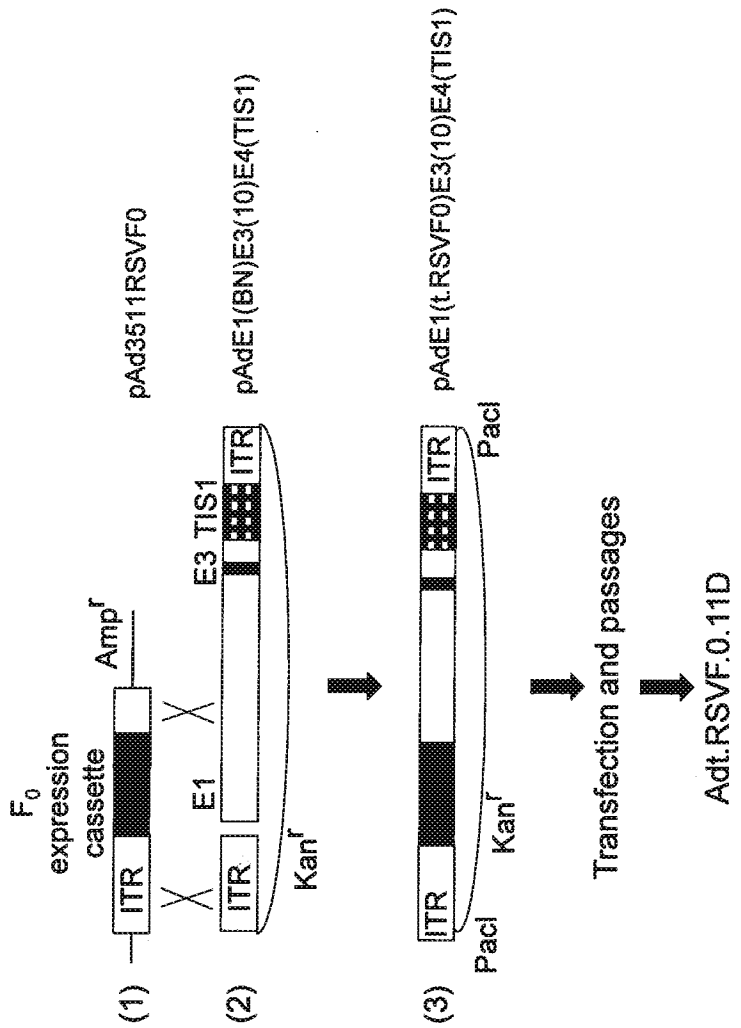

Adenovirus
E1
E3
E4
E1 region replaced with transgene expression cassette
E3 region deletion
E4 region replaced with TIS1
Ad5.11D vector
Poly(A) site
cDNA
Promoter
TIS1
E4 region replaced with IVa2 and pIX
Ad5.4344 vector
Poly(A) site
cDNA
Promoter
muM polyA
R-IVa2 - pIX

FIG. 4


pAd3511RSVF0
F_0 expression cassette
Amp^r
ITR
E1
E3
TIS1
ITR
Kan^r
(1)
(2)
pAdE1(BN)E3(10)E4(TIS1)
pAdE1(t.RSVF0)E3(10)E4(TIS1)
PacI
PacI
(3)
Transfection and passages
Adt.RSVF.0.11D

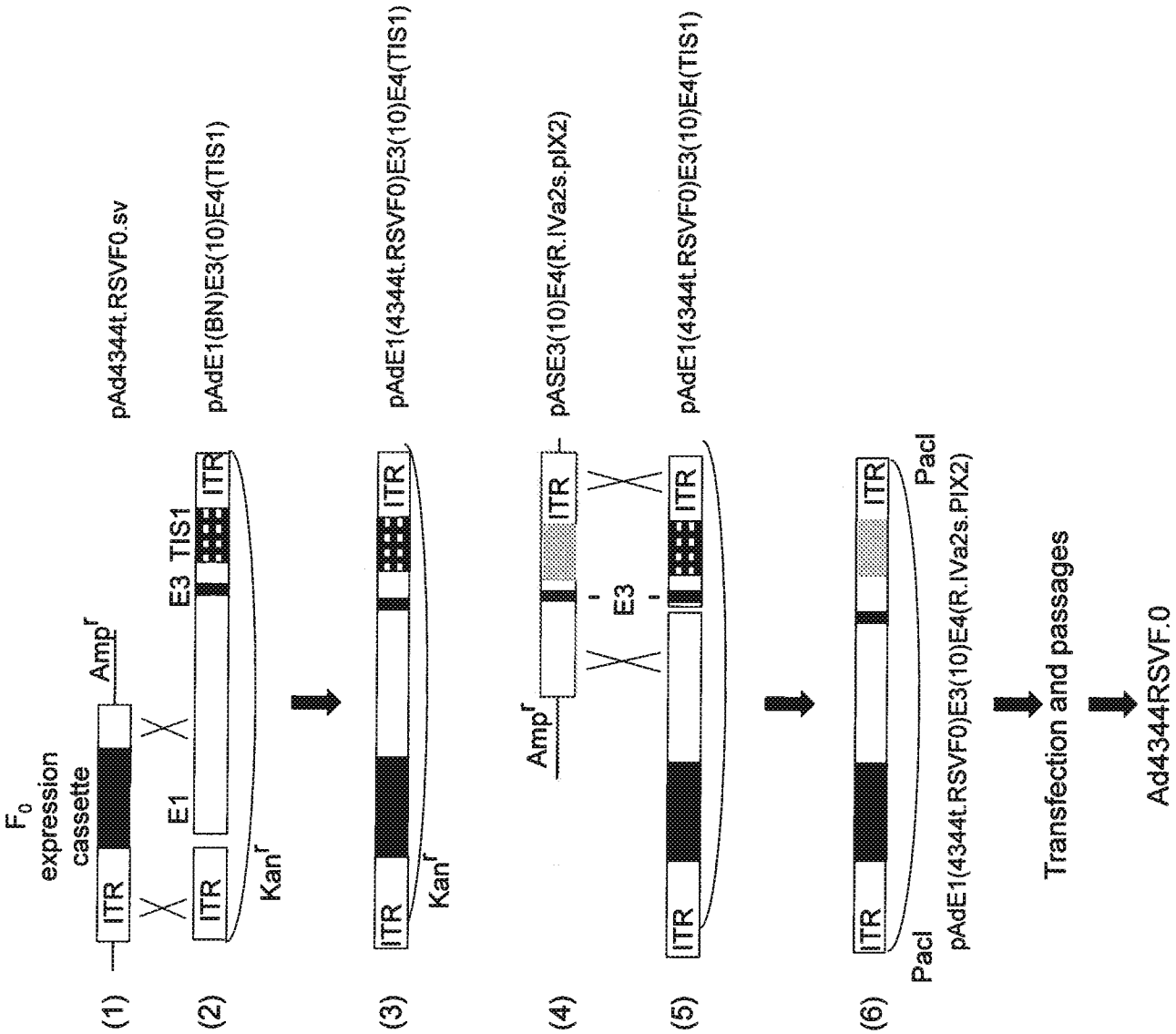

F0 expression cassette
Amp^r
ITR
pAd43441.RSVF0.sv
(1)
E1
E3
TIS1
ITR
ITR
Kan^r
pAdE1(BN)E3(10)E4(TIS1)
(2)
ITR
ITR
Kan^r
pAdE1(4344t.RSVF0)E3(10)E4(TIS1)
(3)
Amp^r
ITR
pASE3(10)E4(R.IVa2s.pIX2)
(4)
E3
ITR
ITR
pAdE1(4344t.RSVF0)E3(10)E4(TIS1)
(5)
ITR
ITR
PacI
PacI
pAdE1(4344t.RSVF0)E3(10)E4(R.IVa2s.PIX2)
(6)
Transfection and passages
Ad4344RSVF.0

FIG. 5

FIG. 6

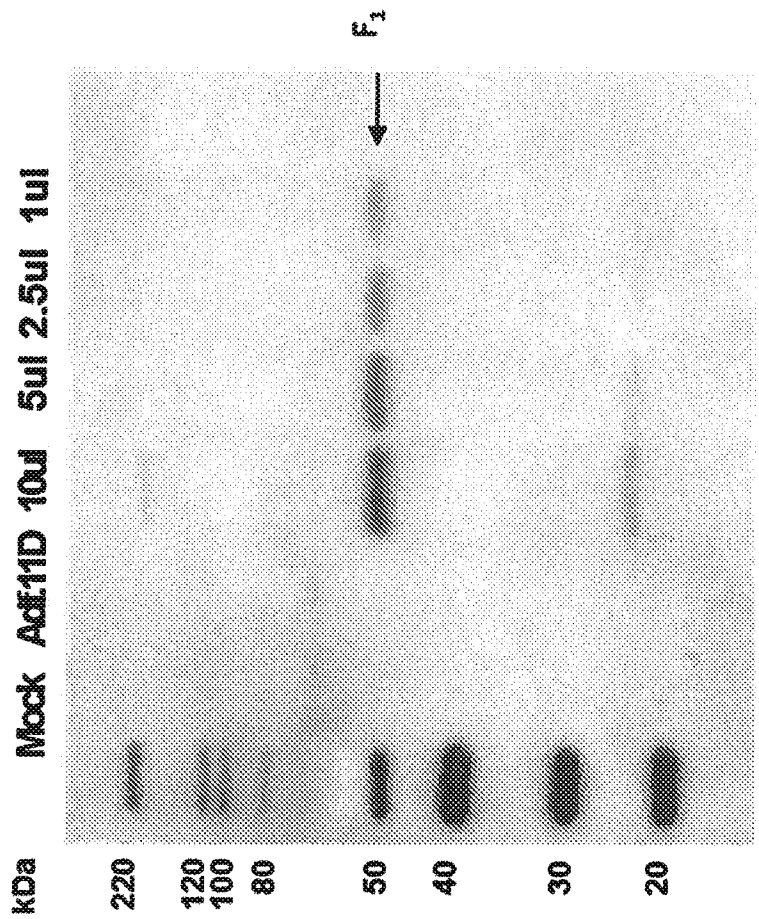

Ad5-F
Mock
Ad E1 1D
10ul
5ul
2.5ul
1ul
F1
kDa
220
120
100
80
50
40
30
20

FIG. 7

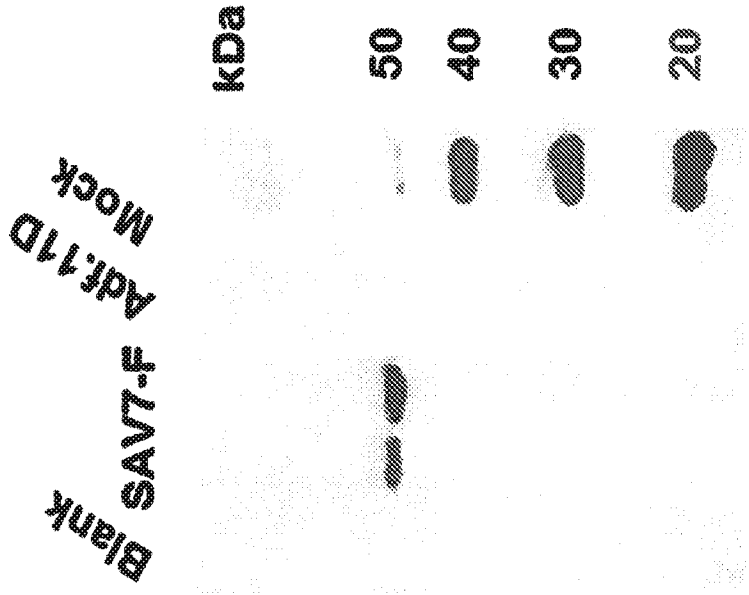


FIG. 8

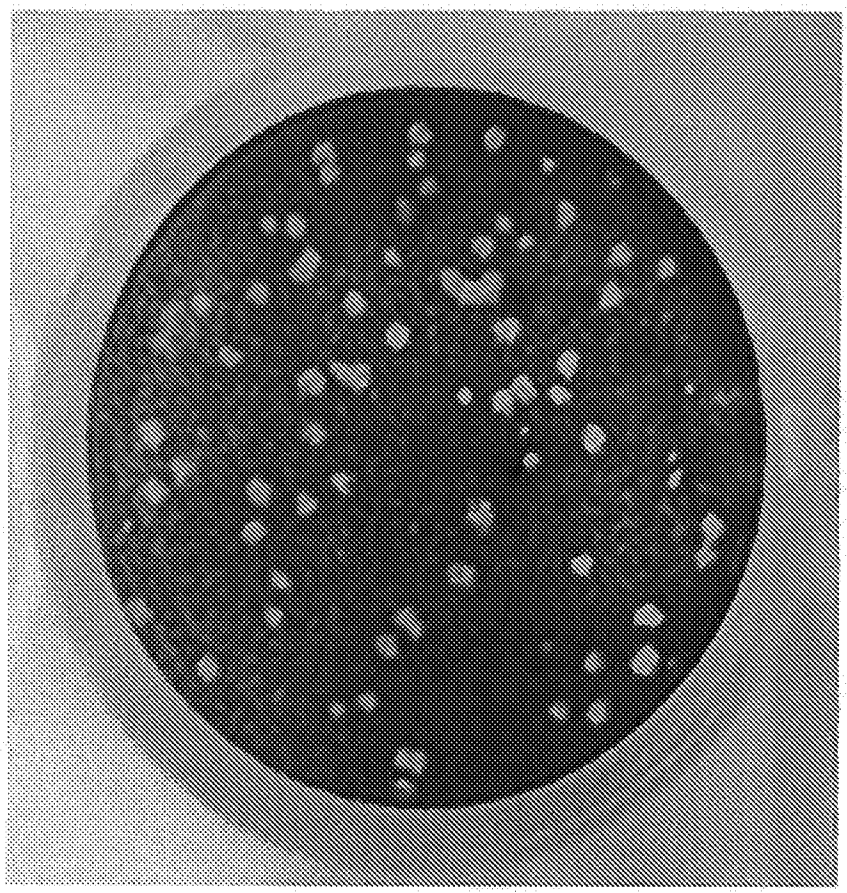


FIG. 9

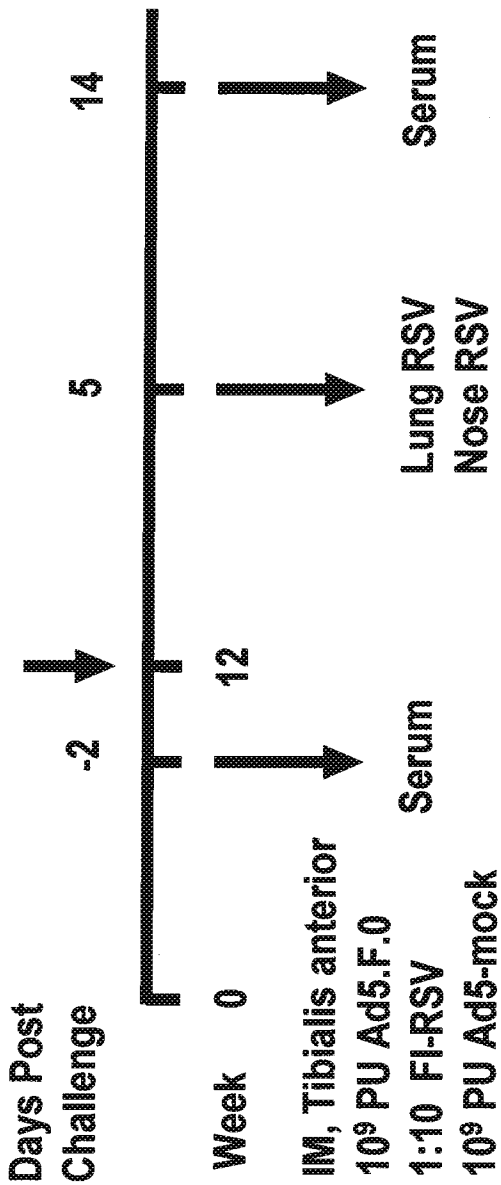

Days Post Challenge
Week
0
12
-2
5
14
IM, Tibialis anterior
10⁹ PU Ad5.F.0
1:10 FI-RSV
10⁹ PU Ad5-mock
N=5 per group
Serum
RSV Challenge
Lung RSV
Nose RSV
Serum

FIG. 10

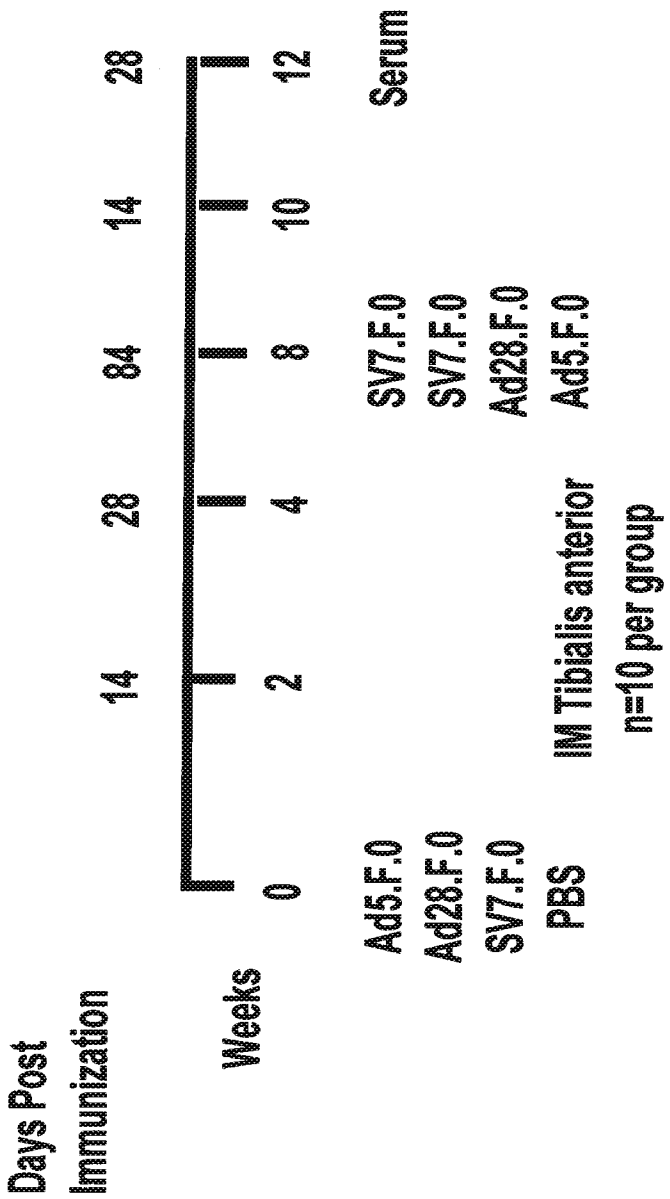

Days Post Immunization
14
28
84
14
28
Weeks
0
2
4
8
10
12
Ad5.F.0
Ad28.F.0
SV7.F.0
PBS
SV7.F.0
SV7.F.0
Ad28.F.0
Ad5.F.0
Serum
IM Tibialis anterior
n=10 per group

FIG. 11

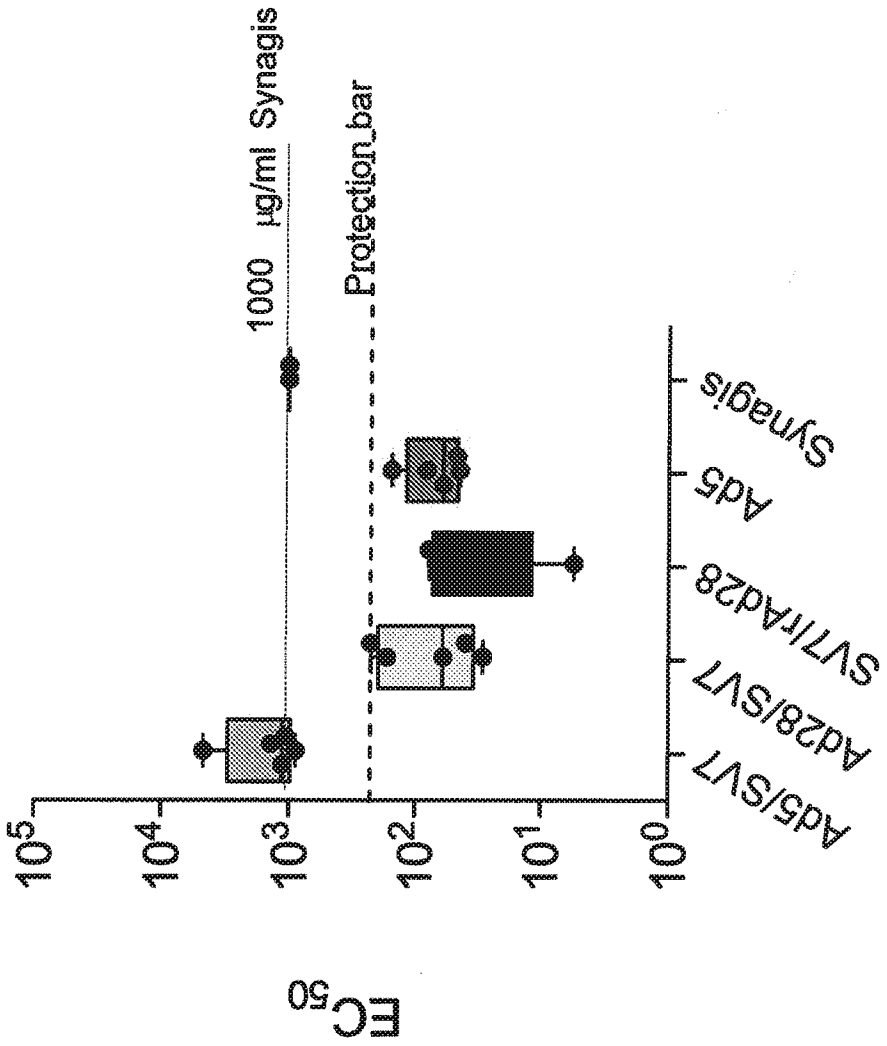

1000 µg/ml Synagis
Protection_bar
Ad5/SVT
Ad28/SVT
SVT/rAd28
Ad5
Synagis
10^0
10^1
10^2
10^3
10^4
10^5
EC_{50}

FIG. 12

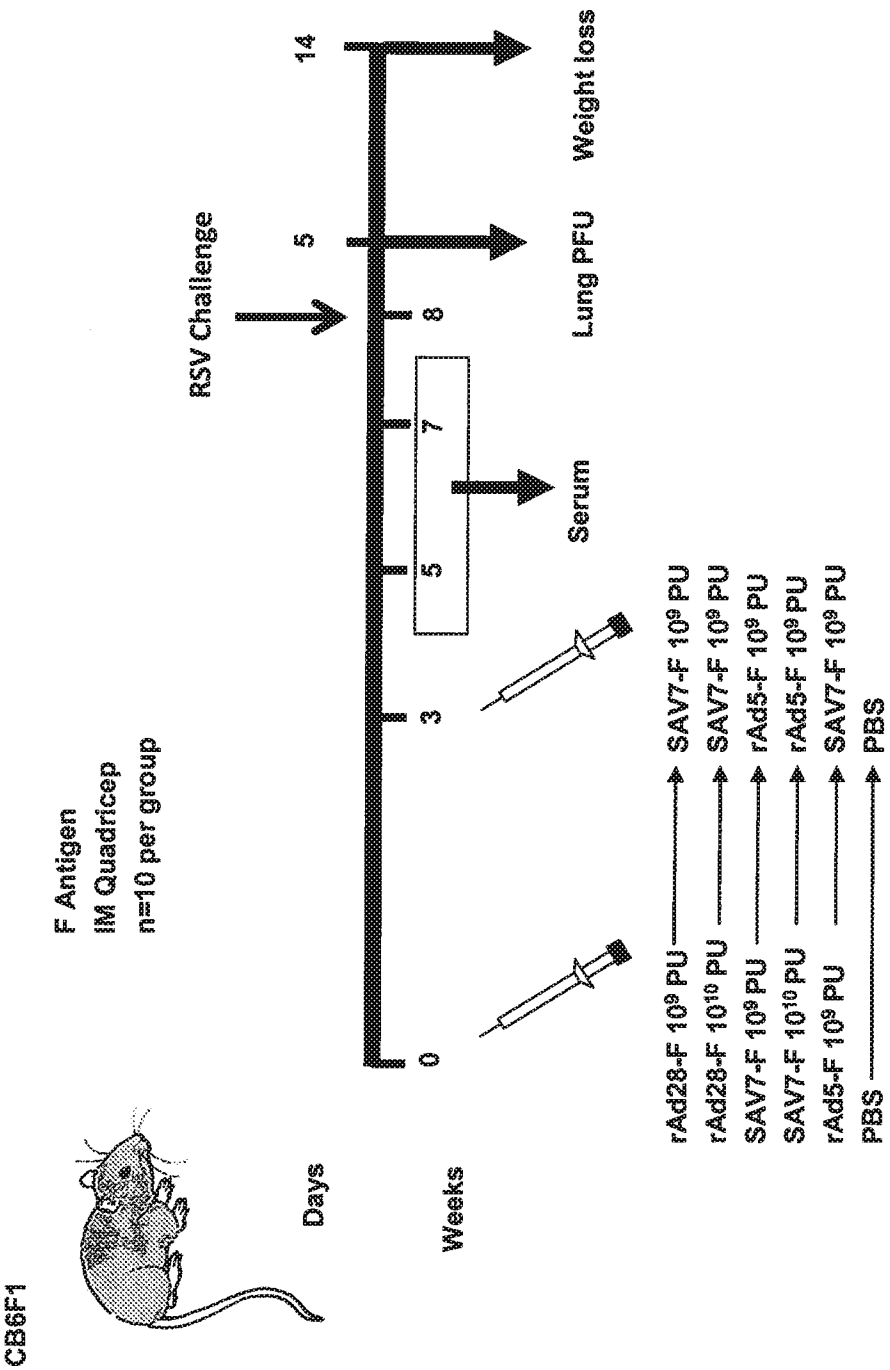

CB6F1
F Antigen
IM Quadricep
n=10 per group
Days
Weeks
0
3
5
7
8
14
RSV Challenge
Serum
Lung PFU
Weight loss
rAd28-F 10^9 PU
rAd28-F 10^10 PU
SAV7-F 10^9 PU
SAV7-F 10^10 PU
rAd5-F 10^9 PU
PBS
SAV7-F 10^9 PU
SAV7-F 10^9 PU
rAd5-F 10^9 PU
rAd5-F 10^9 PU
SAV7-F 10^9 PU
PBS

FIG. 13(A)

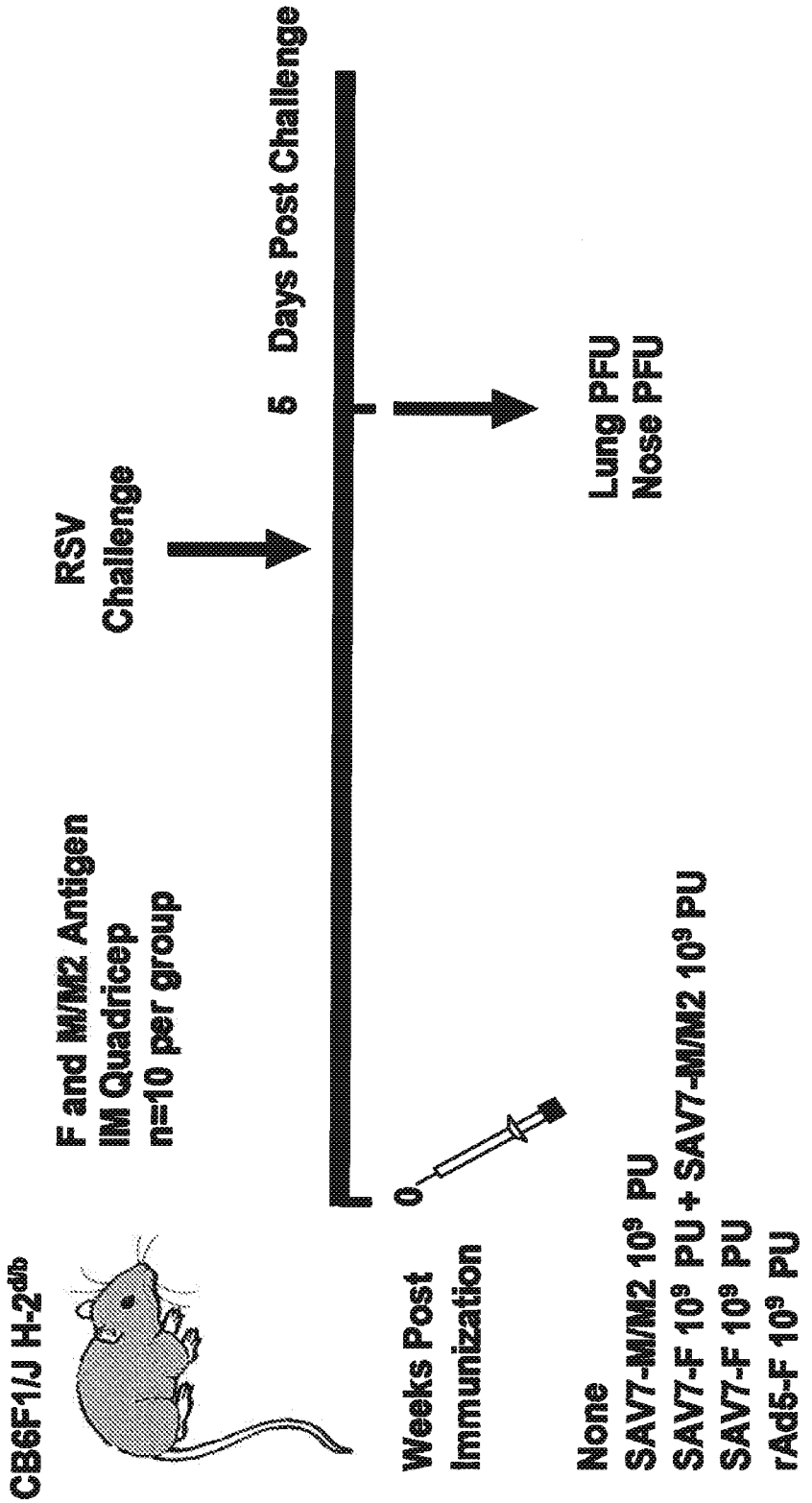

CB6F1/J H-2db
F and M/M2 Antigen
IM Quadricep
n=10 per group
RSV
Challenge
5 Days Post Challenge
Lung PFU
Nose PFU
0
Weeks Post
Immunization
None
SAV7-M/M2 10^9 PU
SAV7-F 10^9 PU + SAV7-M/M2 10^9 PU
SAV7-F 10^9 PU
rAd5-F 10^9 PU

FIG. 13(B)

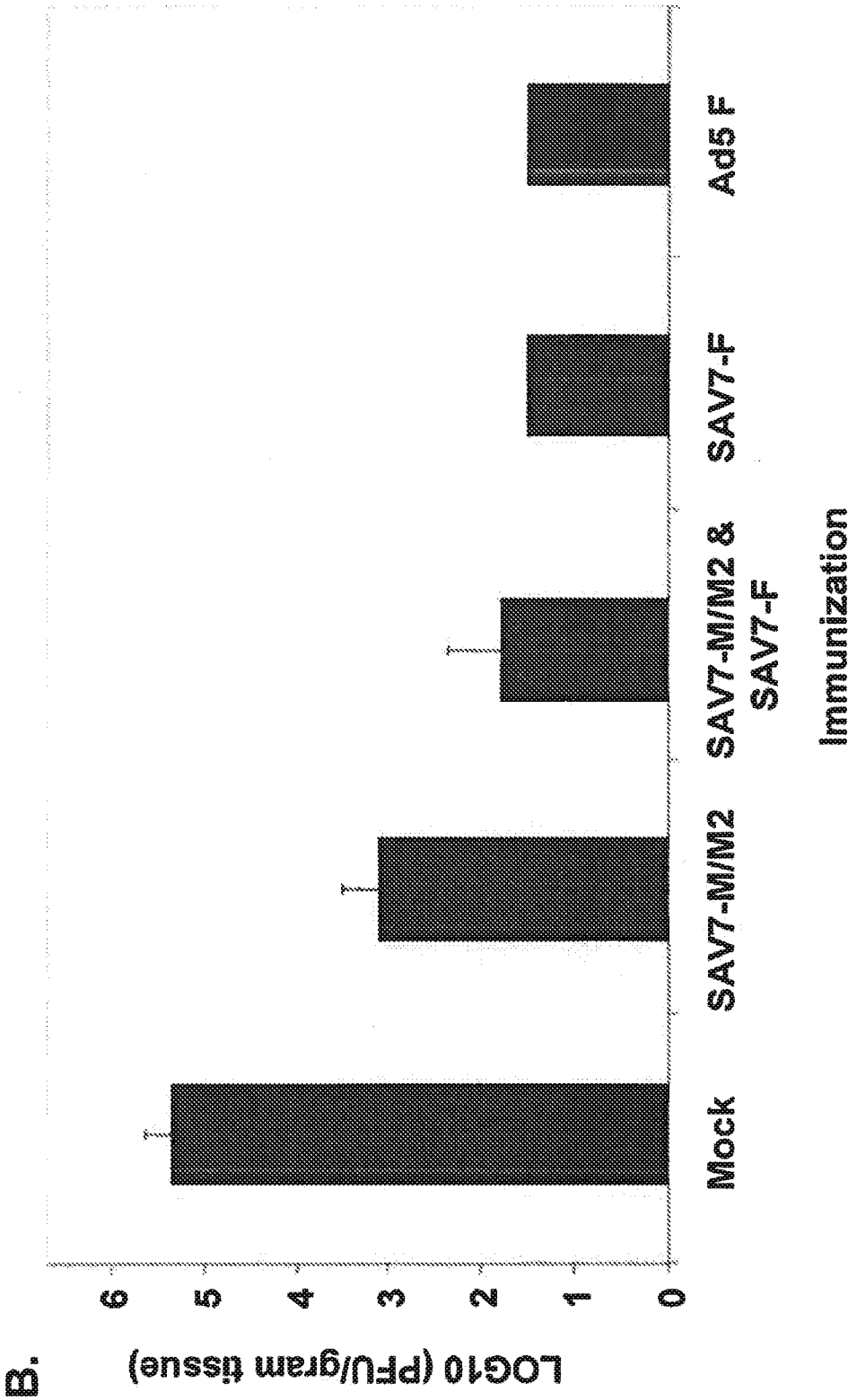

B.
LOG10 (PFU/gram tissue)
0
1
2
3
4
5
6
Mock
SAV7-M/M2
SAV7-M/M2 & SAV7-F
SAV7-F
Ad5 F
Immunization

FIG. 13(C)

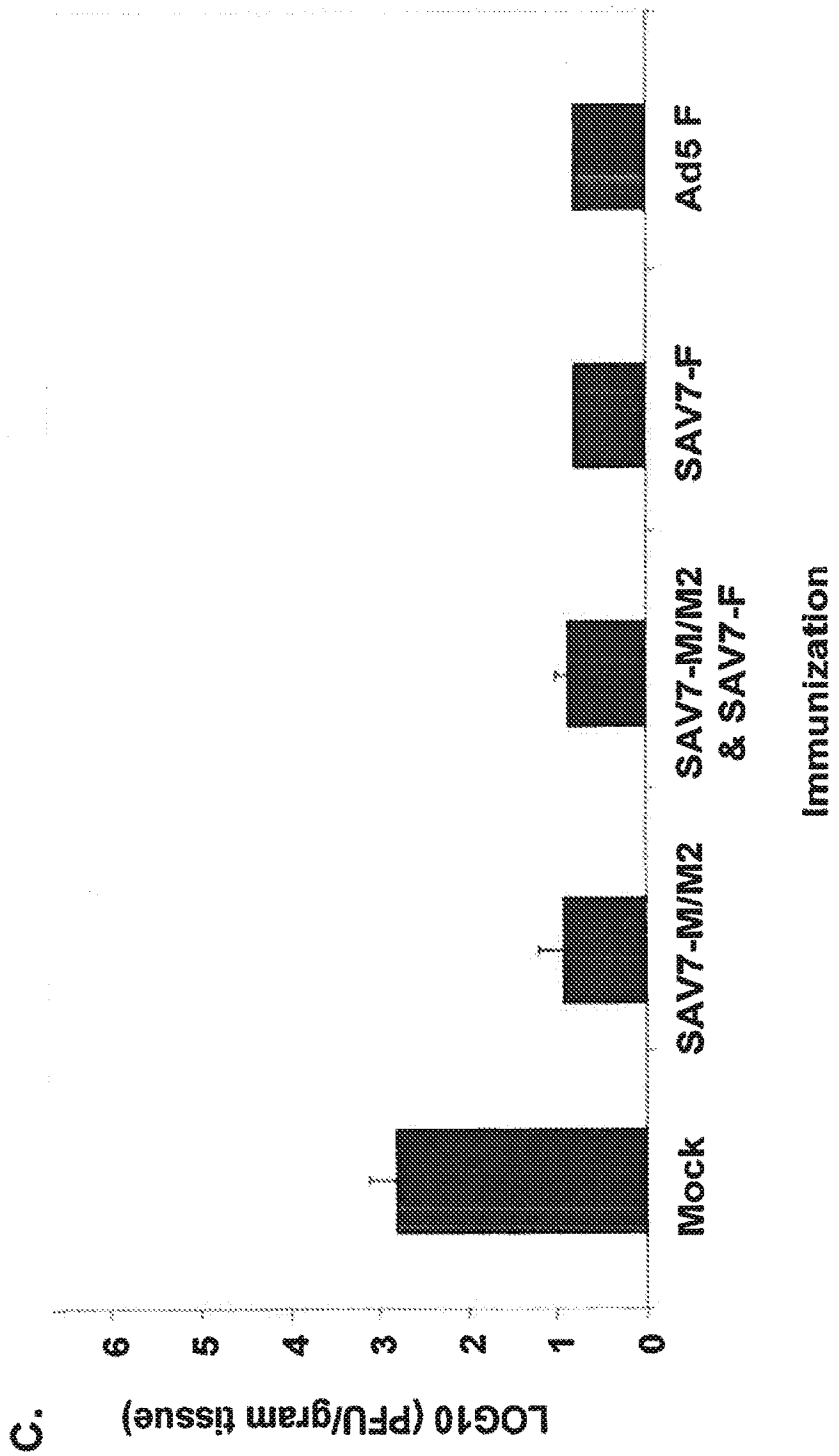

C.
LOG10 (PFU/gram tissue)
0
1
2
3
4
5
6
Mock
SAV7-M/M2
SAV7-M/M2 & SAV7-F
SAV7-F
Ad5 F
Immunization

FIG. 14(A)

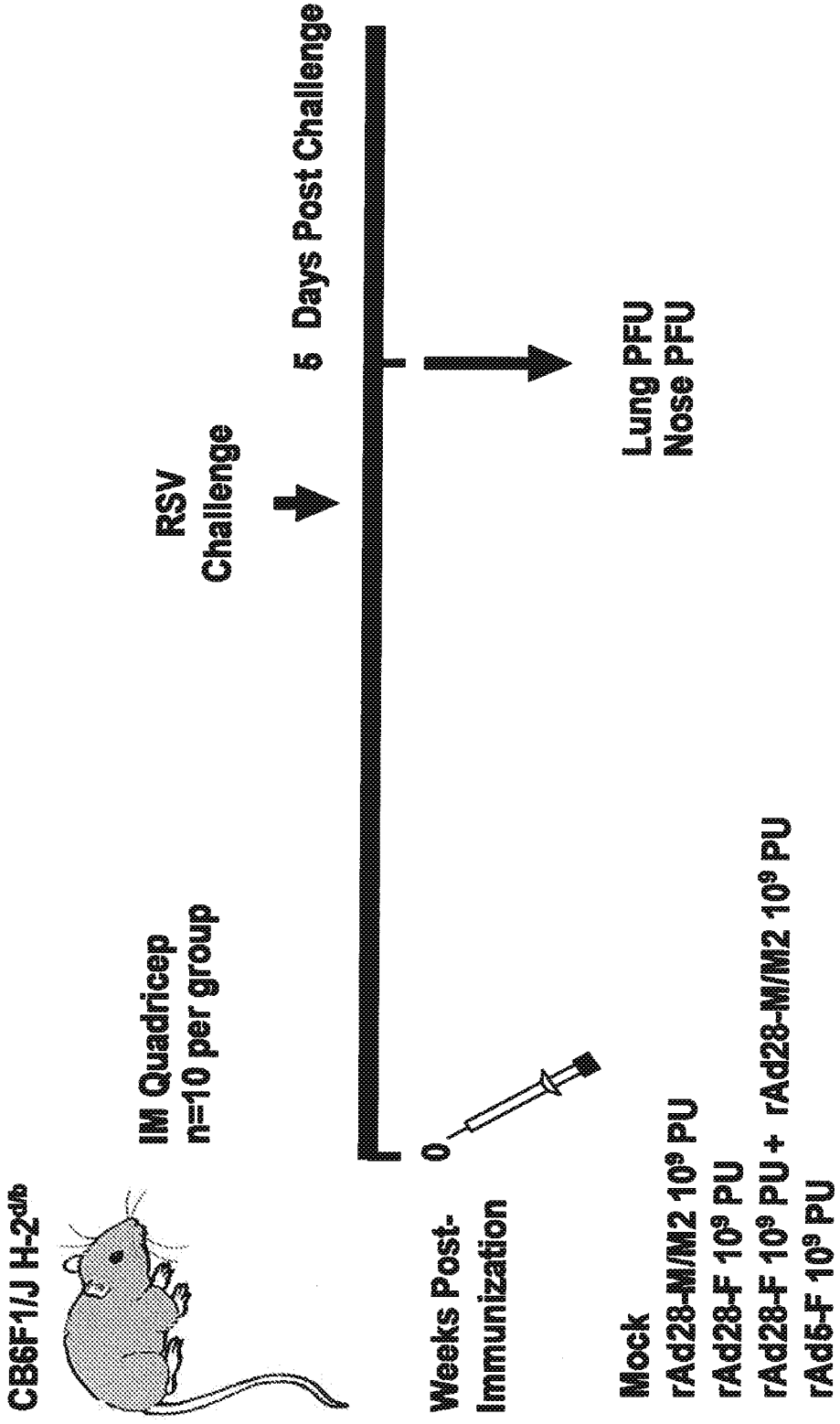

CB6F1/J H-2d/b
IM Quadricep
n=10 per group
RSV
Challenge
5 Days Post Challenge
Lung PFU
Nose PFU
0
Weeks Post-
Immunization
Mock
rAd28-M/M2 10^9 PU
rAd28-F 10^9 PU
rAd28-F 10^9 PU + rAd28-M/M2 10^9 PU
rAd5-F 10^9 PU

FIG. 14(B)

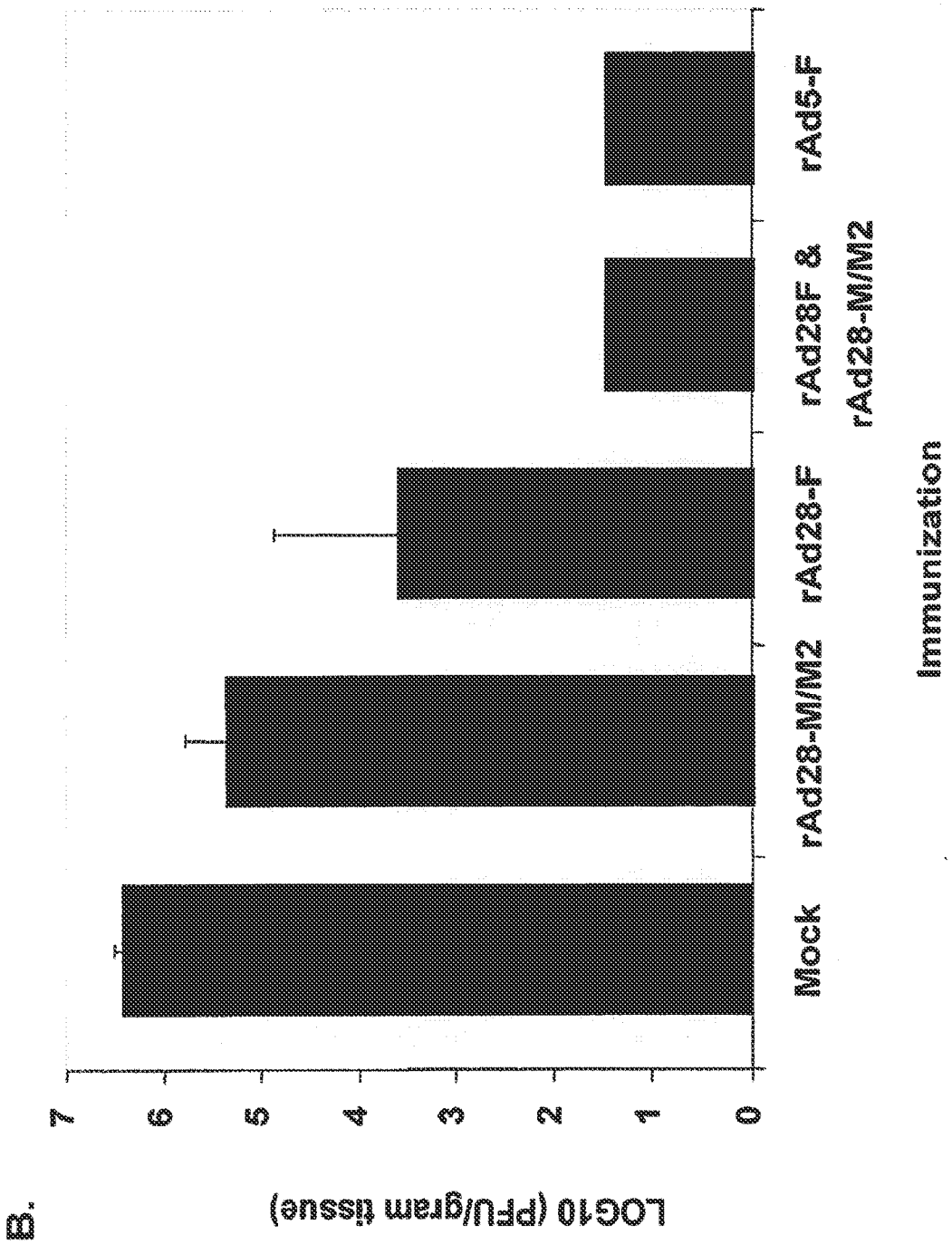

B.
LOG10 (PFU/gram tissue)
0
1
2
3
4
5
6
7
Mock
rAd28-M/M2
rAd28-F
rAd28F & rAd28-M/M2
rAd5-F
Immunization

FIG. 14(C)

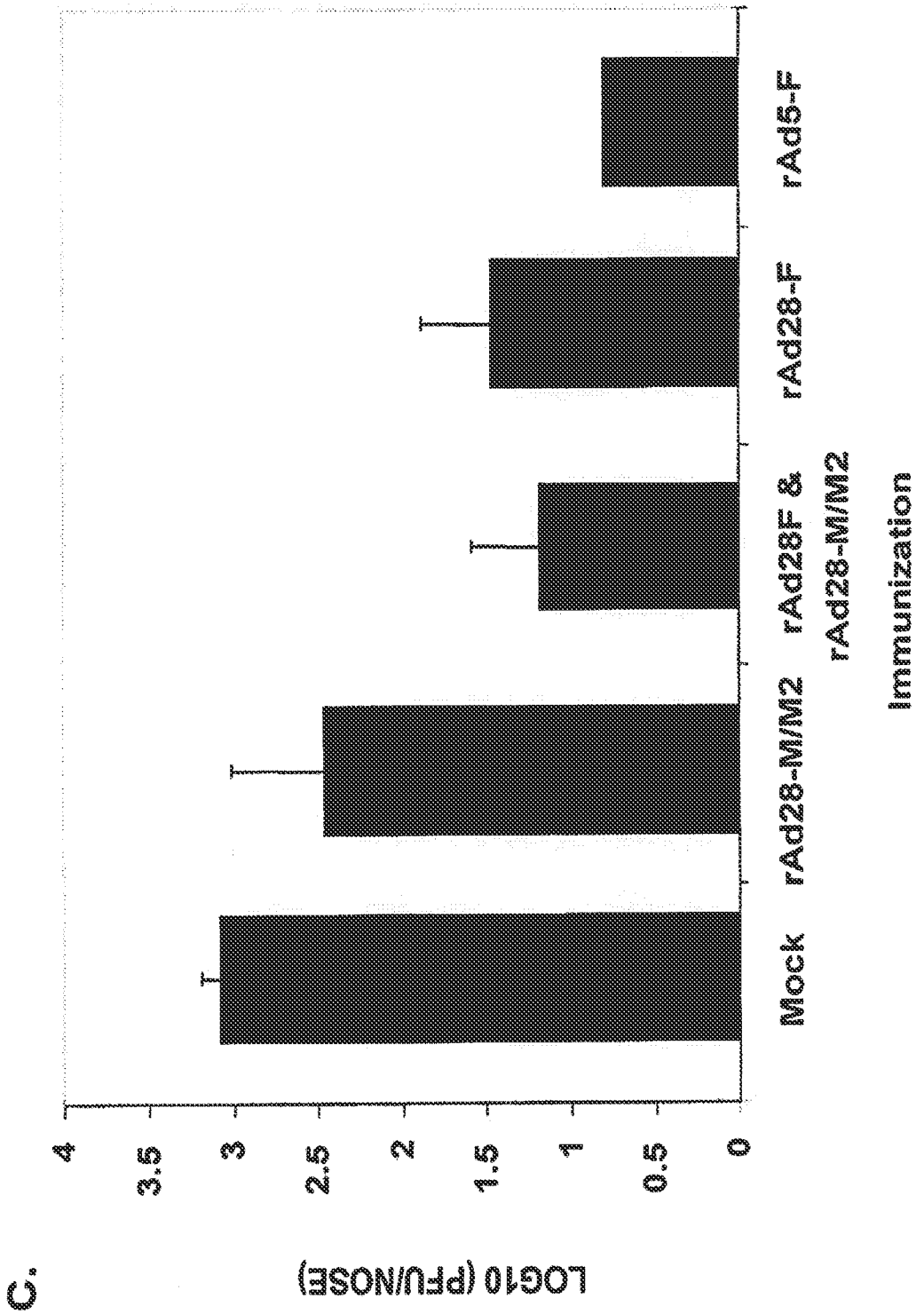

C.
LOG10 (PFU/NOSE)
4
3.5
3
2.5
2
1.5
1
0.5
0
Mock
rAd28-M/M2
rAd28F & rAd28-M/M2
rAd28-F
rAd5-F
Immunization

FIG. 15

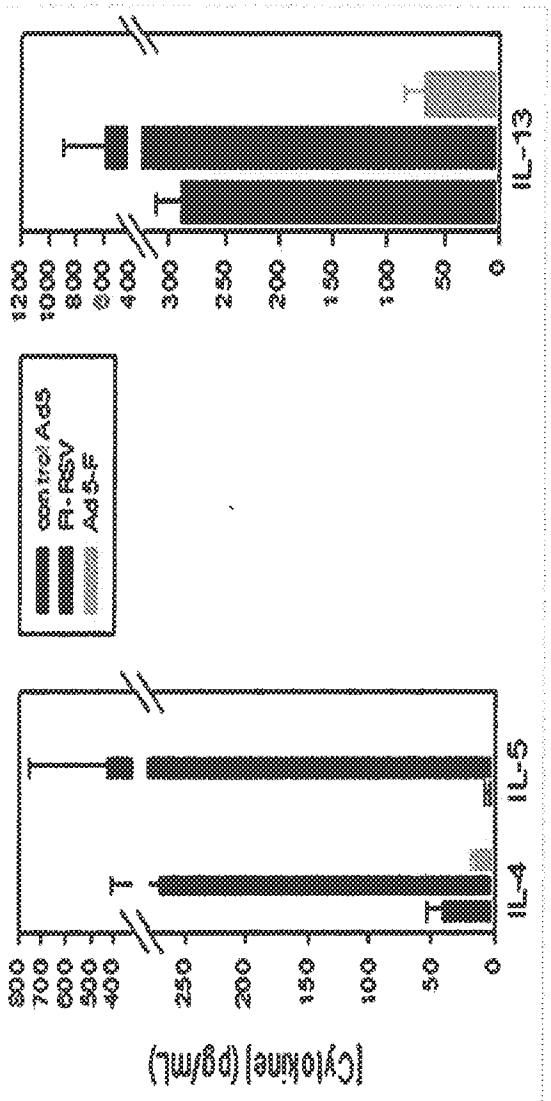


FIG. 16

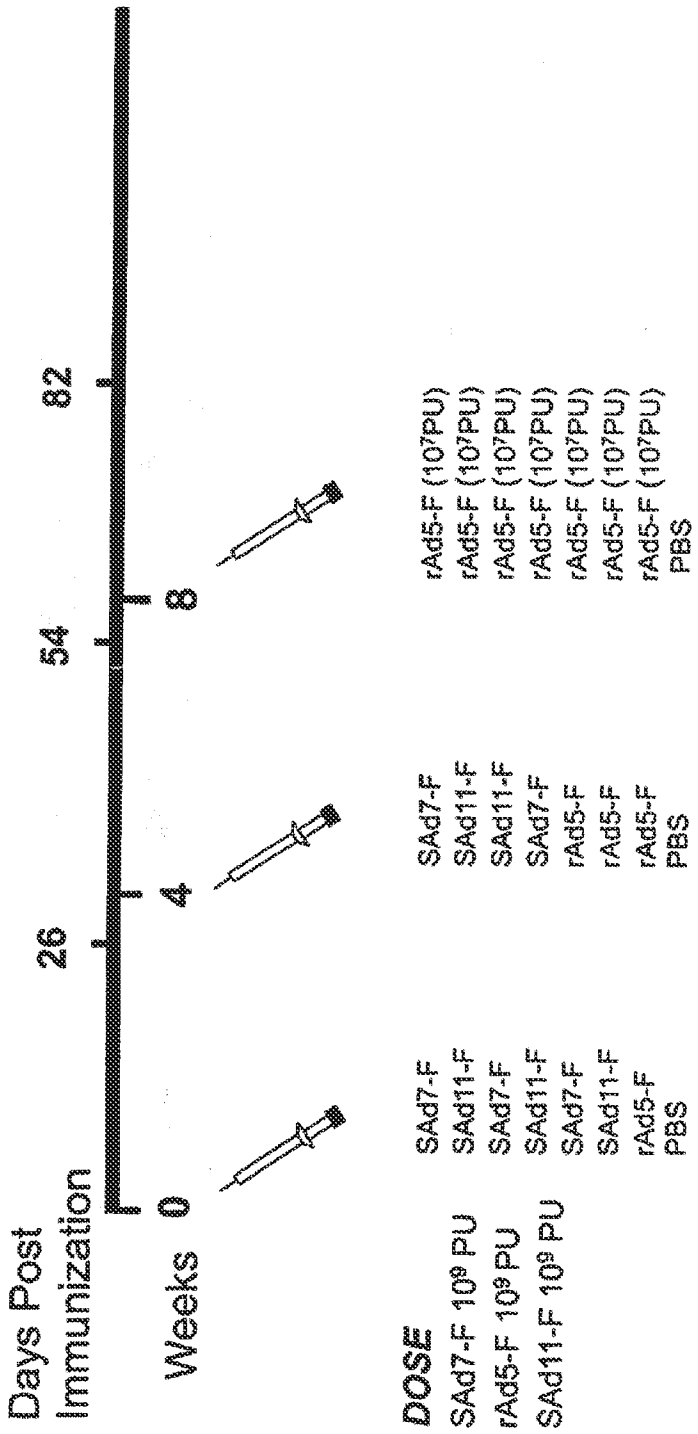

Days Post Immunization
Weeks
0
4
8
26
54
82
Serum
Serum
Serum
DOSE
SAd7-F 10⁹ PU
rAd5-F 10⁹ PU
SAd11-F 10⁹ PU
SAd7-F
SAd11-F
SAd7-F
SAd11-F
SAd7-F
SAd11-F
rAd5-F
PBS
SAd7-F
SAd11-F
SAd11-F
SAd7-F
rAd5-F
rAd5-F
rAd5-F
PBS
rAd5-F (10⁷PU)
rAd5-F (10⁷PU)
rAd5-F (10⁷PU)
rAd5-F (10⁷PU)
rAd5-F (10⁷PU)
rAd5-F (10⁷PU)
rAd5-F (10⁷PU)
PBS

FIG. 17

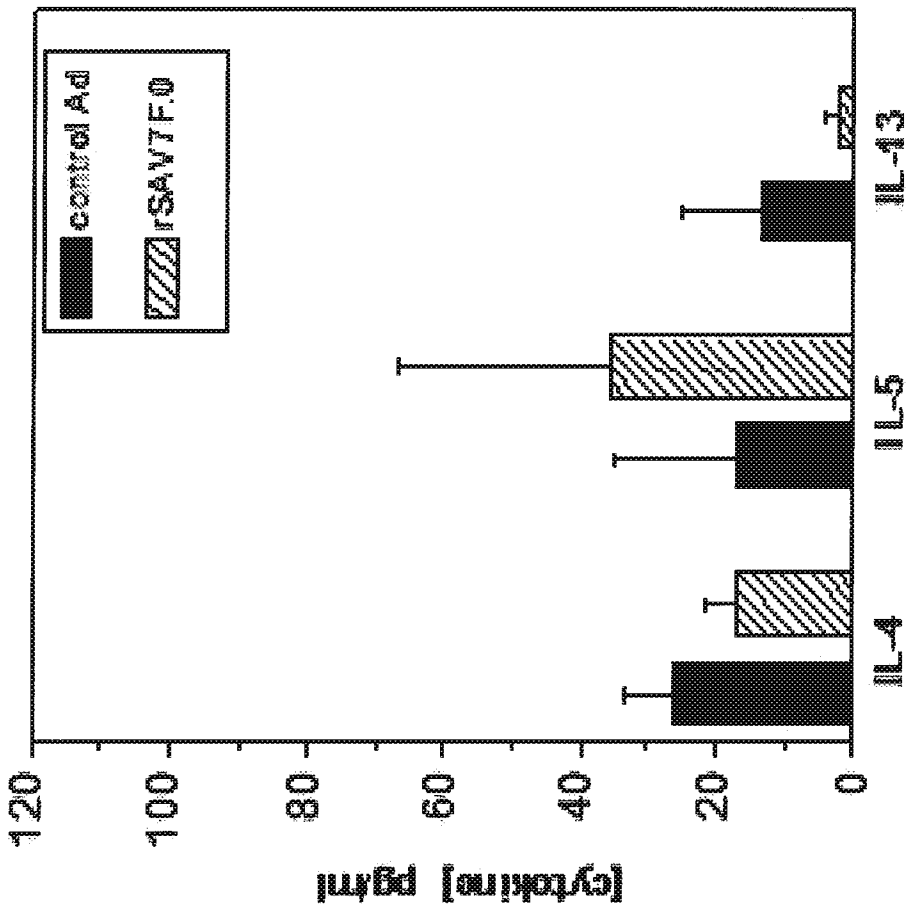

control Ad
rSAV7F.0
120
100
80
60
40
20
0
[cytokine] pg/ml
IL-4
IL-5
IL-13

FIG. 18

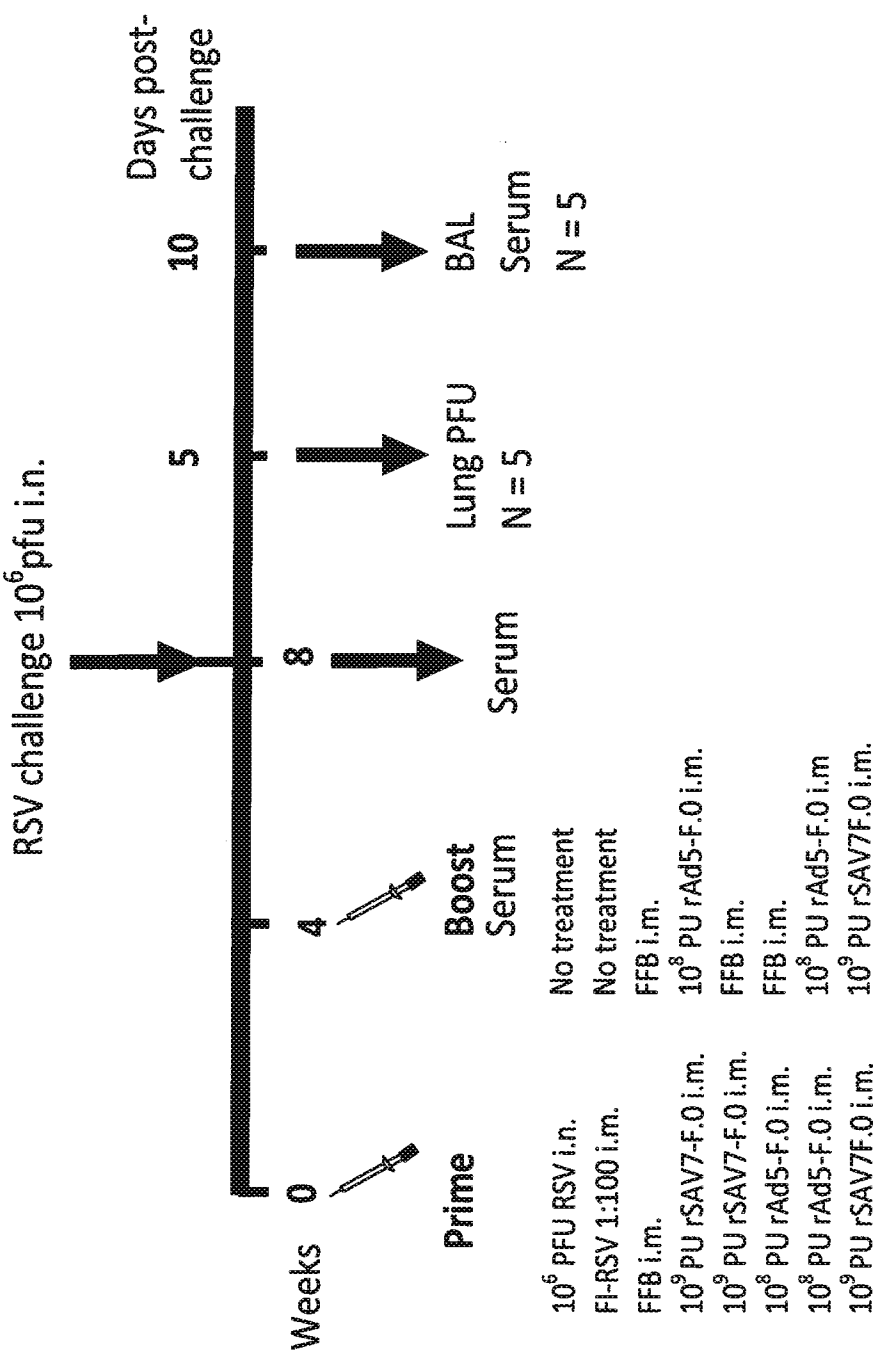

Days post-challenge
RSV challenge 10^6pfu i.n.
Weeks 0 4 8
5 10
Prime
Boost
Serum
Serum
Lung PFU
N = 5
BAL
Serum
N = 5
10^6 PFU RSV i.n.
FI-RSV 1:100 i.m.
FFB i.m.
10^9 PU rSAV7-F.0 i.m.
10^9 PU rSAV7-F.0 i.m.
10^8 PU rAd5-F.0 i.m.
10^8 PU rAd5-F.0 i.m.
10^9 PU rSAV7F.0 i.m.
No treatment
No treatment
FFB i.m.
10^8 PU rAd5-F.0 i.m.
FFB i.m.
FFB i.m.
10^8 PU rAd5-F.0 i.m
10^9 PU rSAV7F.0 i.m.

FIG. 19

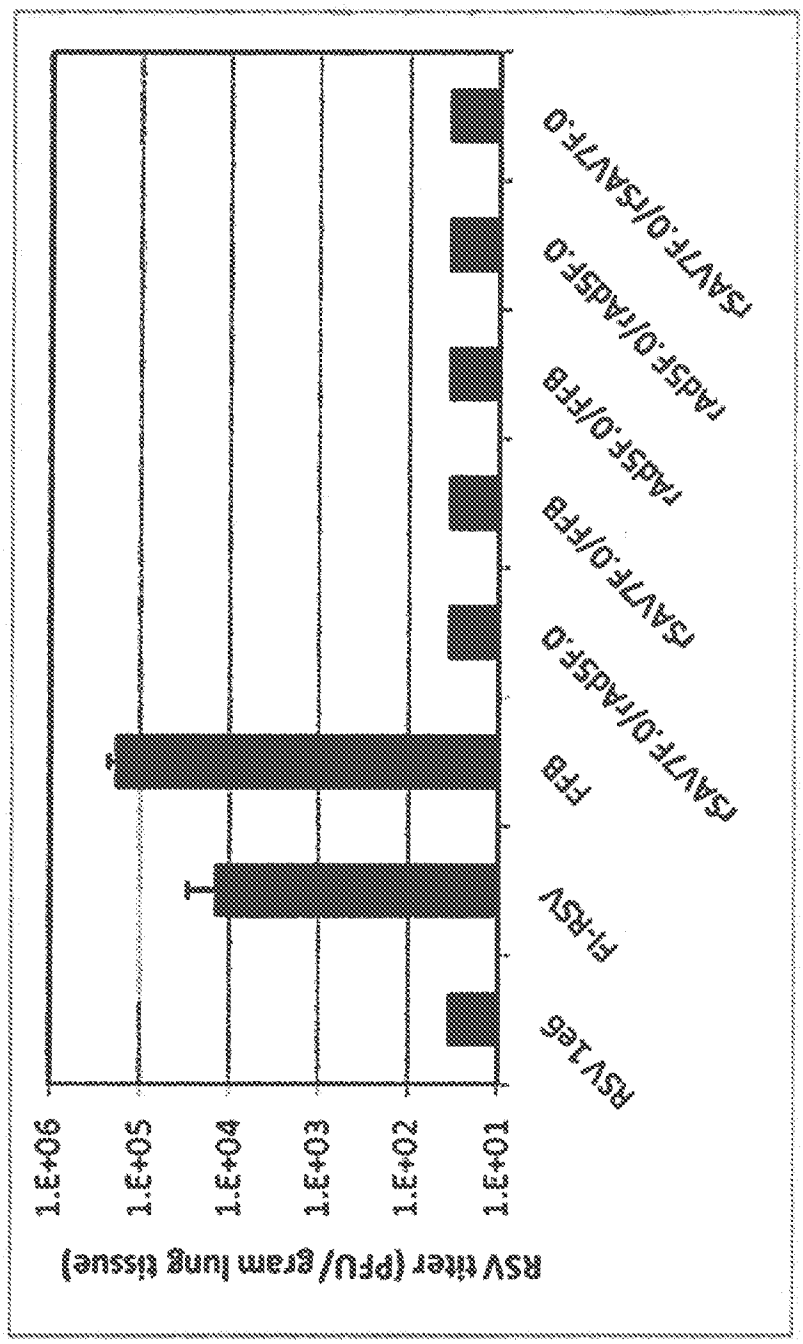

RSV titer (PFU/ gram lung tissue)
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
RSV Leo
Ft-RSV
FFB
rSAVTF.0/rADSF.0
rSAVTF.0/FFB
rADSF.0/FFB
rADSF.0/rADSF.0
rSAVTF.0/rSAVTF.0

FIG. 20

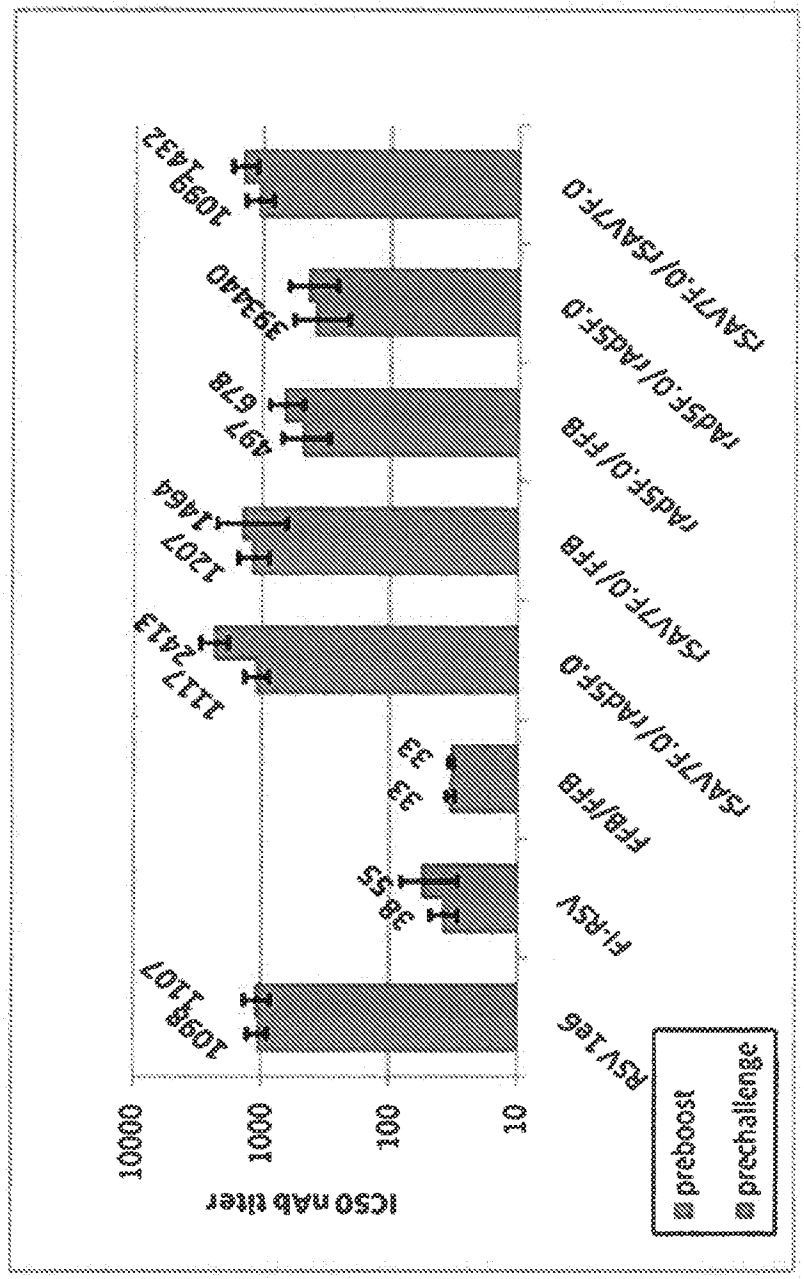

RSV1a6
FIRSV
FFB/FFB
fSAVTF.0/fADSF.0
fADSF.0/FFB
fSAVTF.0/FFB
fADSF.0/fADSF.0
fSAVTF.0/fSAVTF.0
preboost
prechallenge
ICS0 nAb titer
10000
1000
100
10
1098 1107
1 1
38 55
1 1
33 33
1 1
1117 2413
1 1
1207 1464
1 1
497 678
1 1
39340
1 1
1098 4332
1 1

FIG. 21

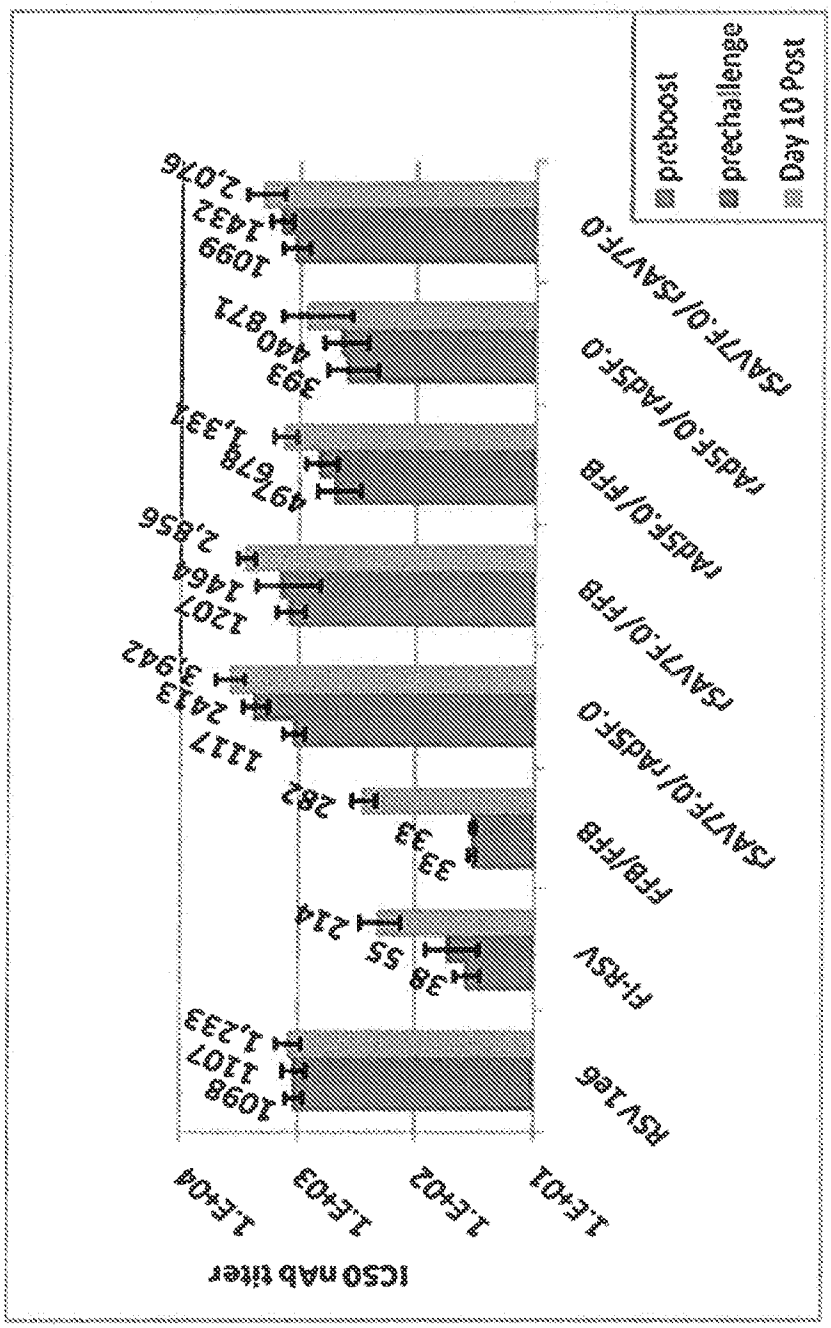

preboost
prechallenge
Day 10 Post
rSAVTF.0/rSAVTF.0
rAdSF.0/rAdSF.0
rAdSF.0/FFB
rSAVTF.0/FFB
rSAVTF.0/rAdSF.0
FFB/FFB
rHRSV
RSV16b
1098
1107
1,233
38
55
214
33
33
282
1117
2413
3,942
1207
1464
2,856
4976
1,331
393
440
871
1099
1432
2,076
1E+01
1E+02
1E+03
1E+04
IC50 nAb titer

FIG. 22

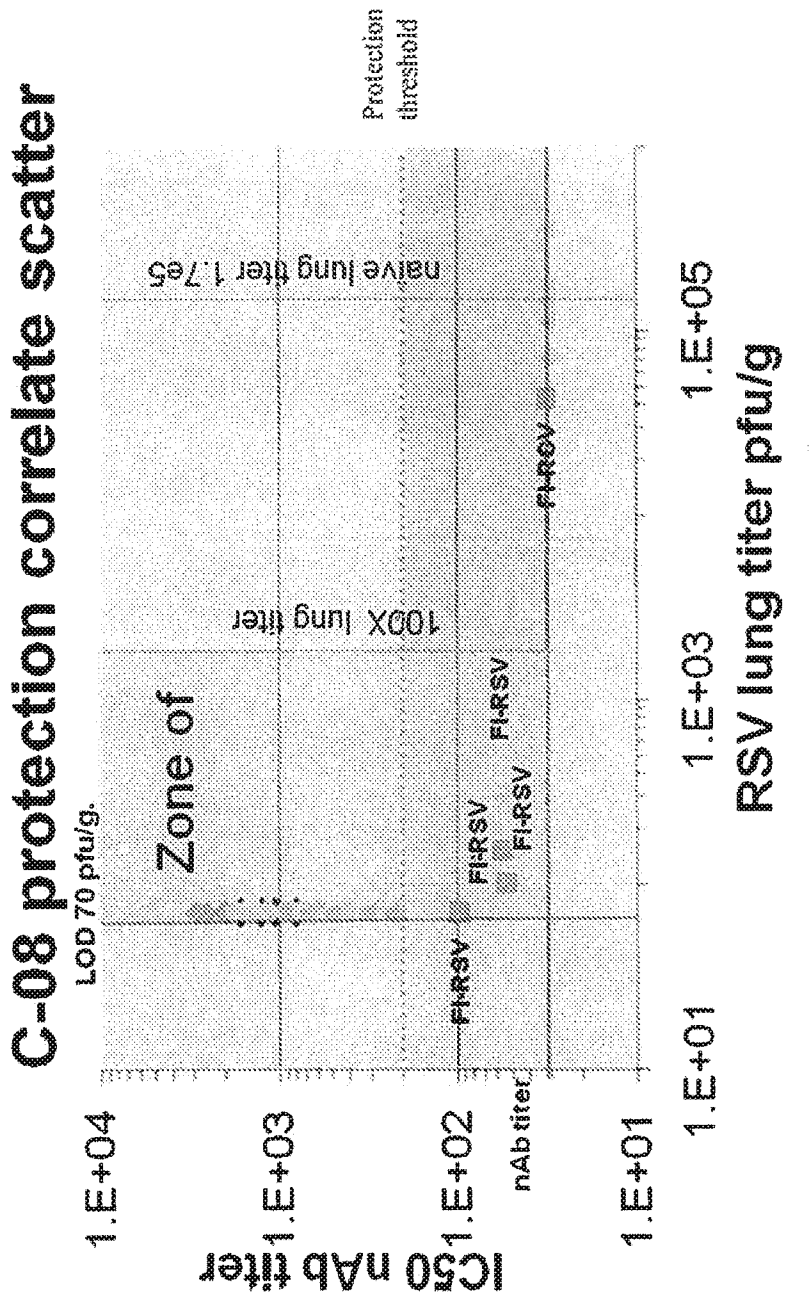

C-08 protection correlate scatter
LOD 70 pfu/g.
Zone of
100X lung titer
naive lung titer 1.7e5
Protection threshold
FI-RSV
FI-RSV
FI-RSV
FI-RSV
nAb titer
IC50 nAb titer
1.E+04
1.E+03
1.E+02
1.E+01
1.E+01
1.E+03
1.E+05
RSV lung titer pfu/g

FIG. 23

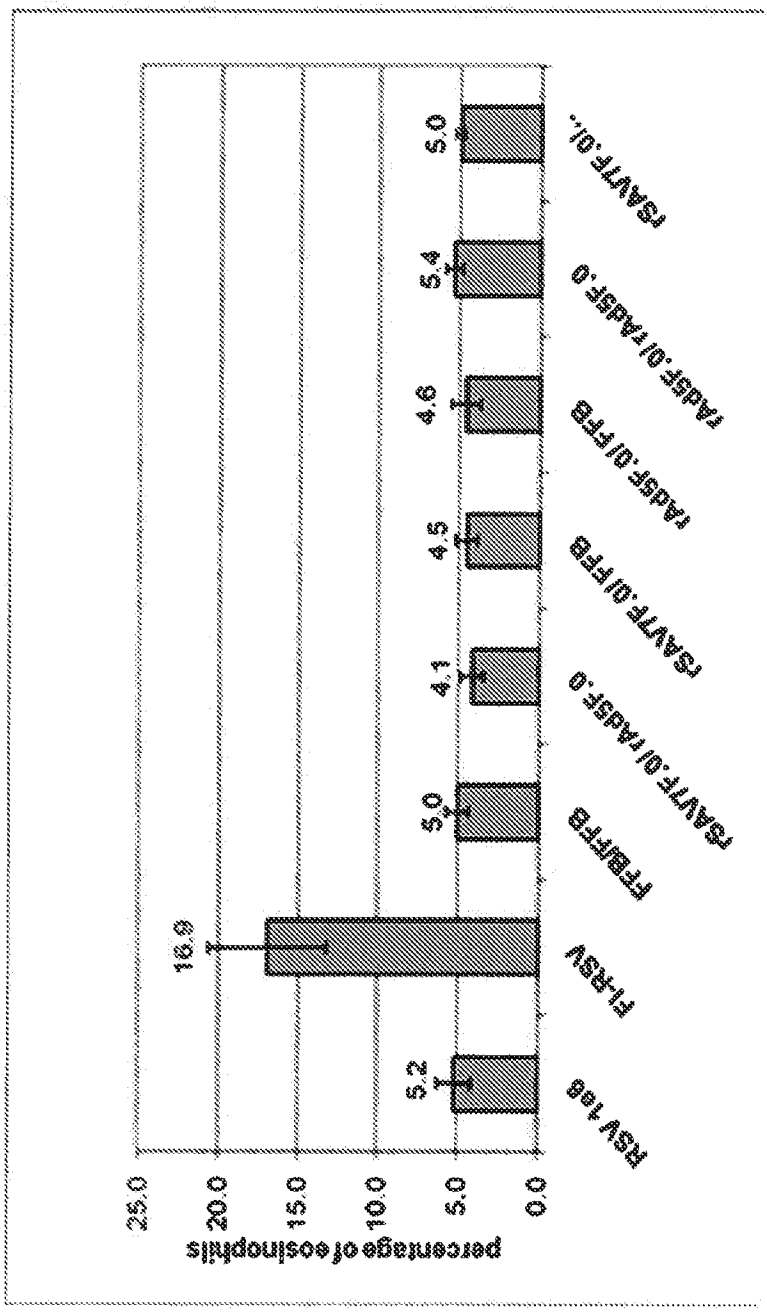

percentage of eosinophils
25.0
20.0
15.0
10.0
5.0
0.0
RSV 1a8
5.2
FI-RSV
16.9
FFB/FFB
5.0
rSAVTF.01/rADSF.0
4.1
rSAVTF.01/FFB
4.5
rADSF.01/FFB
4.6
rADSF.01/rADSF.0
5.4
rSAVTF.01...
5.0

FIG. 24

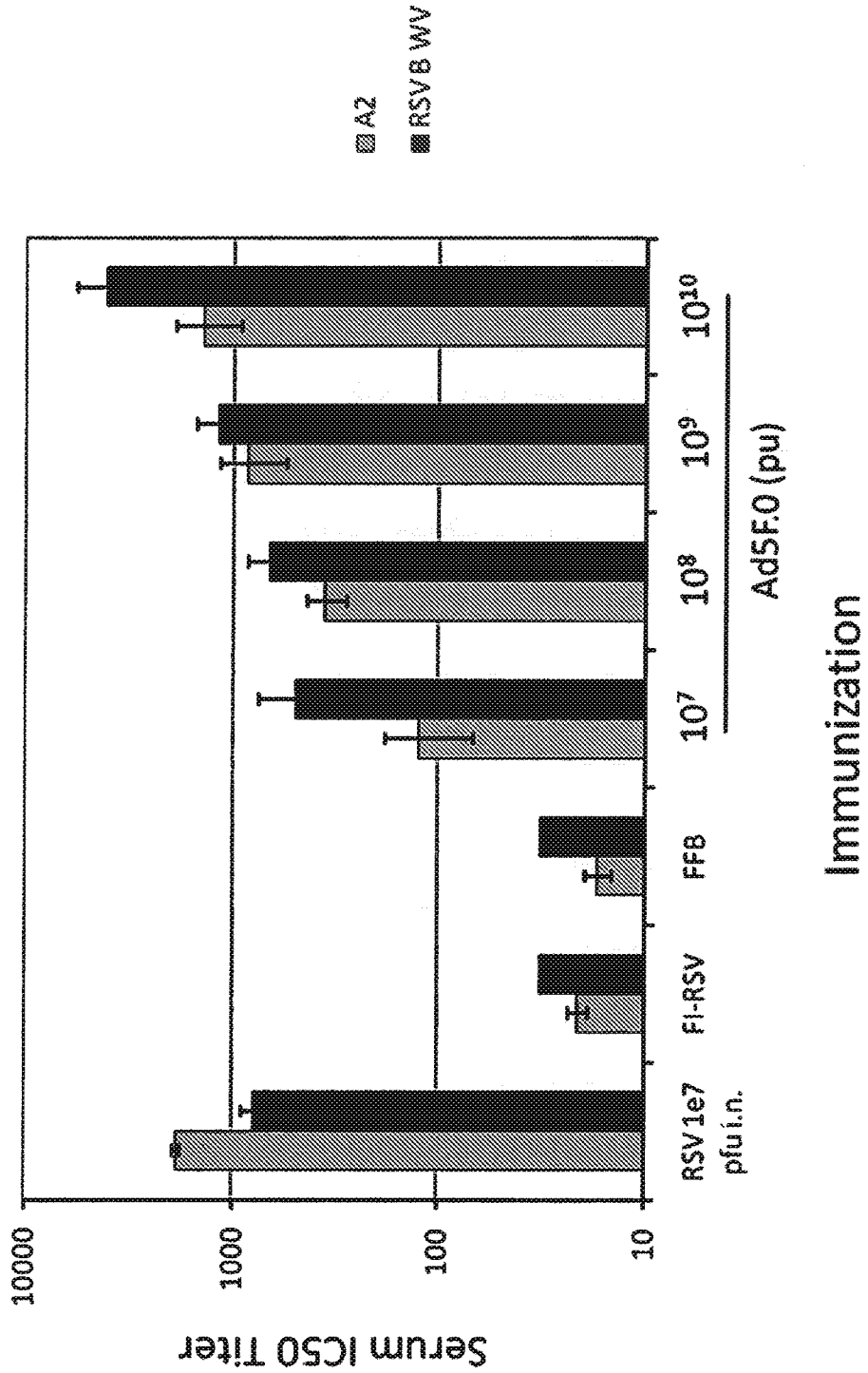

A2
RSV 8 WV
Serum IC50 Titer
10000
1000
100
10
RSV 1e7 pfu i.n.
F1-RSV
FFB
10^7
10^8
10^9
10^10
Ad5F.0 (pu)
Immunization

FIG. 25

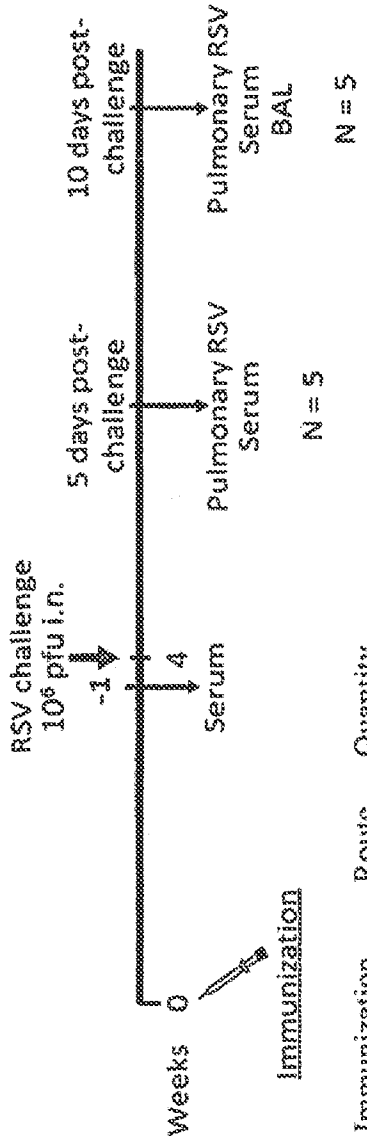

RSV challenge
10⁶ pfu i.n.
-1
4
5 days post-challenge
10 days post-challenge
Pulmonary RSV
Serum
BAL
Pulmonary RSV
Serum
N = 5
N = 5
Weeks 0
Immunization
Immunization Route Quantity
RSV A2 i.n. 1 x 10⁶ PFU
FTB i.m. n/a
FI-RSV i.m. 1:100
rAd5F.0 i.m. 10⁷ PU
rAd5F.0 i.m. 10⁹ PU
rAd43344F.0 i.m. 10⁷ PU
rAd43344F.0 i.m. 10⁹ PU

FIG. 26

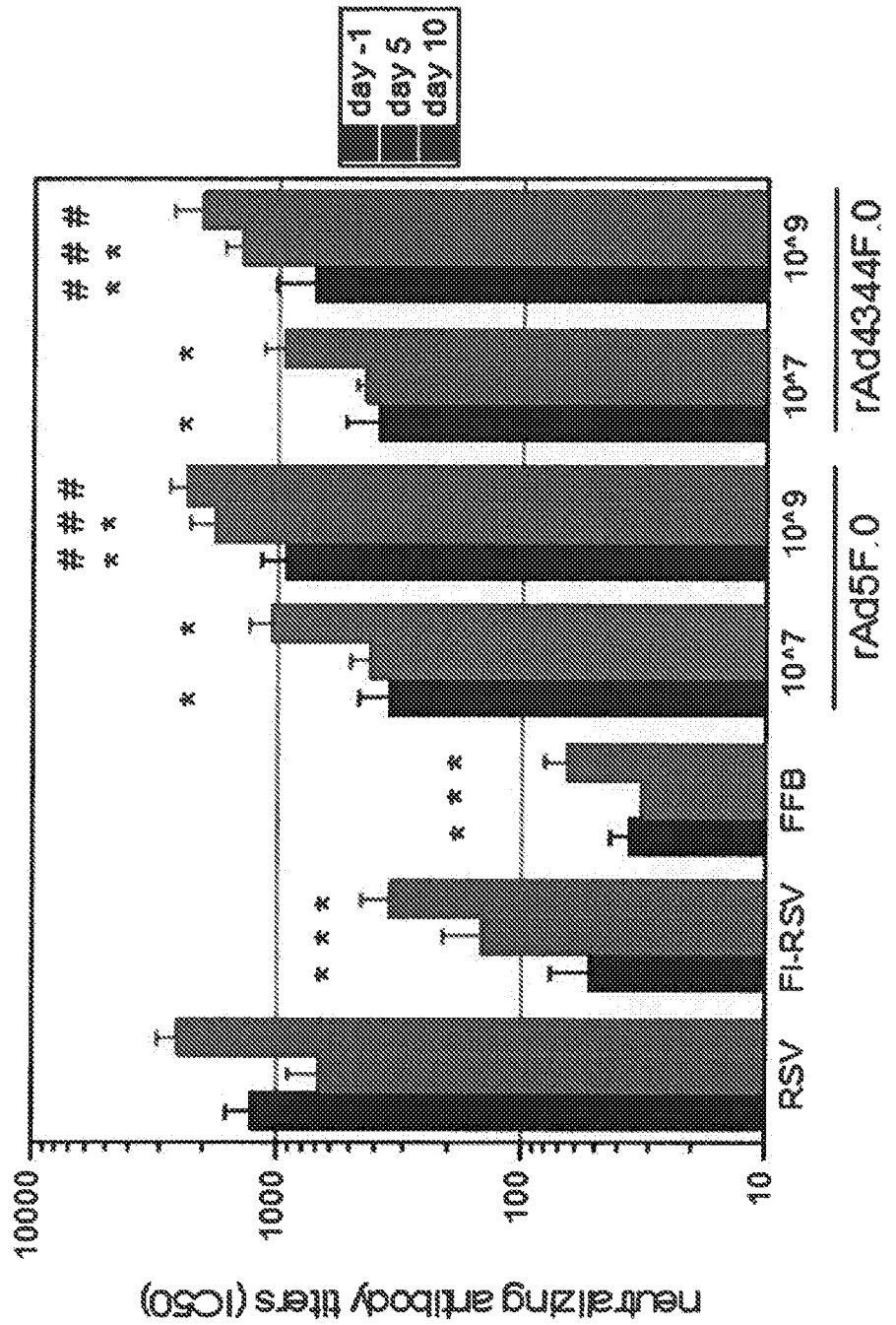

day -1
day 5
day 10
neutralizing antibody titers (IC50)
10000
1000
100
10
RSV
FI-RSV
FFB
10^7
10^9
rAd5F.0
10^7
10^9
rAd43&44F.0

FIG. 27

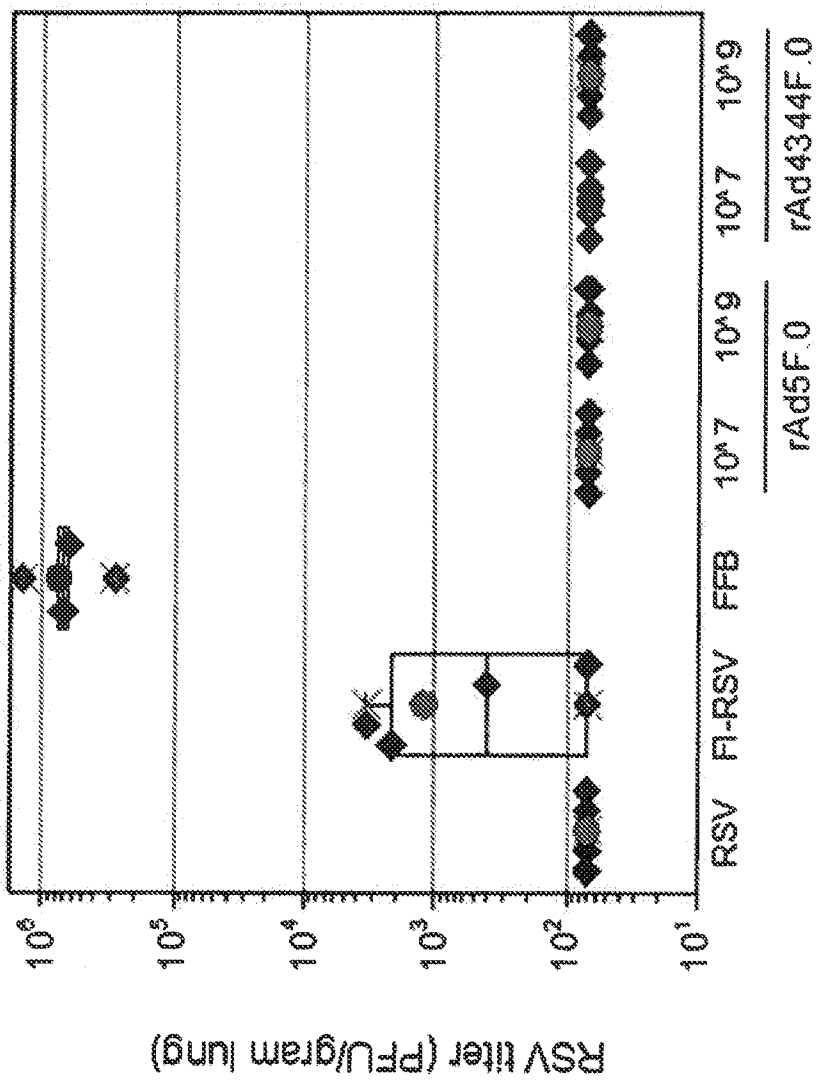

RSV titer (PFU/gram lung)
10^6
10^5
10^4
10^3
10^2
10^1
RSV
A-RSV
FFB
10^7
10^9
10^7
10^9
rAd5F.0
rAd4344F.0

FIG. 28

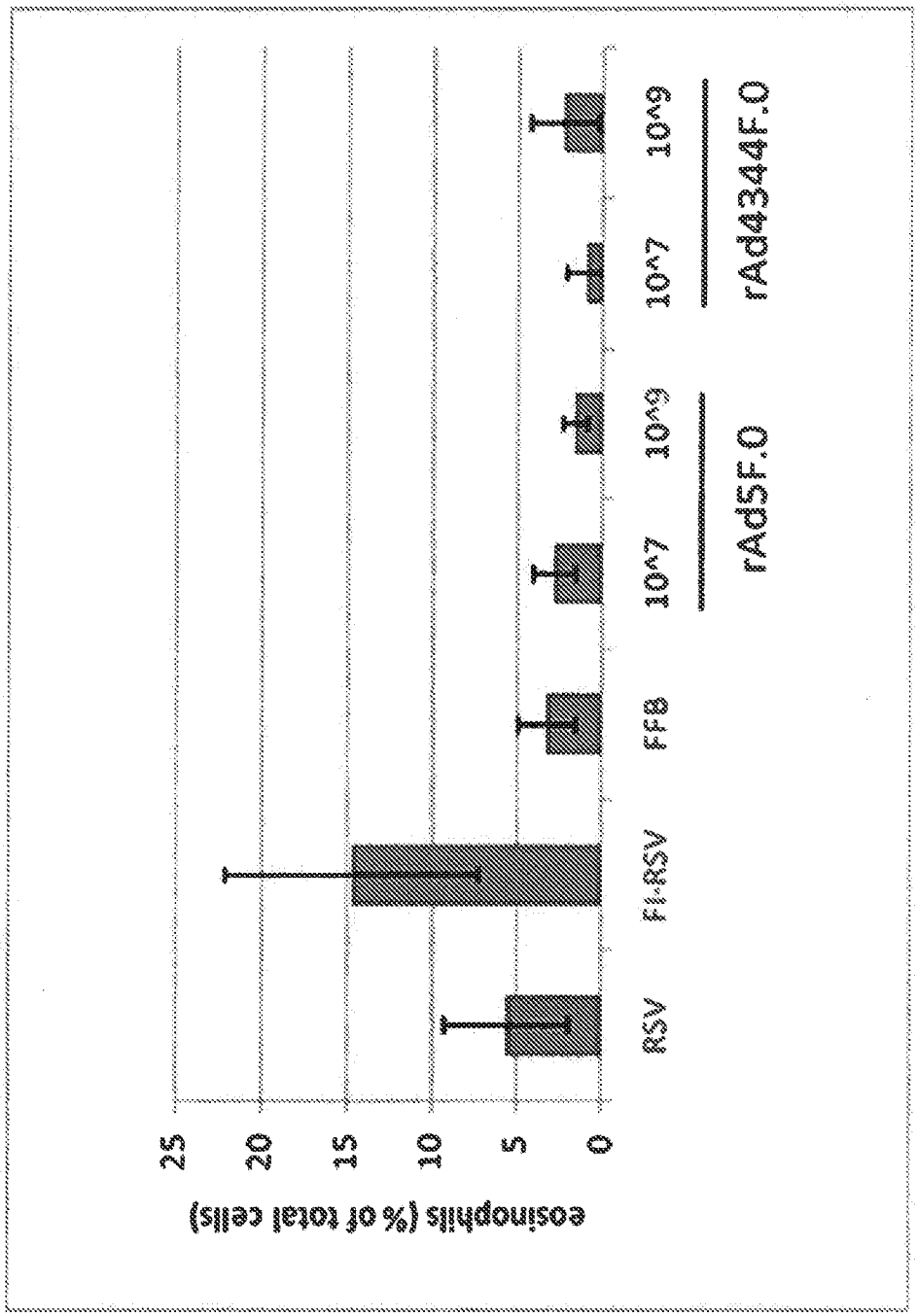

eosinophils (% of total cells)
25
20
15
10
5
0
RSV
F1-RSV
FF8
10^7
10^8
10^7
10^8
rAd5F.0
rAd4344F.0

FIG. 29

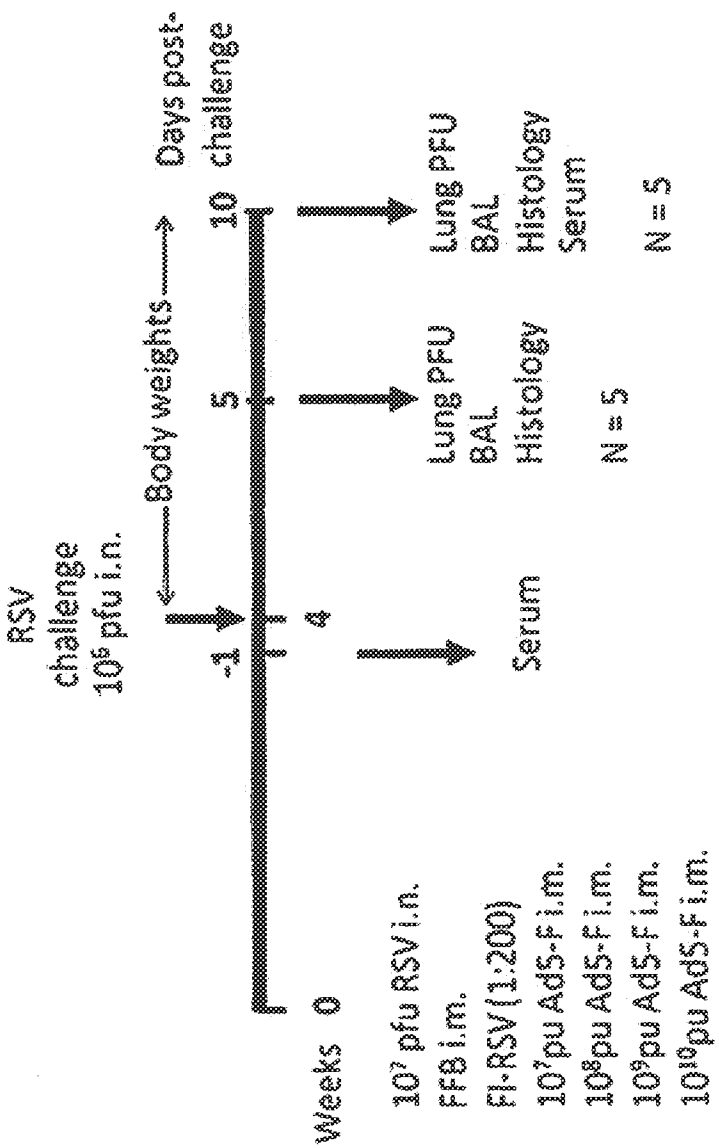

RSV challenge 10⁶ pfu i.n.
Body weights
Days post-challenge
-1
4
5
10
Weeks 0
10⁷ pfu RSV i.n.
FF8 i.m.
FI-RSV(1:200)
10⁷pu Ad5-F i.m.
10⁸pu Ad5-F i.m.
10⁹pu Ad5-F i.m.
10¹⁰pu Ad5-F i.m.
Serum
Lung PFU
BAL
Histology
N = 5
Lung PFU
BAL
Histology
Serum
N = 5

FIG. 30

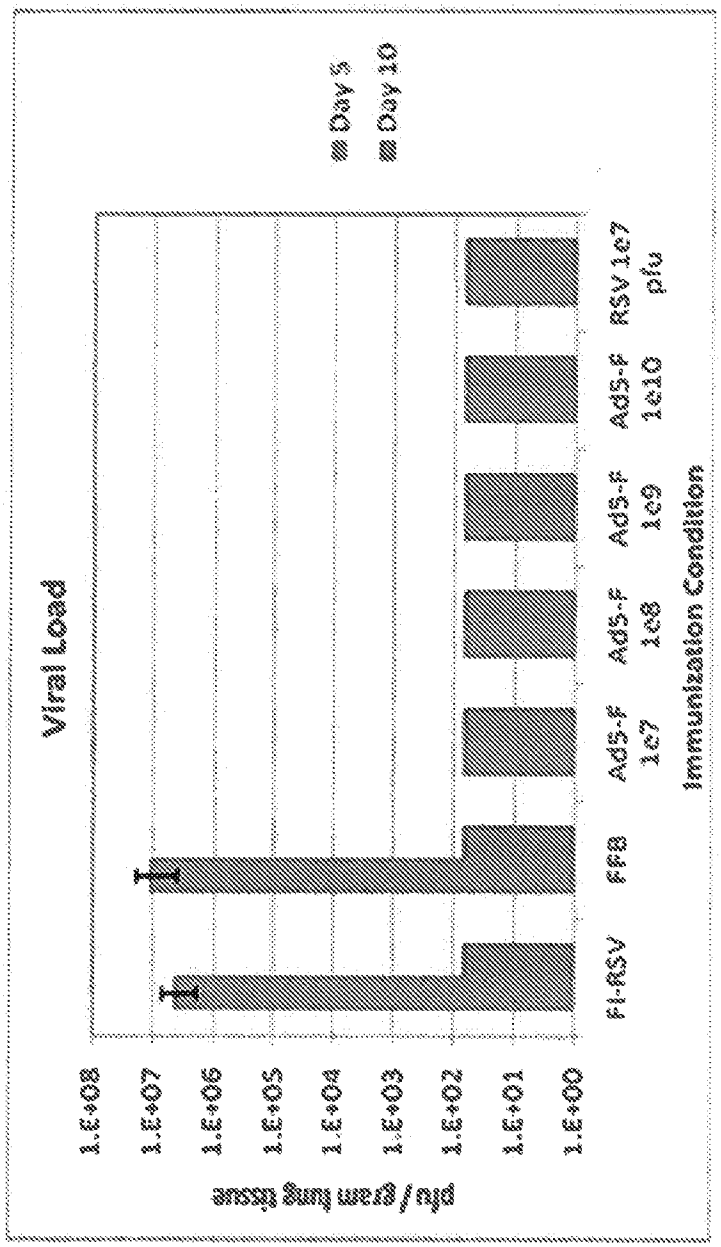

Viral Load
Day 5
Day 10
pfu / gram lung tissue
1.E+08
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
Fl-RSV
FFB
Ad5-F 1e7
Ad5-F 1e8
Ad5-F 1e9
Ad5-F 1e10
RSV 1e7 pfu
Immunization Condition

FIG. 31

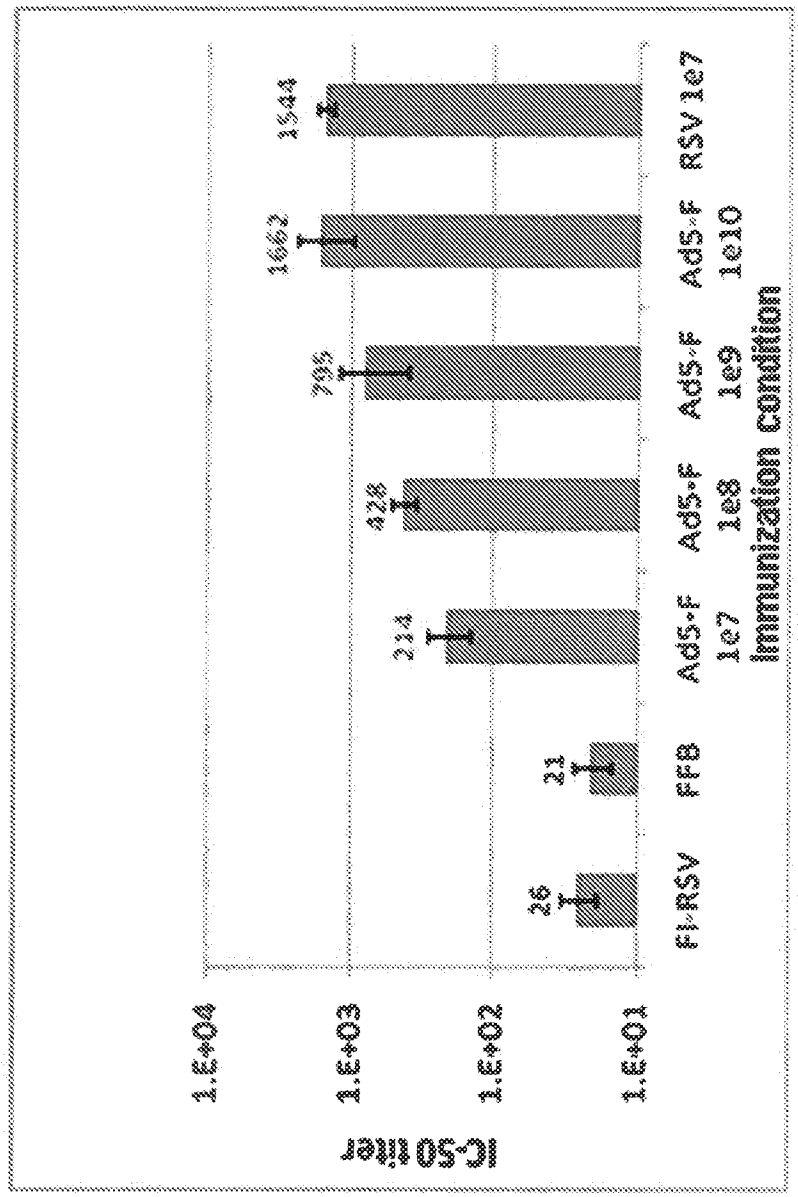

IC50 titer
1.E+04
1.E+03
1.E+02
1.E+01
26
21
224
428
795
1662
1544
F1-RSV
FFB
Ad5-F 1e7
Ad5-F 1e8
Ad5-F 1e9
Ad5-F 1e10
RSV 1e7
Immunization condition

FIG. 32

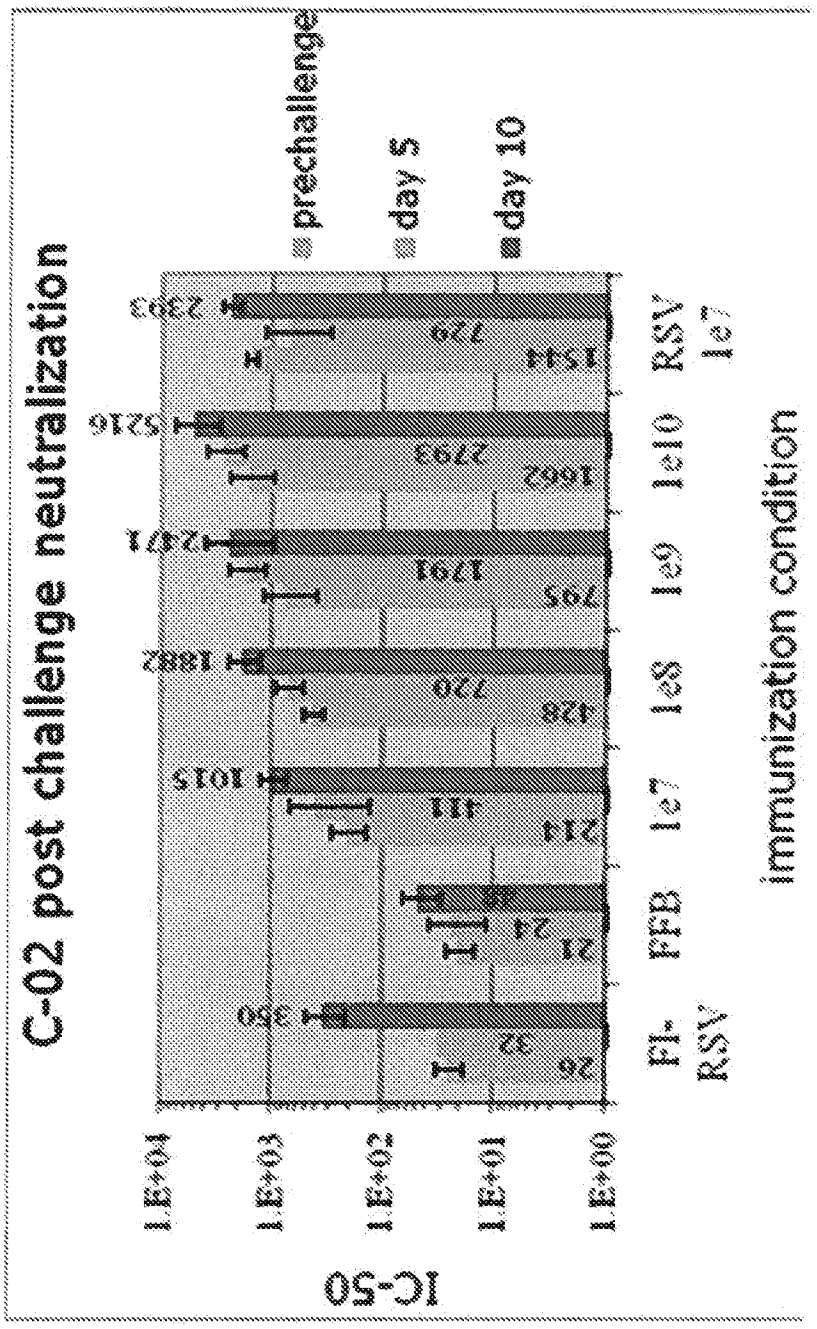

C-02 post challenge neutralization
prechallenge
day 5
day 10
IC-50
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
immunization condition
FI-RSV
FFB
1e7
1e8
1e9
1e10
RSV 1e7
2393
1514
729
5216
1602
2793
4287
1791
795
1882
720
428
1015
111
211
350
32
26

FIG. 33

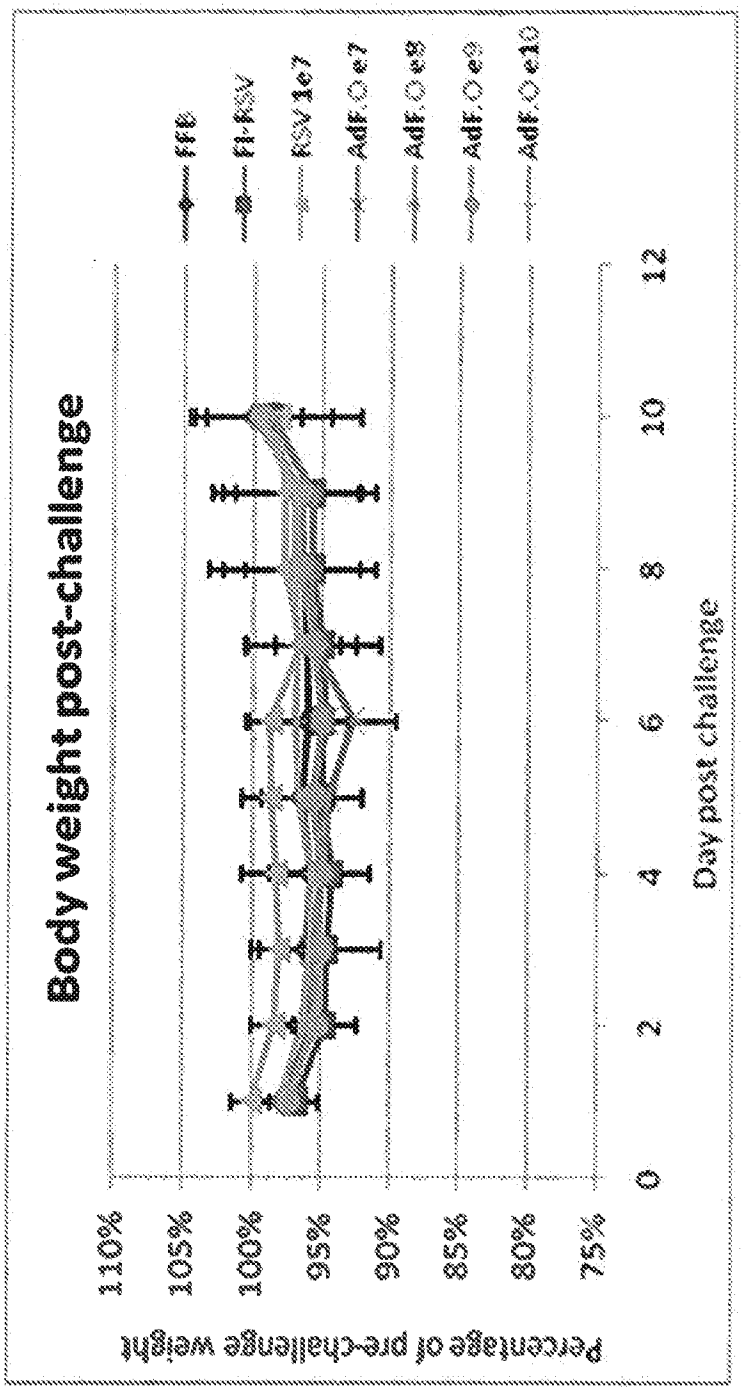

Body weight post-challenge
FFE
FI-RSV
RSV 1e7
AdF.O e7
AdF.O e8
AdF.O e9
AdF.O e10
Percentage of pre-challenge weight
110%
105%
100%
95%
90%
85%
80%
75%
0
2
4
6
8
10
12
Day post challenge

FIG. 34

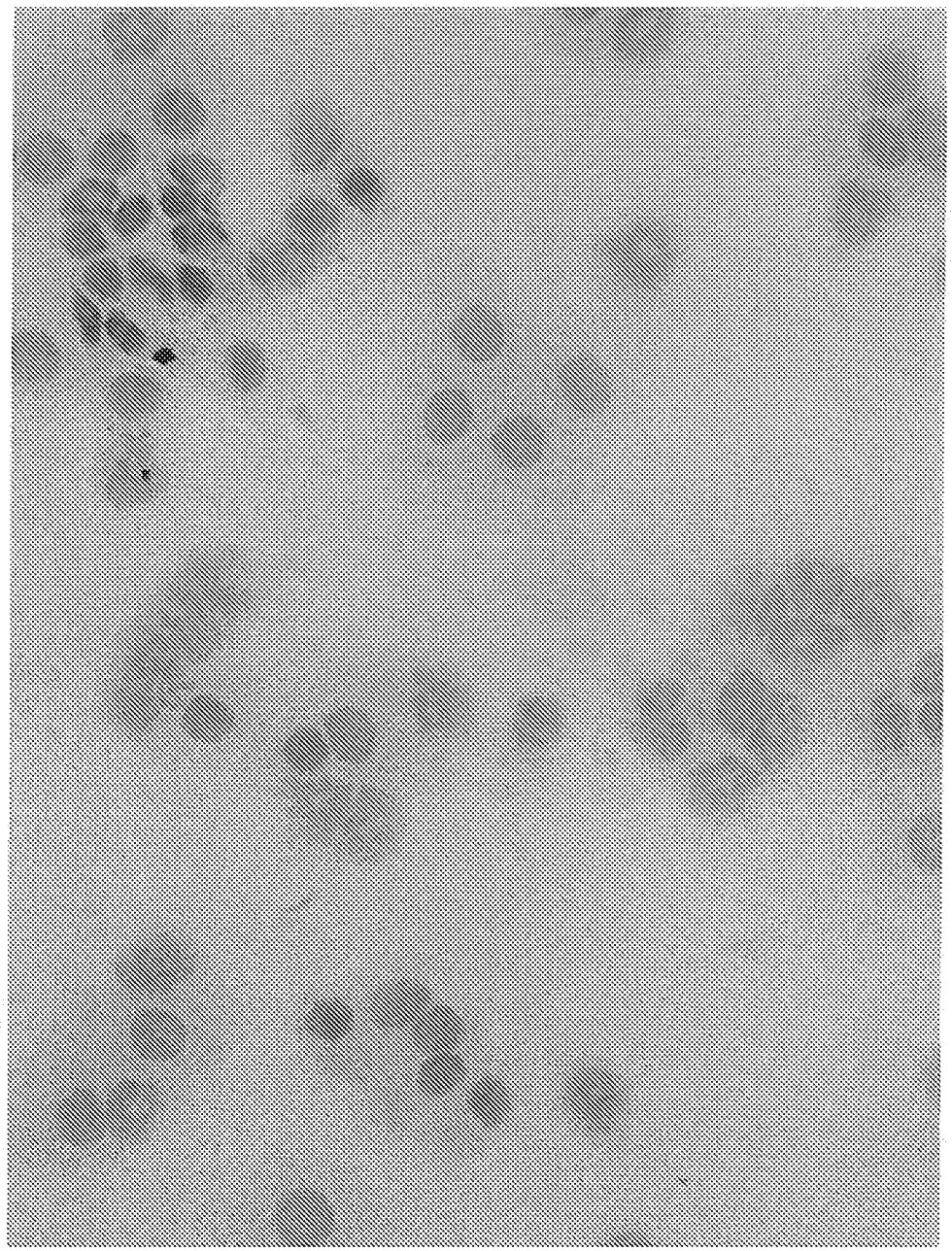


FIG. 35

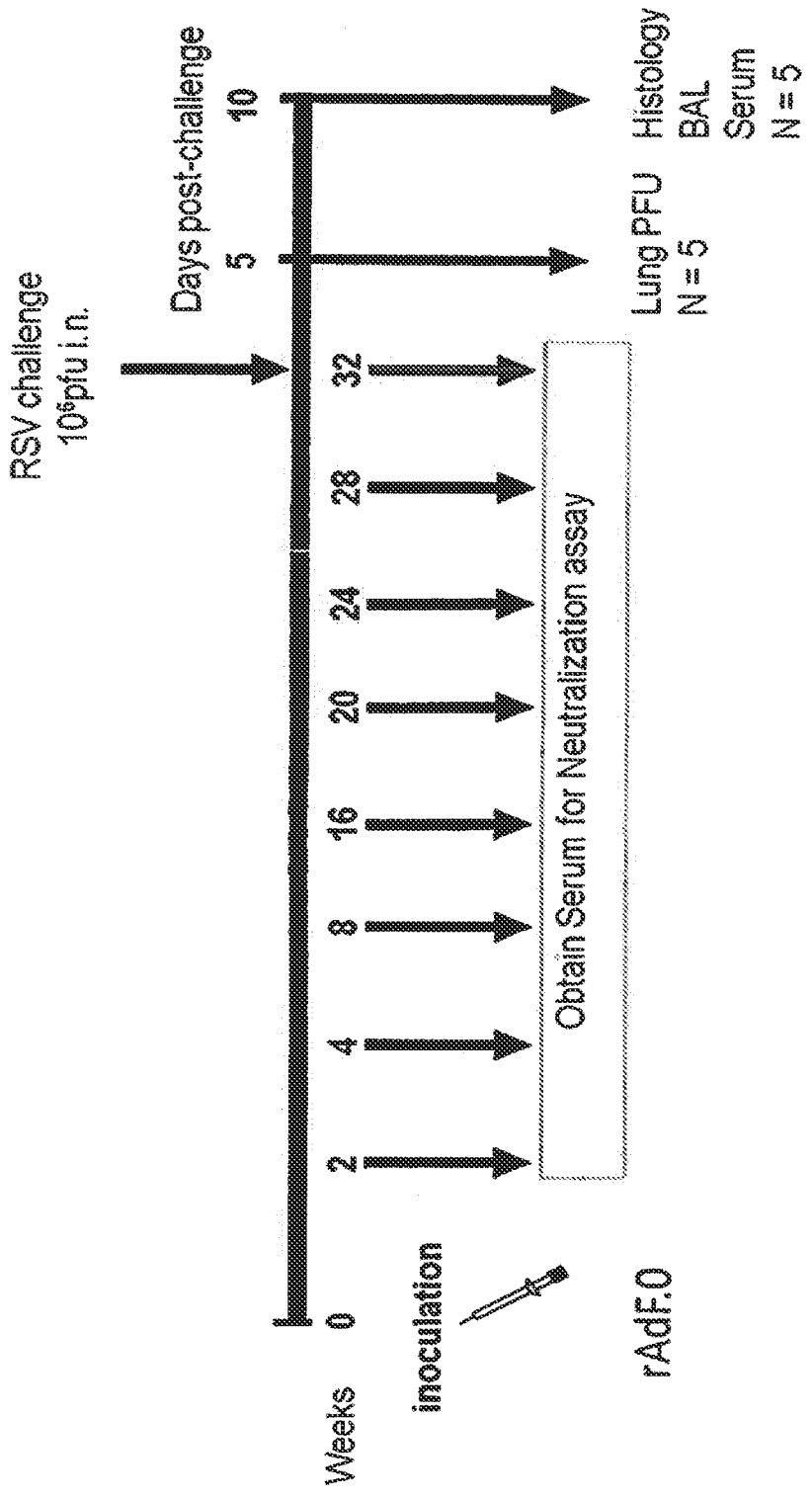

RSV challenge
10^6pfu i.n.
Days post-challenge
5
10
Lung PFU
N = 5
Histology
BAL
Serum
N = 5
32
28
24
20
16
8
4
2
Obtain Serum for Neutralization assay
Weeks
0
inoculation
rAdF.0

FIG. 36

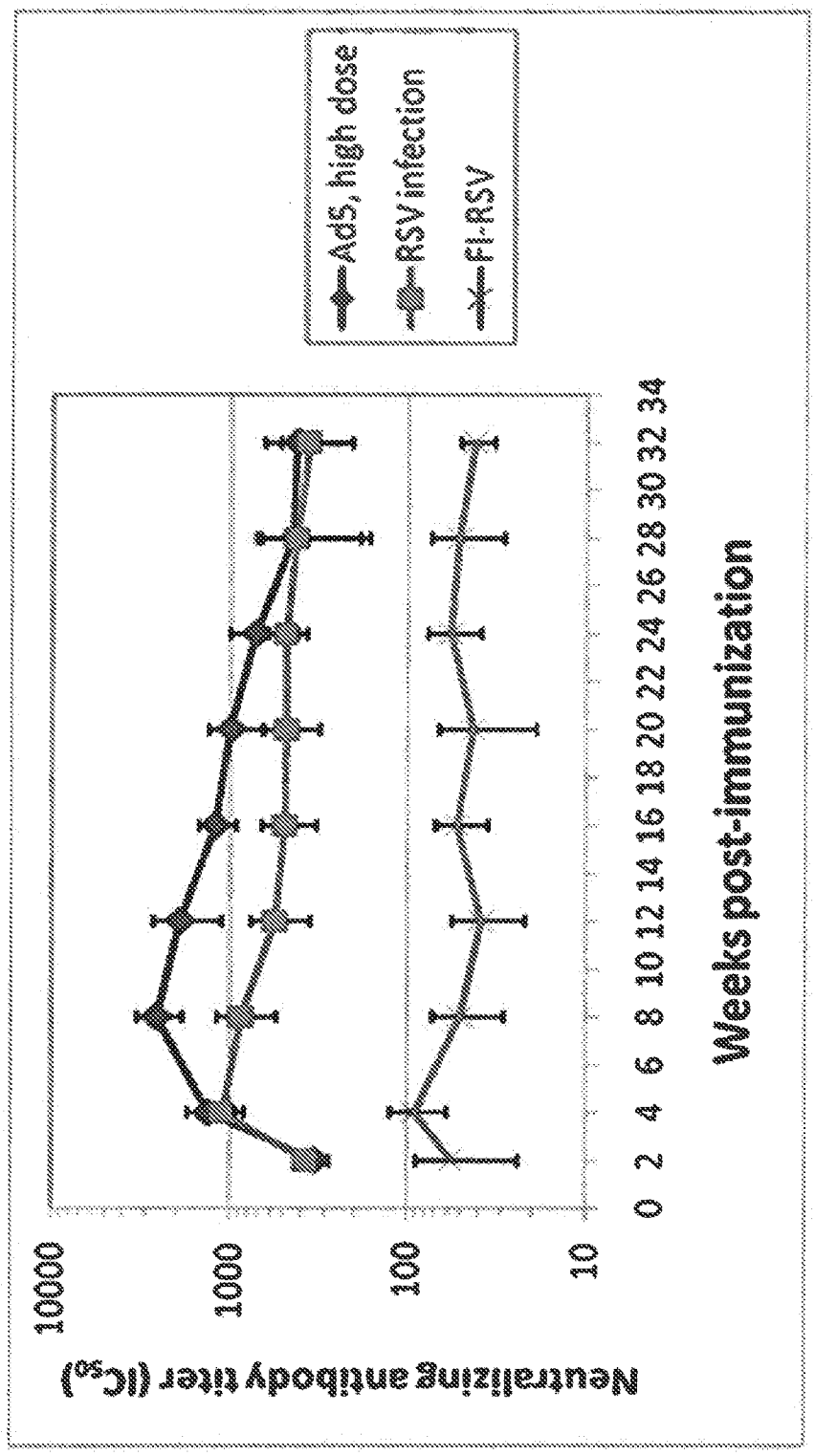

Neutralizing antibody titer (IC_{50})
10000
1000
100
10
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34
Weeks post-immunization
Ad5, high dose
RSV infection
FI-RSV

FIG. 37

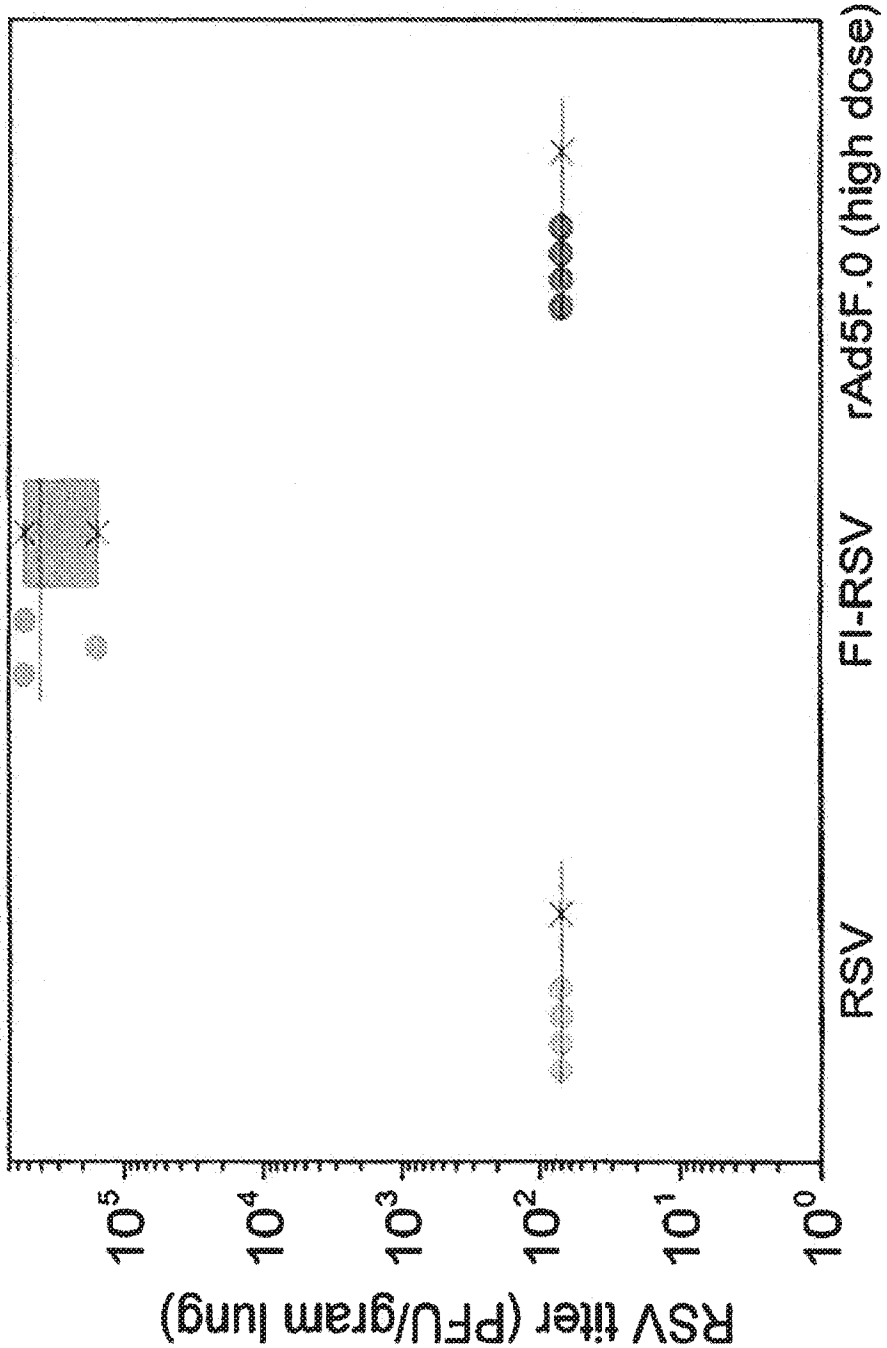

RSV titer (PFU/gram lung)
10^5
10^4
10^3
10^2
10^1
10^0
RSV
FI-RSV
rAd5F.0 (high dose)

FIG. 38

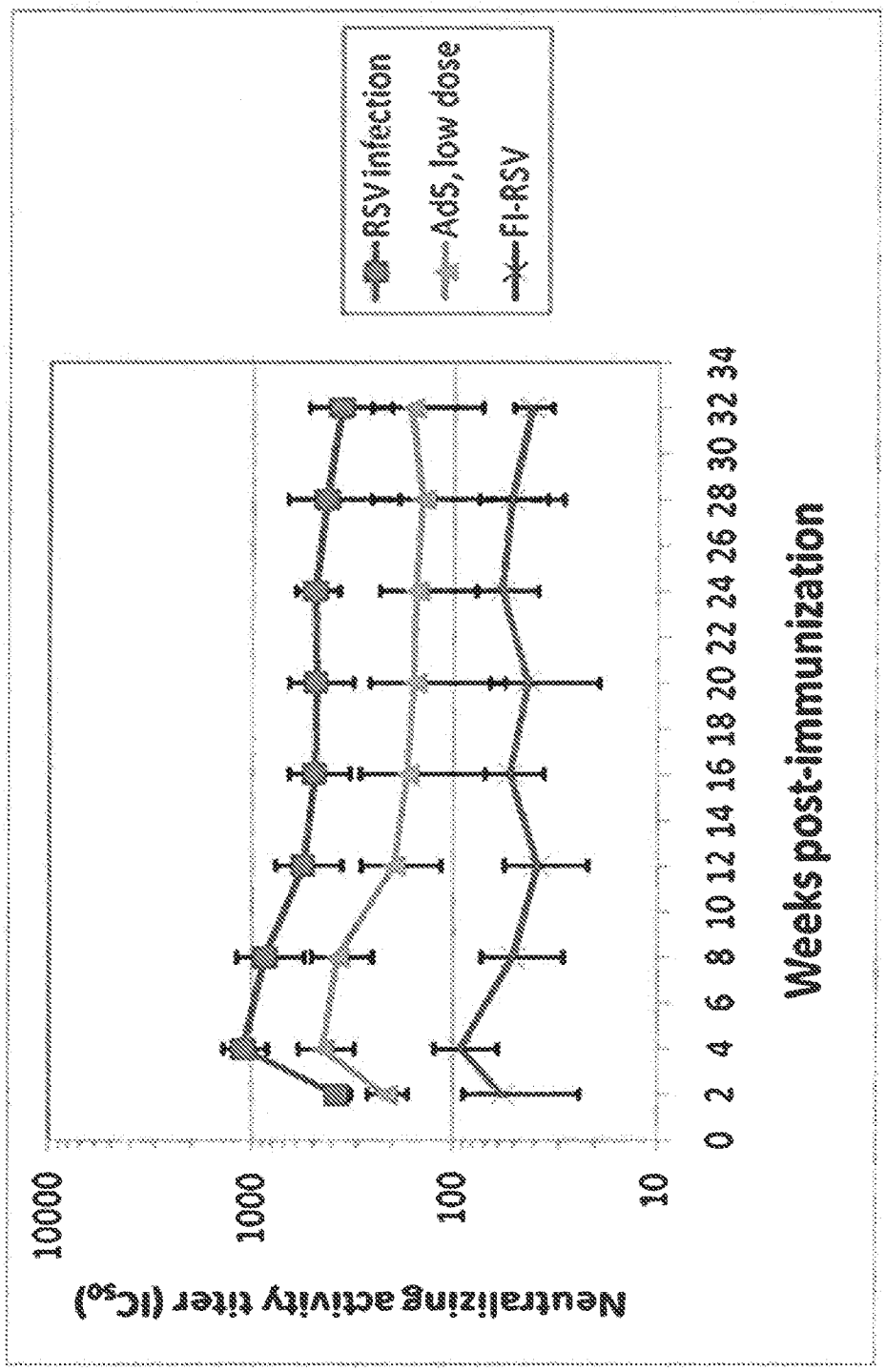

RSV infection
Ads, low dose
FI-RSV
Neutralizing activity titer (IC_{50})
10000
1000
100
10
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34
Weeks post-immunization

FIG. 39

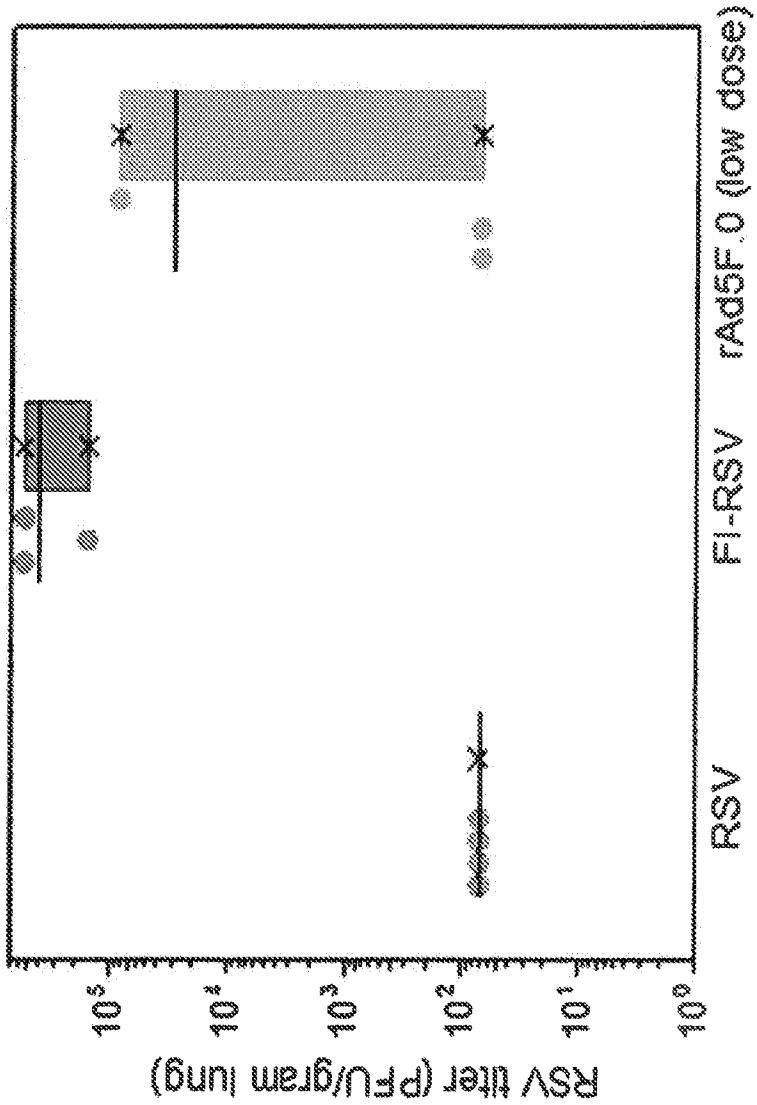

RSV titer (PFU/gram lung)
10^5
10^4
10^3
10^2
10^1
10^0
RSV
FI-RSV
rAd5F.0 (low dose)

FIG. 40

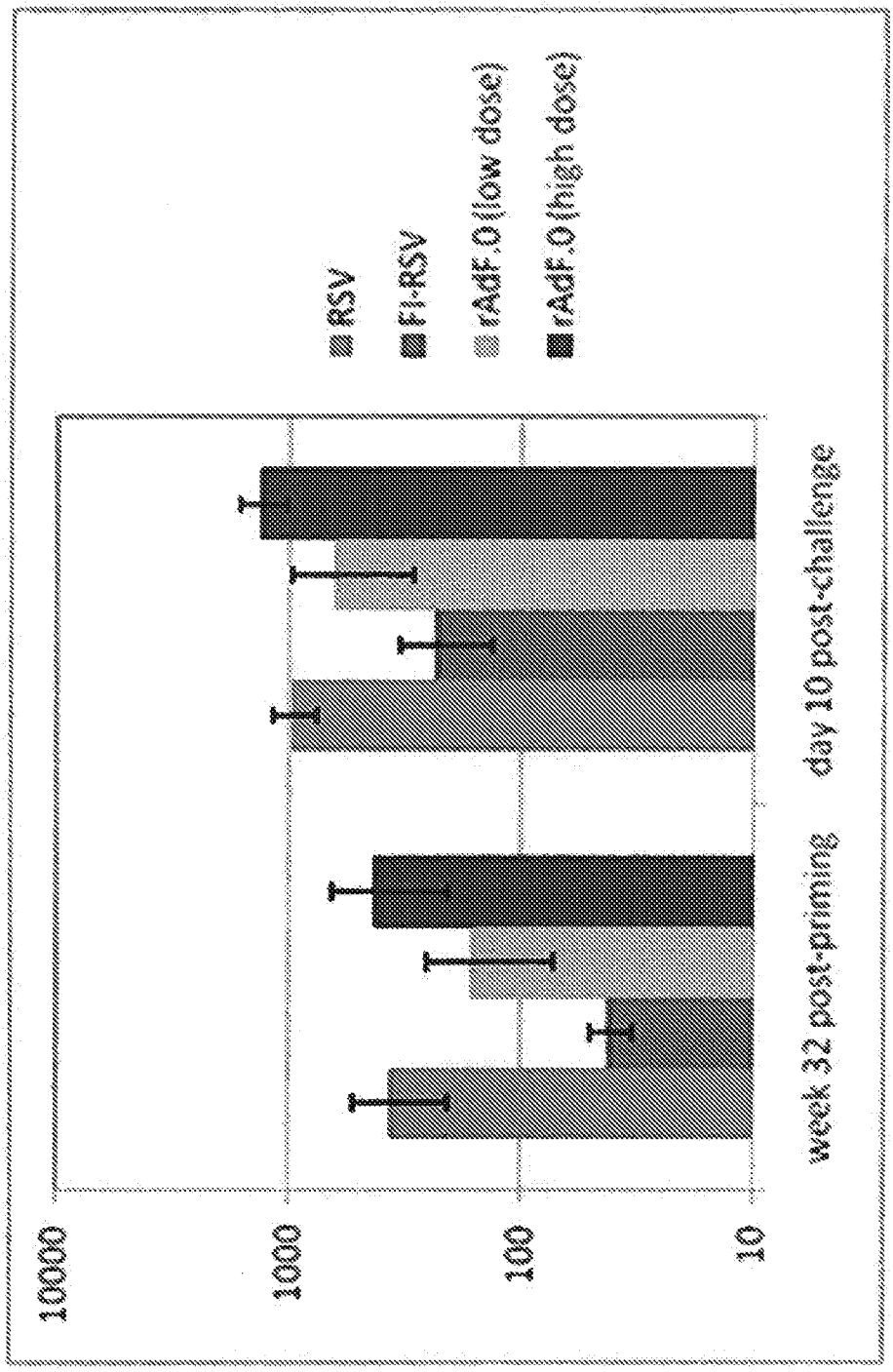

RSV
F1-RSV
rAdF.0 (low dose)
rAdF.0 (high dose)
10000
1000
100
10
week 32 post-priming
day 10 post-challenge

FIG. 41

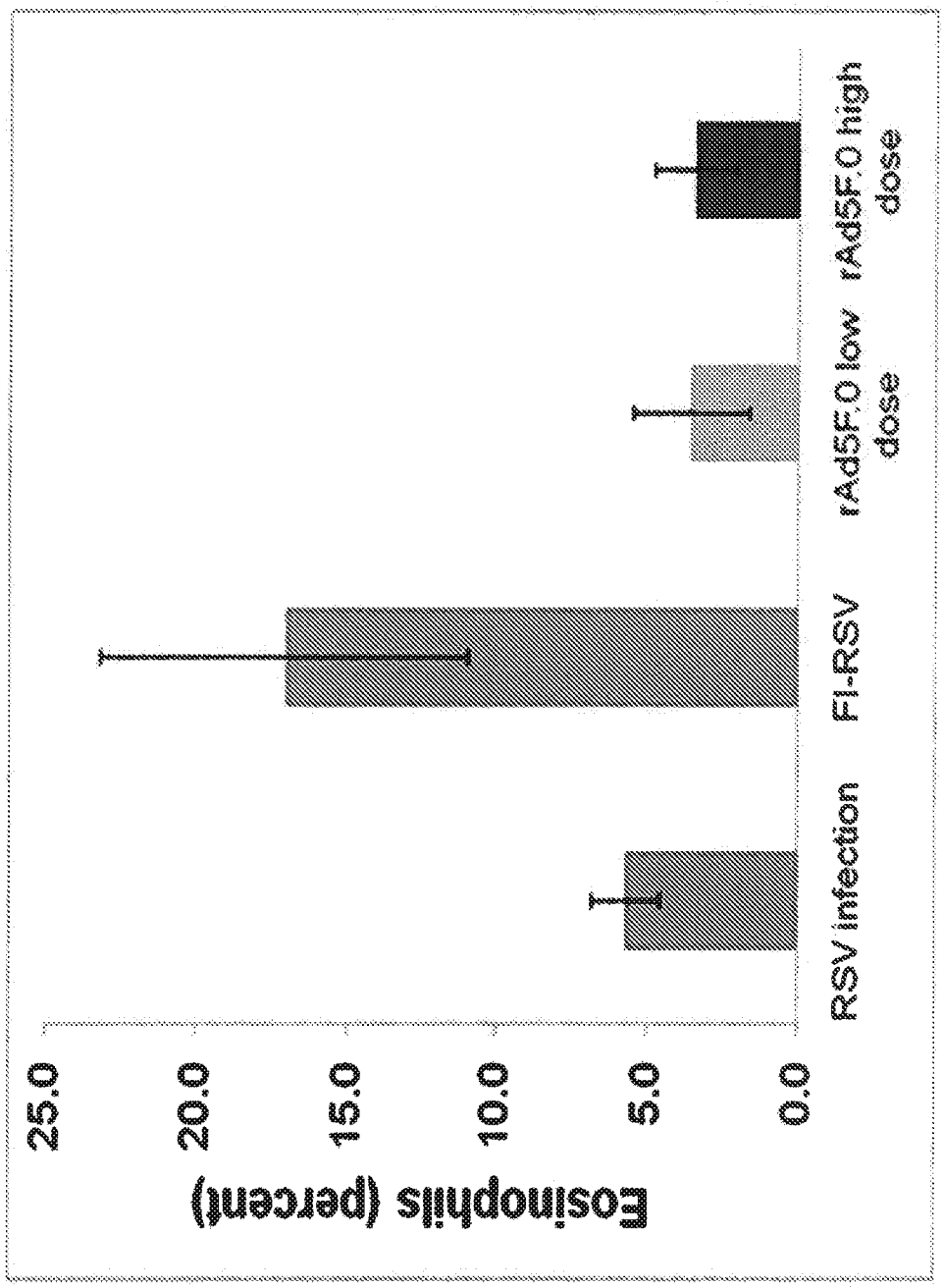

Eosinophils (percent)
25.0
20.0
15.0
10.0
5.0
0.0
RSV infection
FI-RSV
rAd5F.0 low dose
rAd5F.0 high dose

FIG. 42

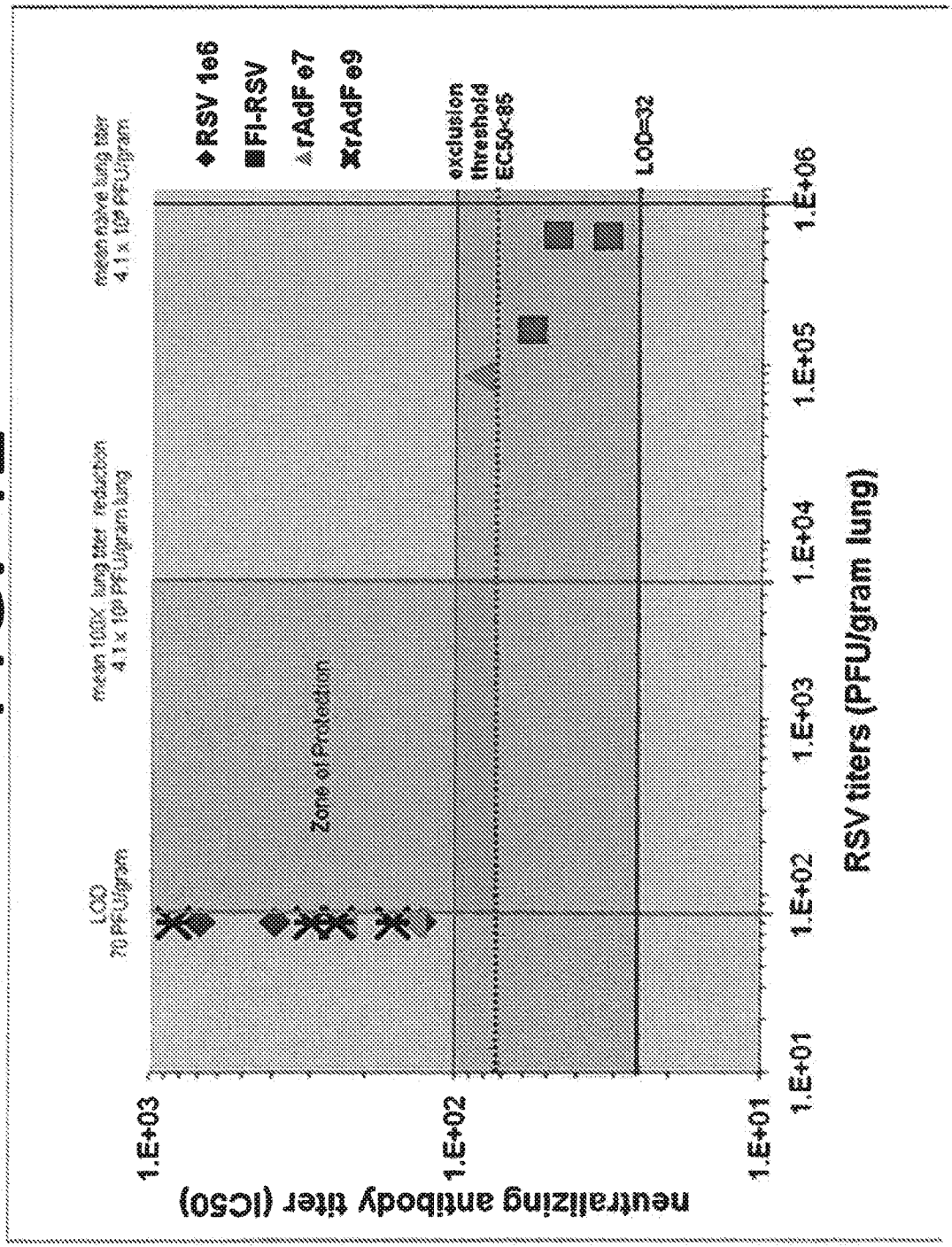

RSV 1e6
FI-RSV
rAdF e7
rAdF e9
exclusion threshold EC50=85
LOD=32
Zone of Protection
LOD 20 PFU/gram
RSV titers (PFU/gram lung)
neutralizing antibody titer (IC50)
1.E+01
1.E+02
1.E+03
1.E+04
1.E+05
1.E+06

FIG. 43

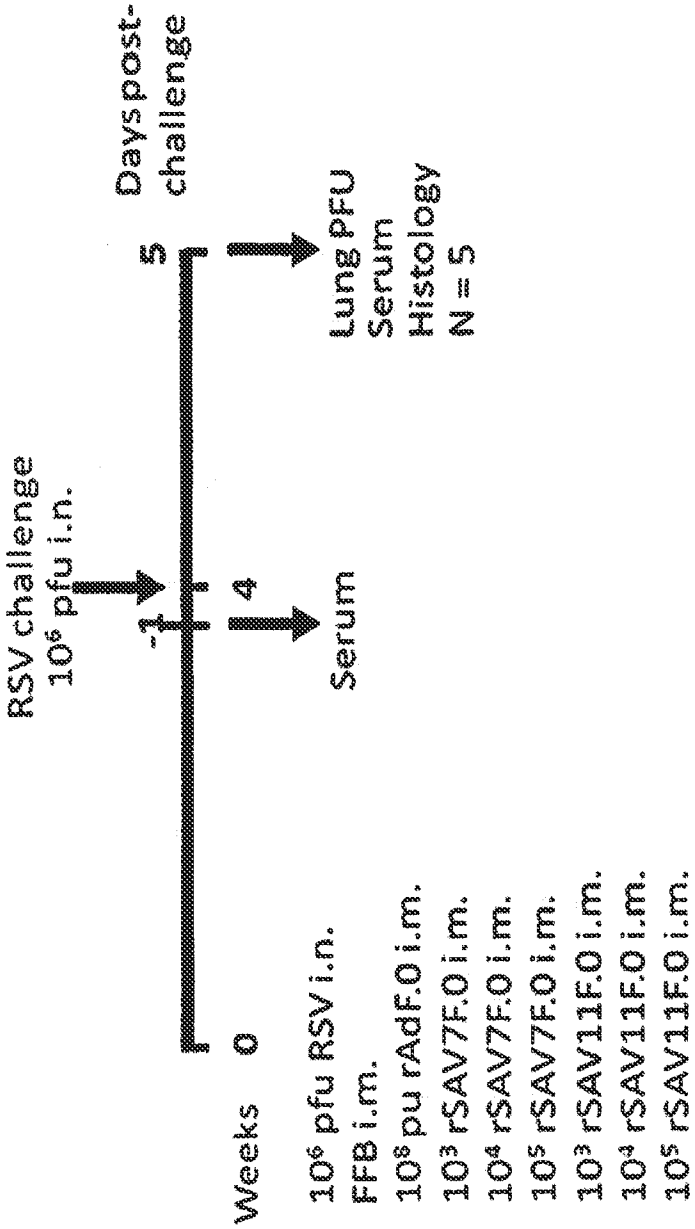

RSV challenge
10⁶ pfu i.n.
Days post-challenge
Weeks 0
-1
4
5
Serum
Lung PFU
Serum
Histology
N = 5
10⁶ pfu RSV i.n.
FFB i.m.
10⁸ pu rAdF.0 i.m.
10³ rSAV7F.0 i.m.
10⁴ rSAV7F.0 i.m.
10⁵ rSAV7F.0 i.m.
10³ rSAV11F.0 i.m.
10⁴ rSAV11F.0 i.m.
10⁵ rSAV11F.0 i.m.

FIG. 44

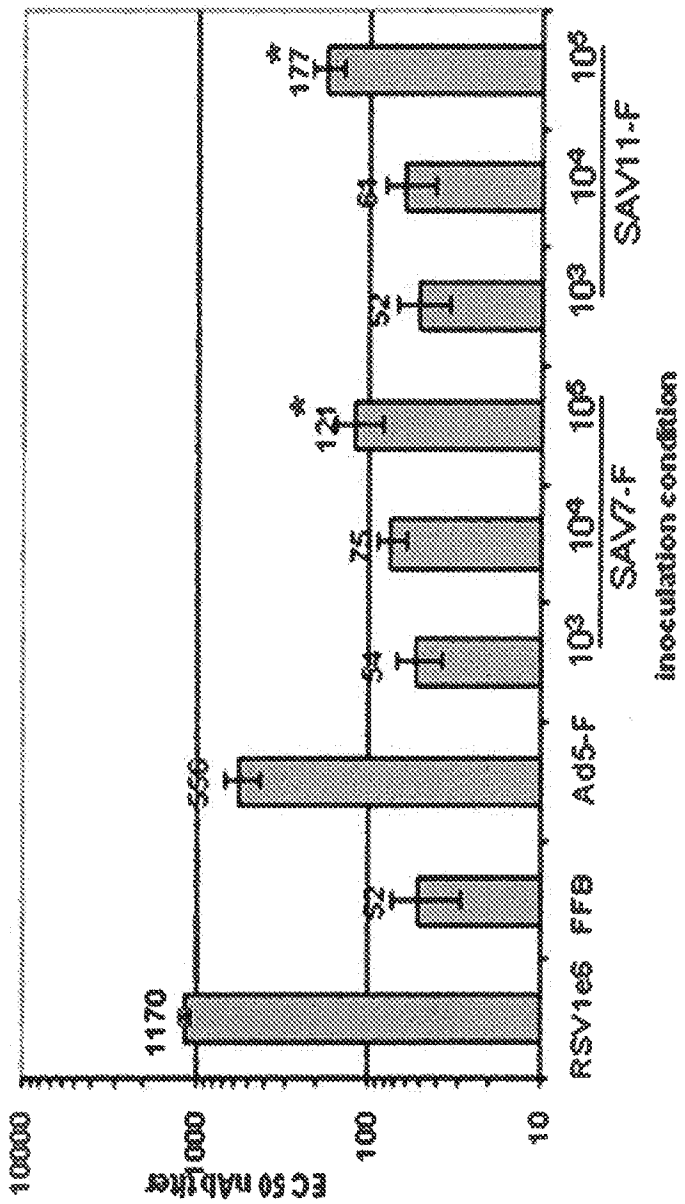

EC50 nAb titer
10000
1000
100
10
1170
32
550
34
75
171
52
64
177
RSV16s FFB A05-F SAV7-F SAV11-F
10³ 10⁴ 10⁵ 10³ 10⁴ 10⁵
inoculation condition

FIG. 45

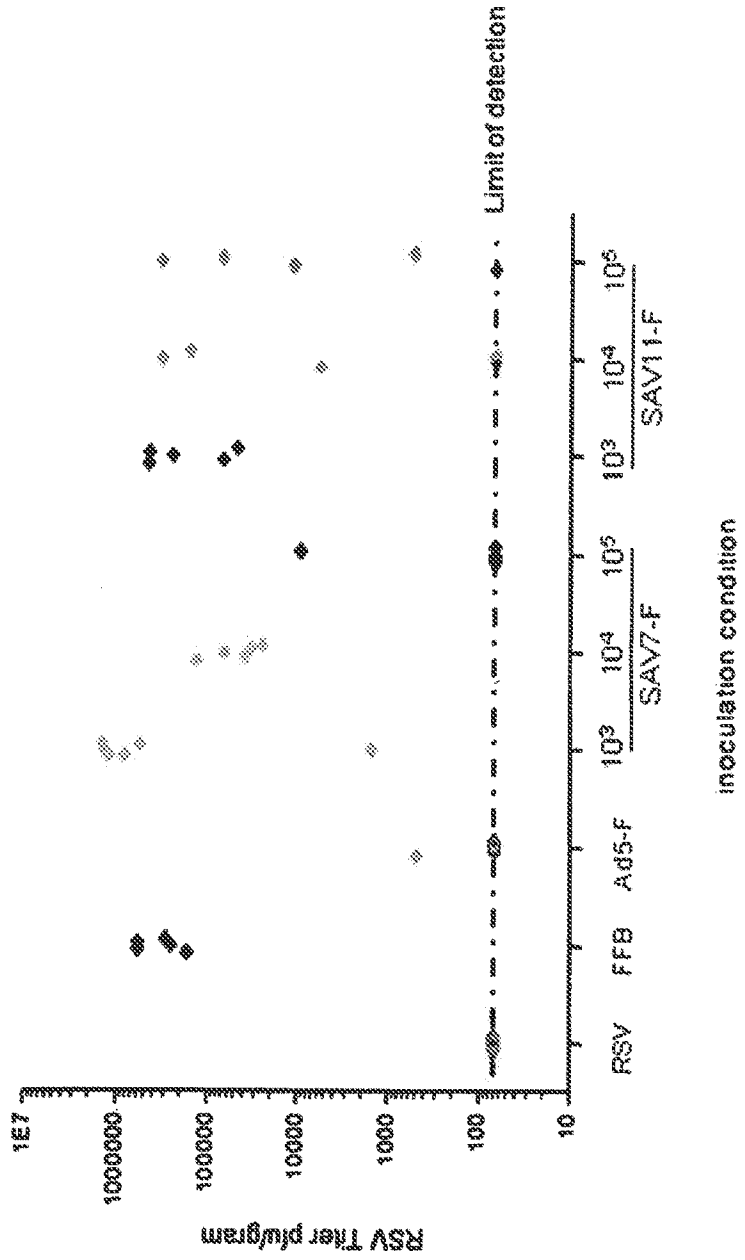

RSV Titer pfu/gram
1E7
1000000
100000
10000
1000
100
10
Limit of detection
RSV
FFB
Ad5-F
10³
10⁴
10⁵
SAV7-F
10³
10⁴
10⁵
SAV11-F
inoculation condition

FIG. 46

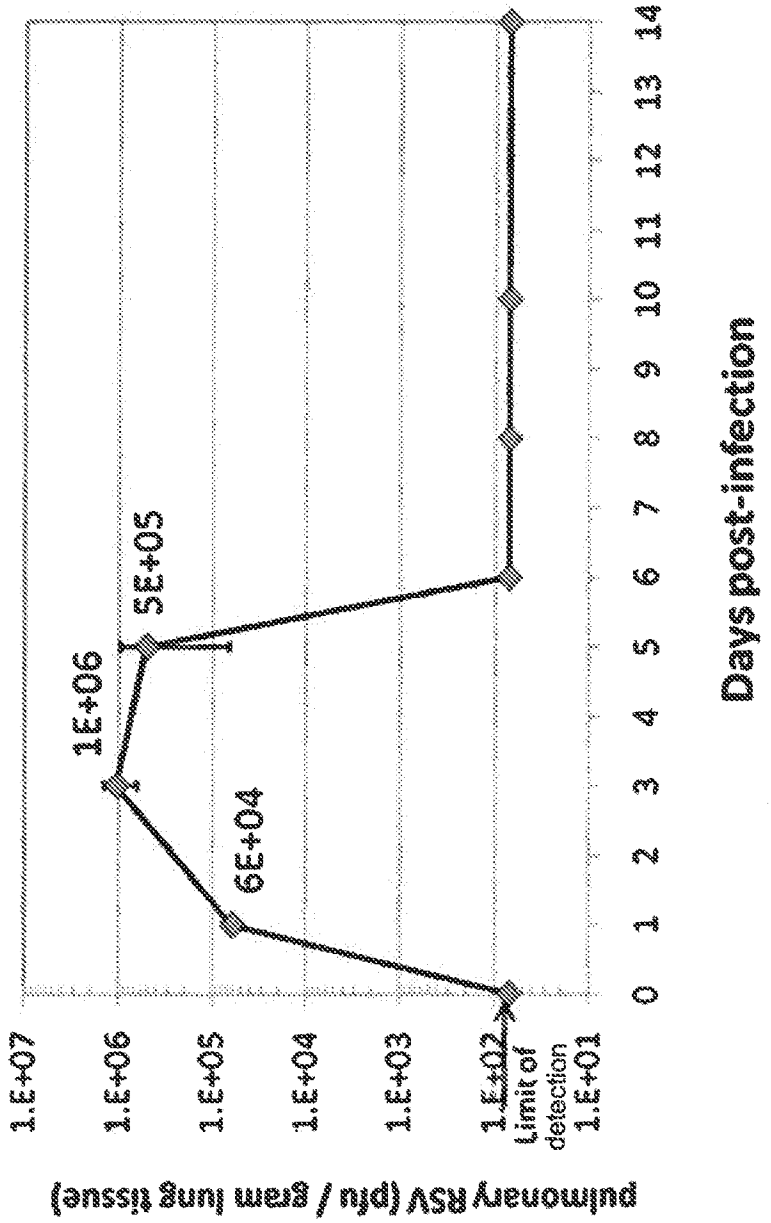

pulmonary RSV (pfu / gram lung tissue)
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
Limit of detection
1.E+01
1E+06
5E+05
6E+04
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Days post-infection

FIG. 47

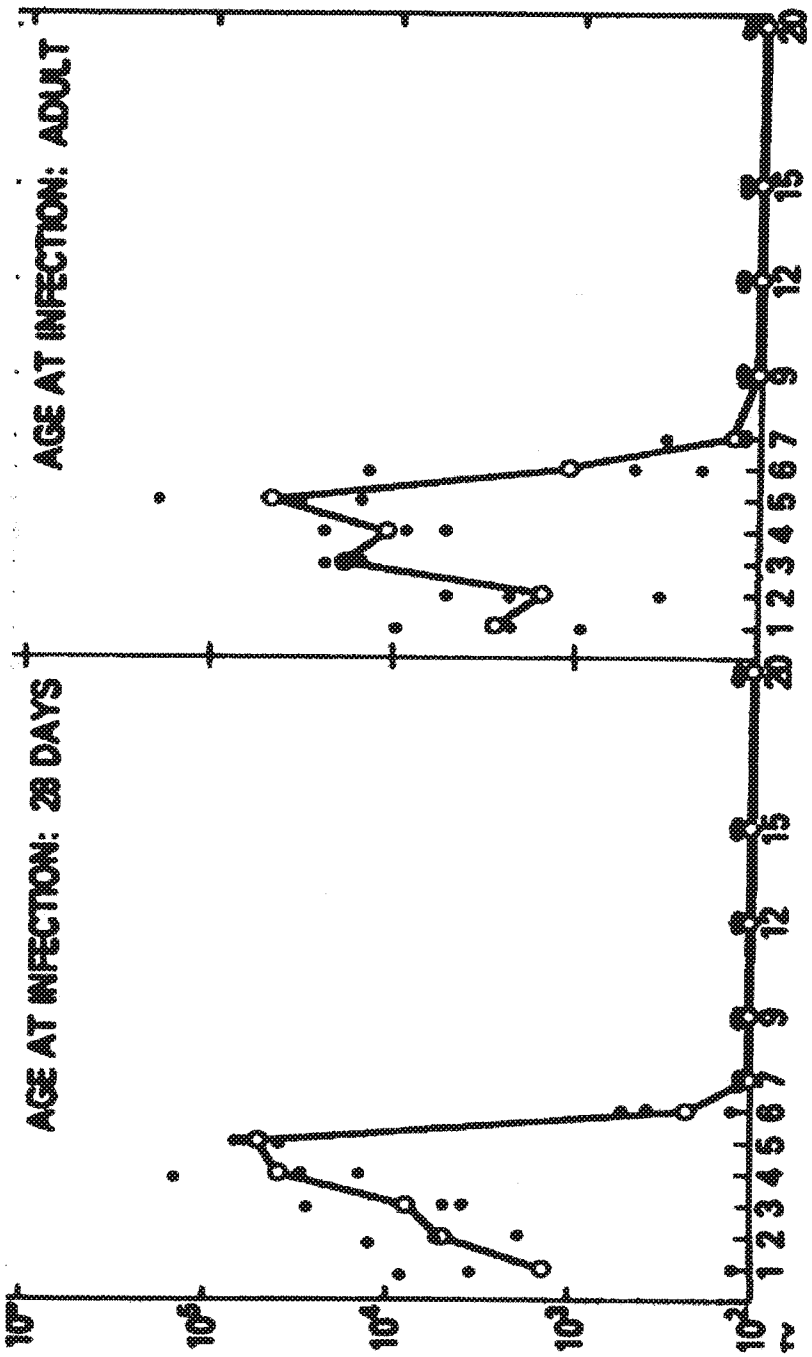

AGE AT INFECTION: ADULT
AGE AT INFECTION: 28 DAYS
10^7
10^6
10^5
10^4
10^3
10^2
1 2 3 4 5 6 7 9 12 15 20 25
1 2 3 4 5 6 7 9 12 15 20 25
T

FIG. 48

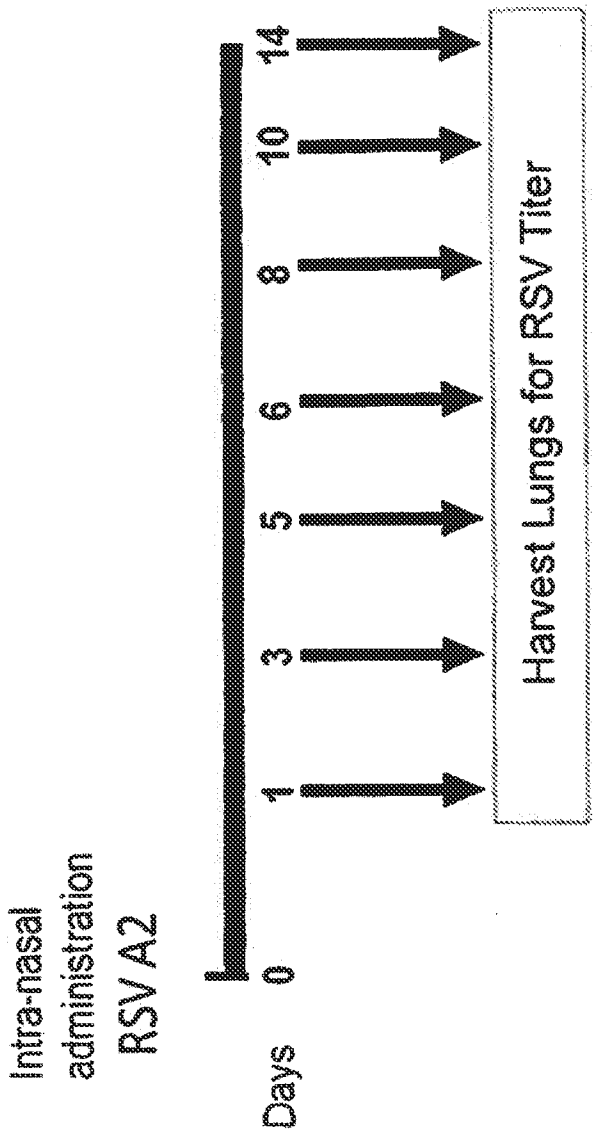

Intra-nasal
administration
RSV A2
Days
0
1
3
5
6
8
10
14
Harvest Lungs for RSV Titer

FIG. 49

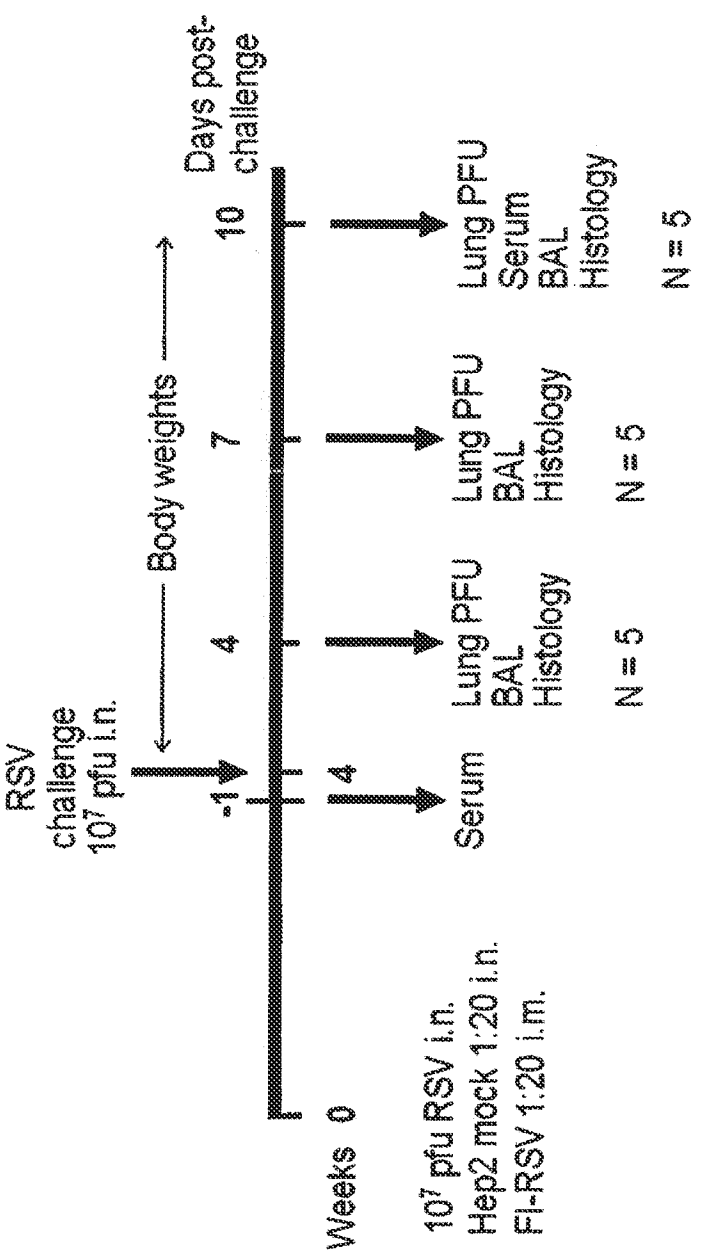

Days post-challenge
Body weights
RSV challenge 10^7 pfu i.n.
Weeks 0
-1
4
4
7
10
10^7 pfu RSV i.n.
Hep2 mock 1:20 i.n.
FI-RSV 1:20 i.m.
Serum
Lung PFU
BAL
Histology
N = 5
Lung PFU
BAL
Histology
N = 5
Lung PFU
Serum
BAL
Histology
N = 5

FIG. 50

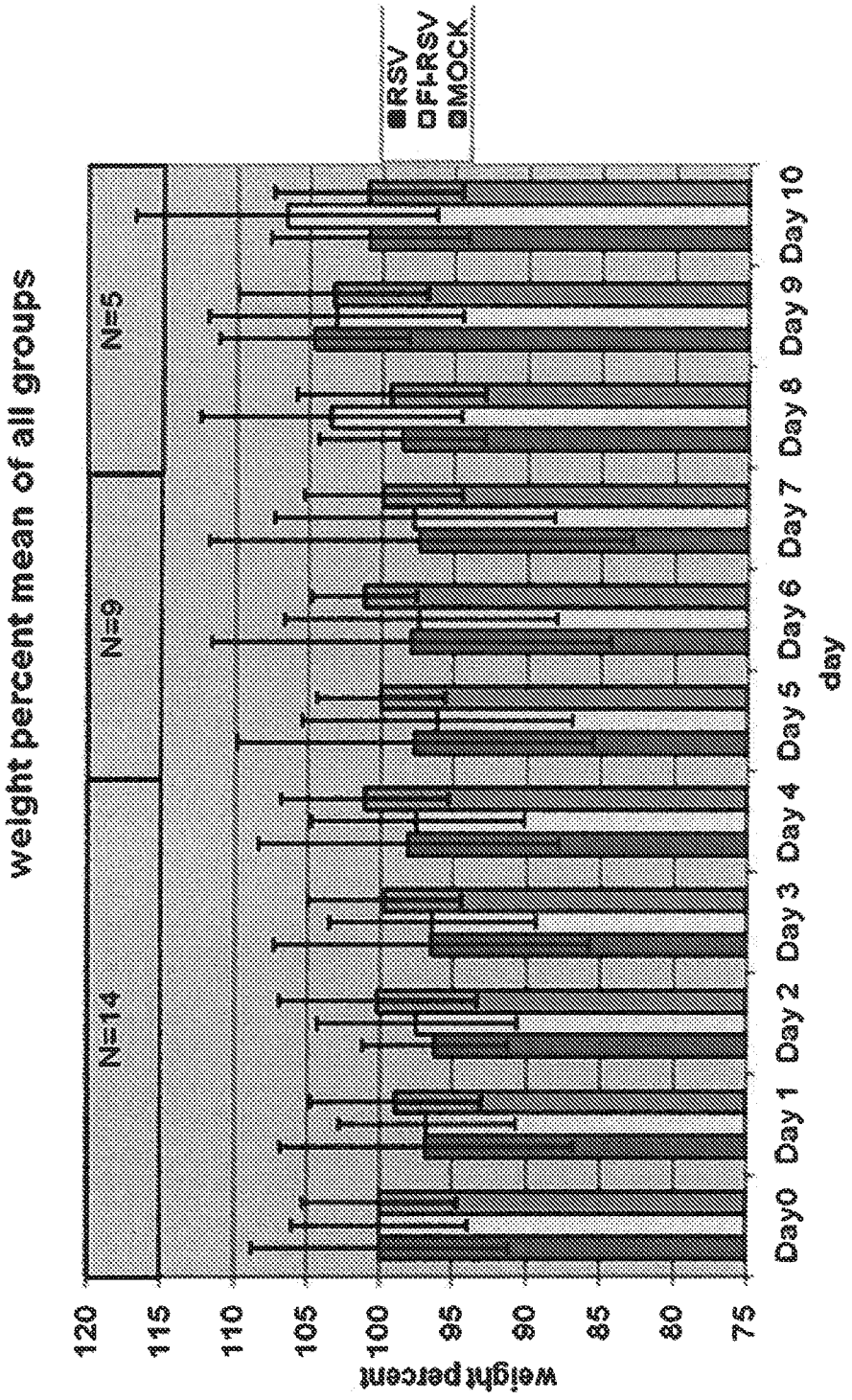

weight percent mean of all groups
N=14
N=9
N=5
RSV
FI-RSV
MOCK
120
115
110
105
100
95
90
85
80
75
weight percent
Day0 Day 1 Day 2 Day 3 Day 4 Day 5 Day 6 Day 7 Day 8 Day 9 Day 10
day

FIG. 51

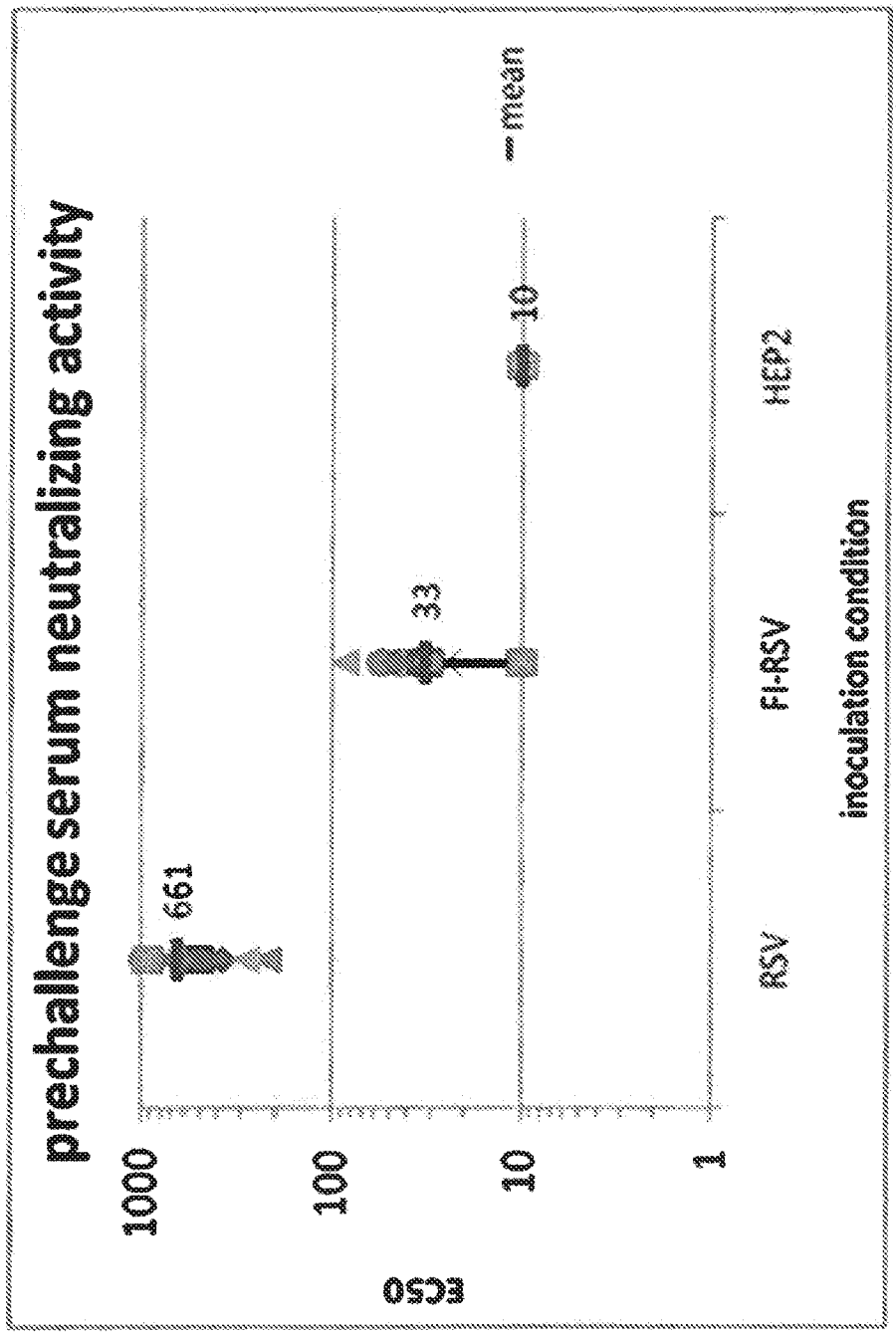

prechallenge serum neutralizing activity
EC50
1000
100
10
1
661
33
10
mean
RSV
FI-RSV
HEP2
inoculation condition

FIG. 52

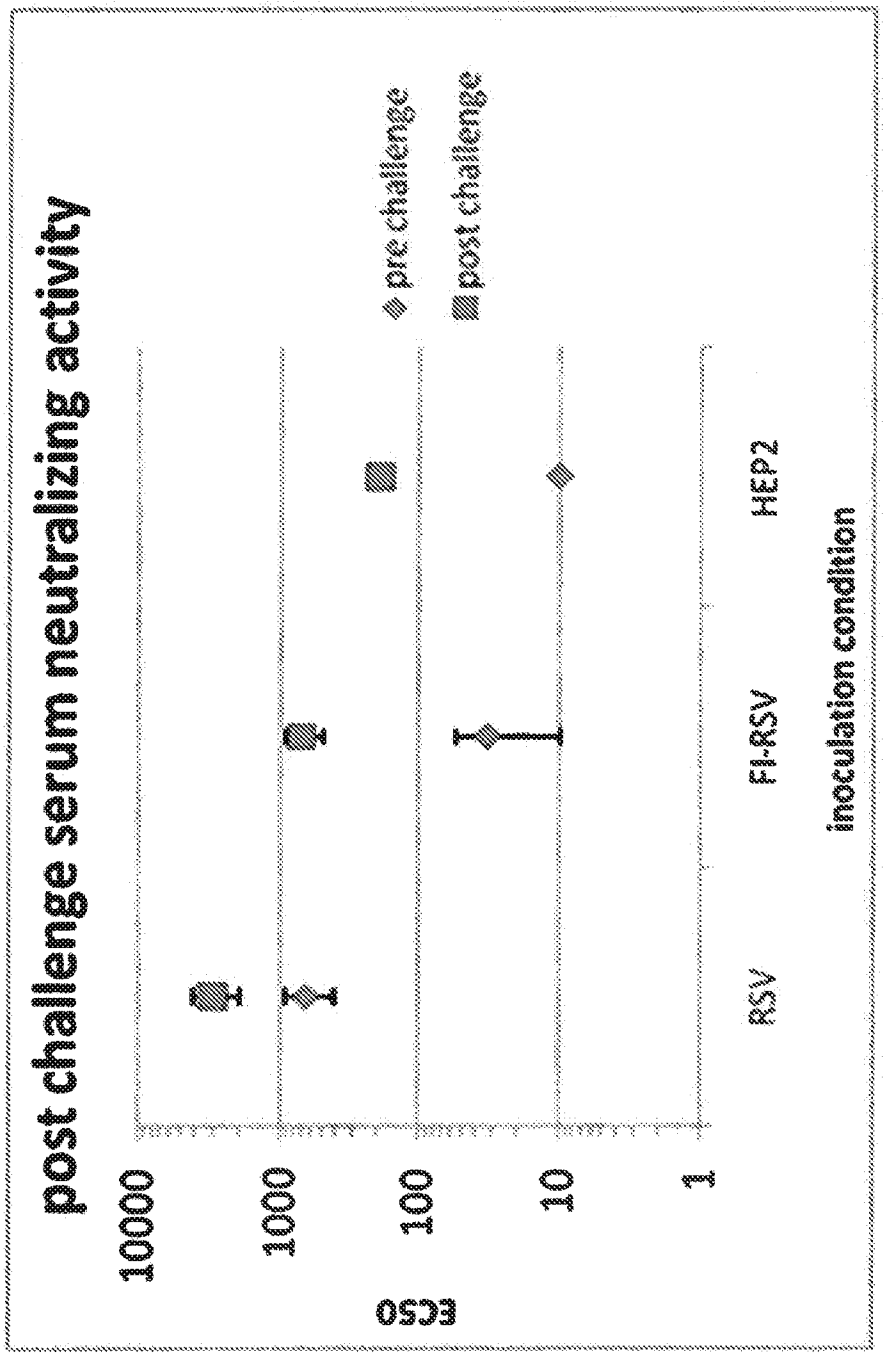

post challenge serum neutralizing activity
pre challenge
post challenge
EC50
10000
1000
100
10
1
RSV
FI-RSV
HEP2
inoculation condition

FIG. 53

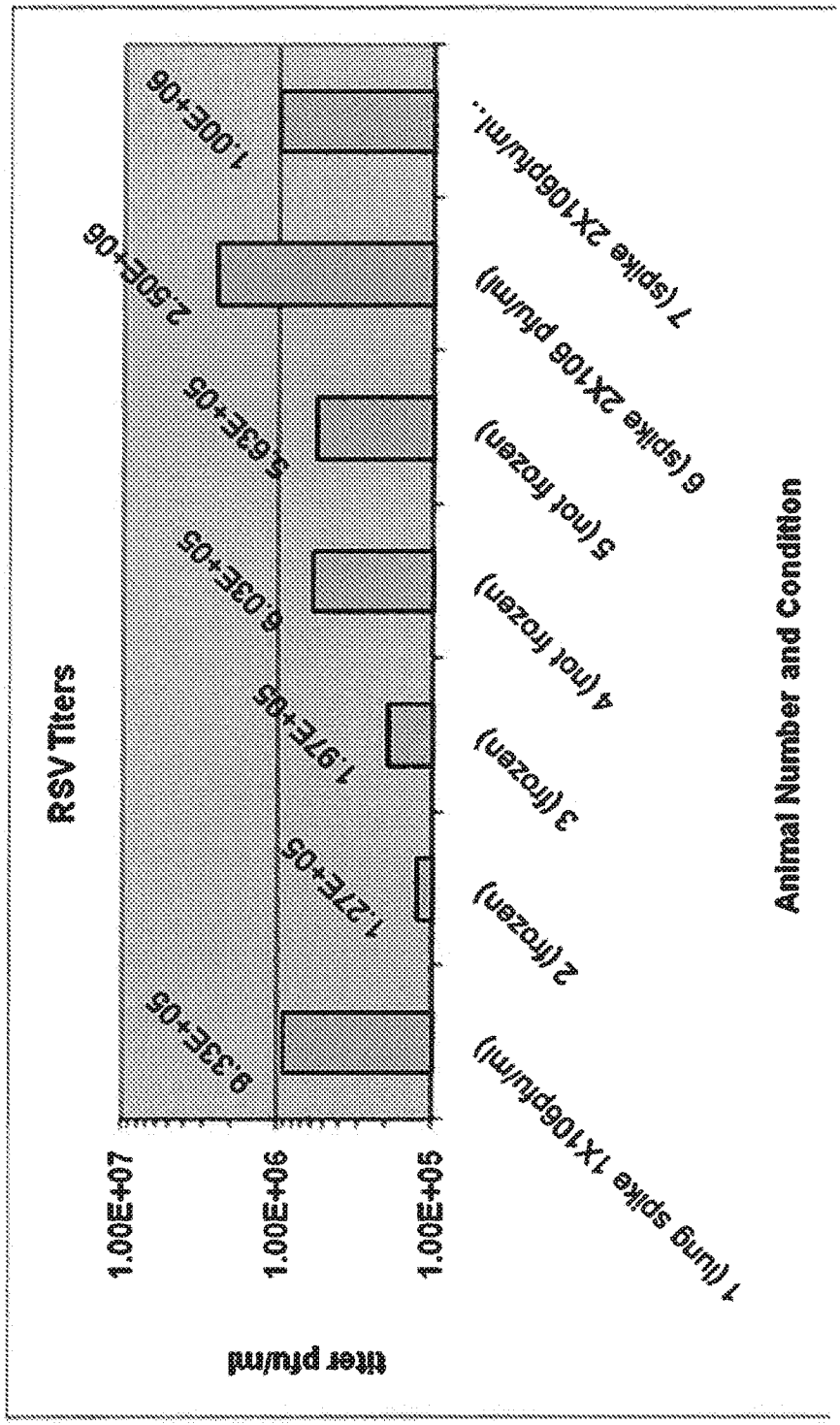

RSV Titers
titer pfu/ml
1.00E+07
1.00E+06
1.00E+05
9.33E+05
1.27E+05
1.97E+05
6.03E+05
5.63E+05
2.50E+06
1.00E+06
1 (1ung spike 1X106pfu/ml)
2 (frozen)
3 (frozen)
4 (not frozen)
5 (not frozen)
6 (spike 2X10B pfu/ml)
7 (spike 2X106pfu/ml..
Animal Number and Condition

FIG. 54

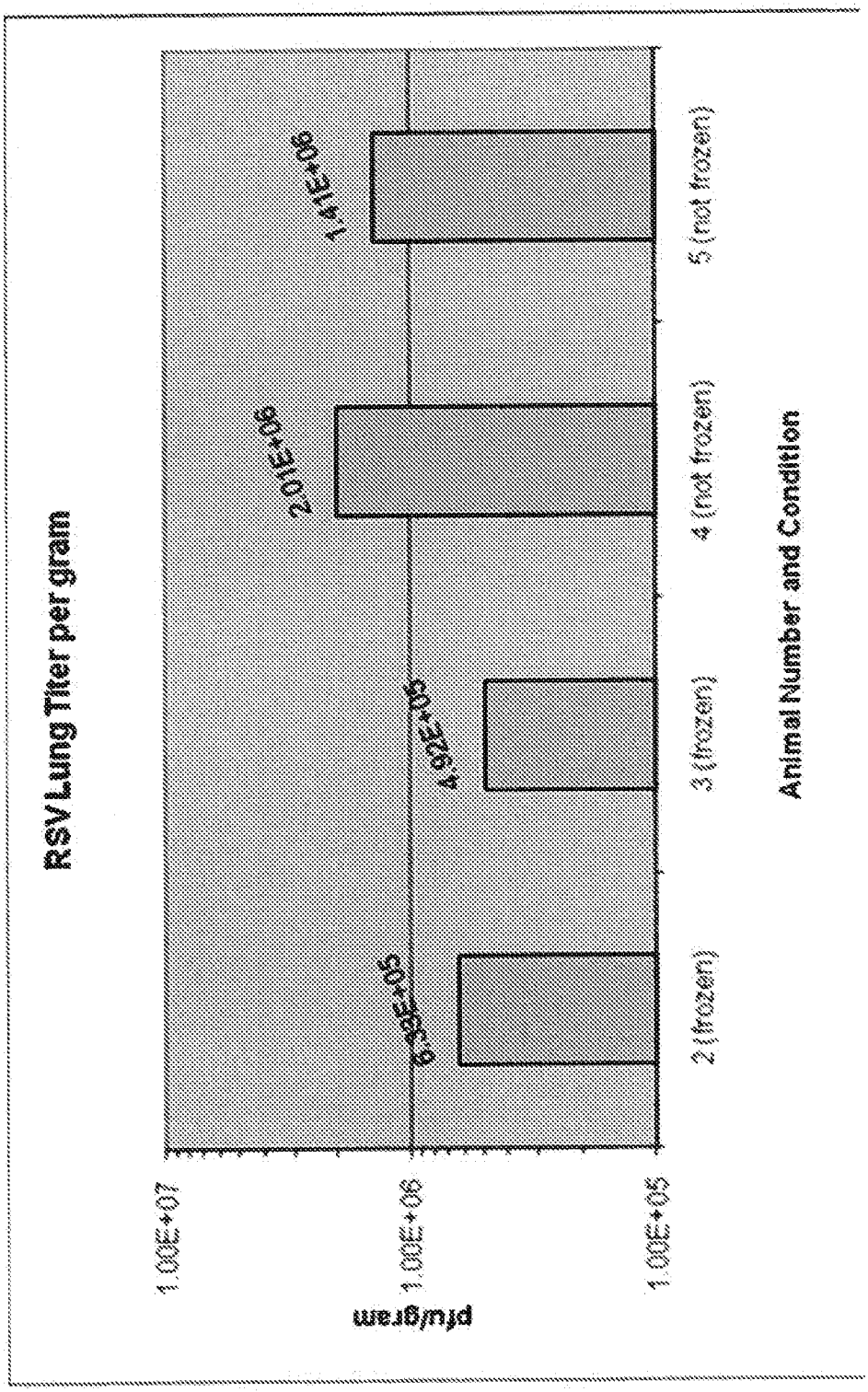

RSV Lung Titer per gram
pfu/gram
1.00E+07
1.00E+06
1.00E+05
6.63E+05
4.92E+05
2.01E+06
1.41E+06
2 (frozen)
3 (frozen)
4 (not frozen)
5 (not frozen)
Animal Number and Condition

FIG. 55

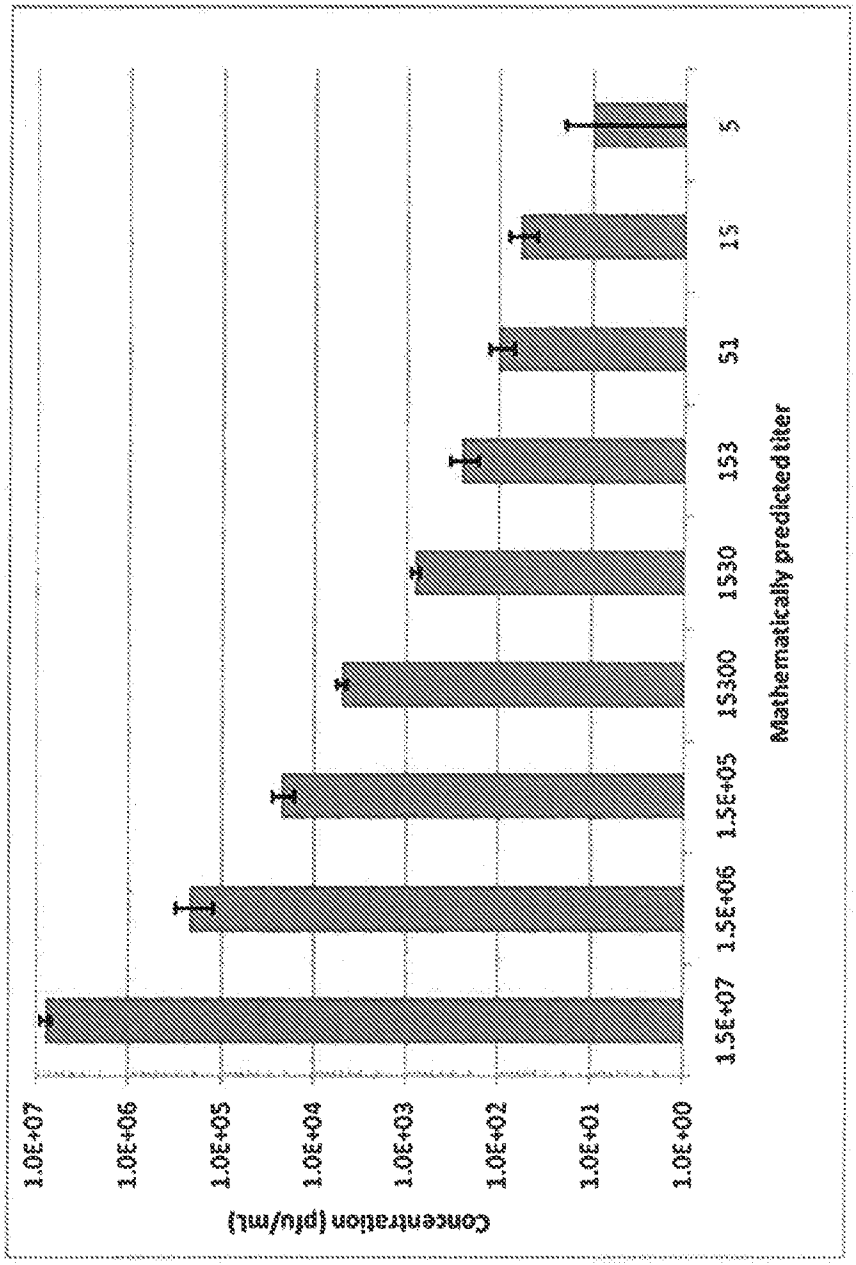

Concentration (pfu/mL)
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
1.5E+07
1.5E+06
1.5E+05
15300
1530
153
51
15
5
Mathematically predicted titer

FIG. 56

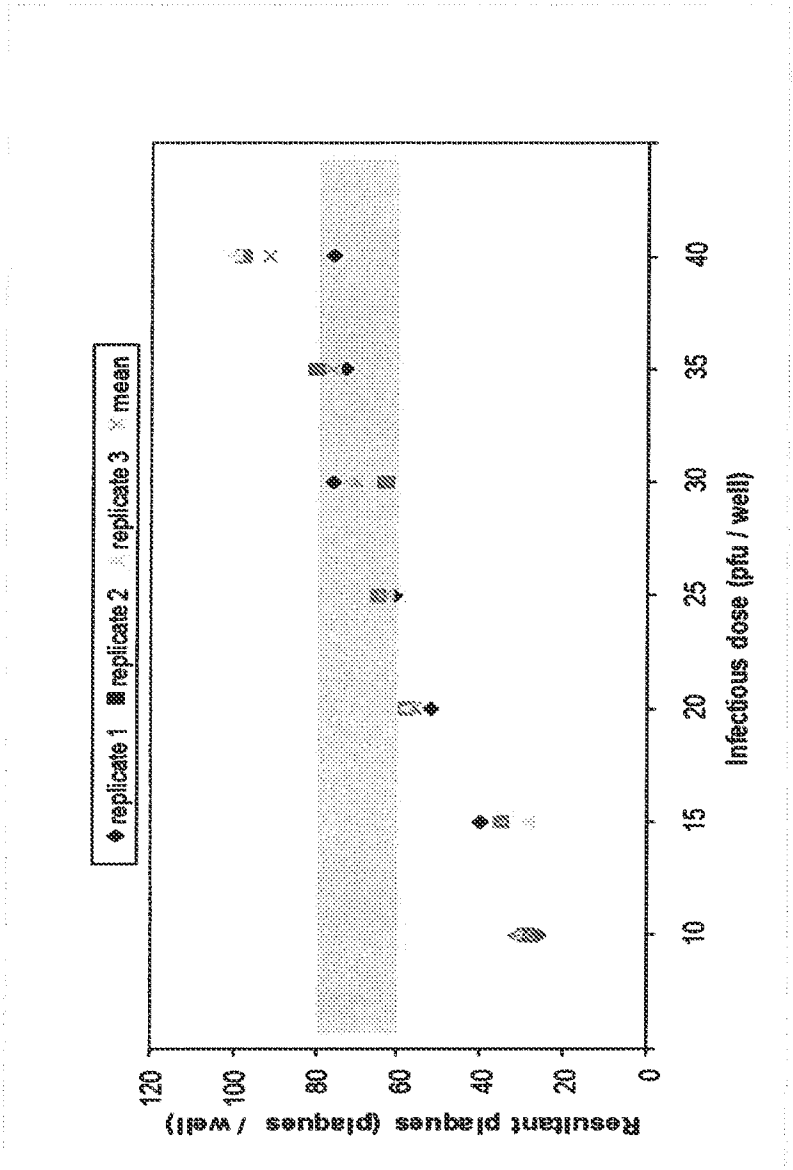

replicate 1
replicate 2
replicate 3
mean
Resultant plaques (plaques / well)
Infectious dose (pfu / well)
0
20
40
60
80
100
120
10
15
20
25
30
35
40

FIG. 57

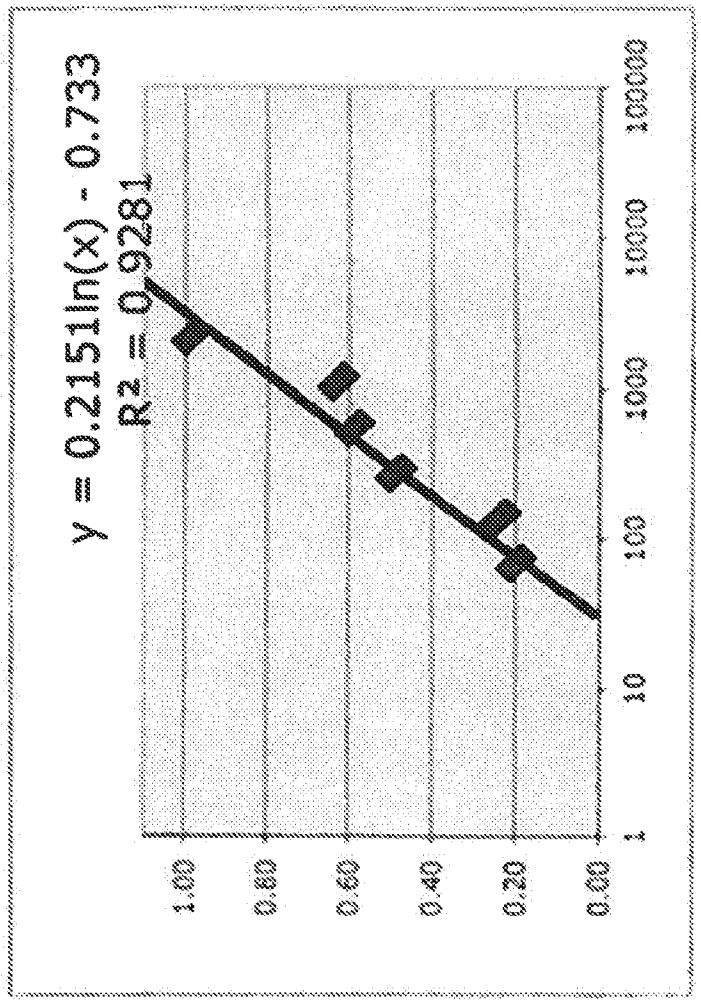

y = 0.2151ln(x) - 0.733
R² = 0.9281
1.00
0.80
0.60
0.40
0.20
0.00
1
10
100
1000
10000
100000

FIG. 58(A)

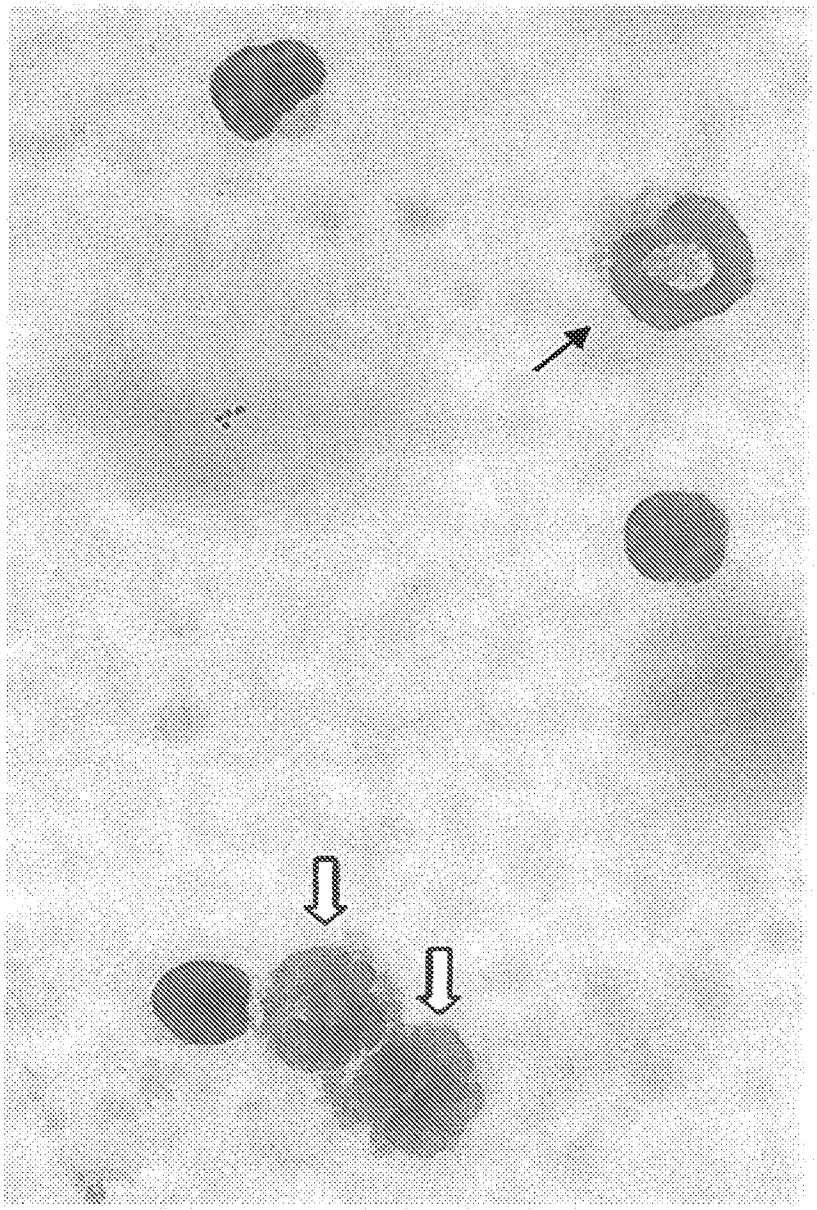


FIG. 58(B)

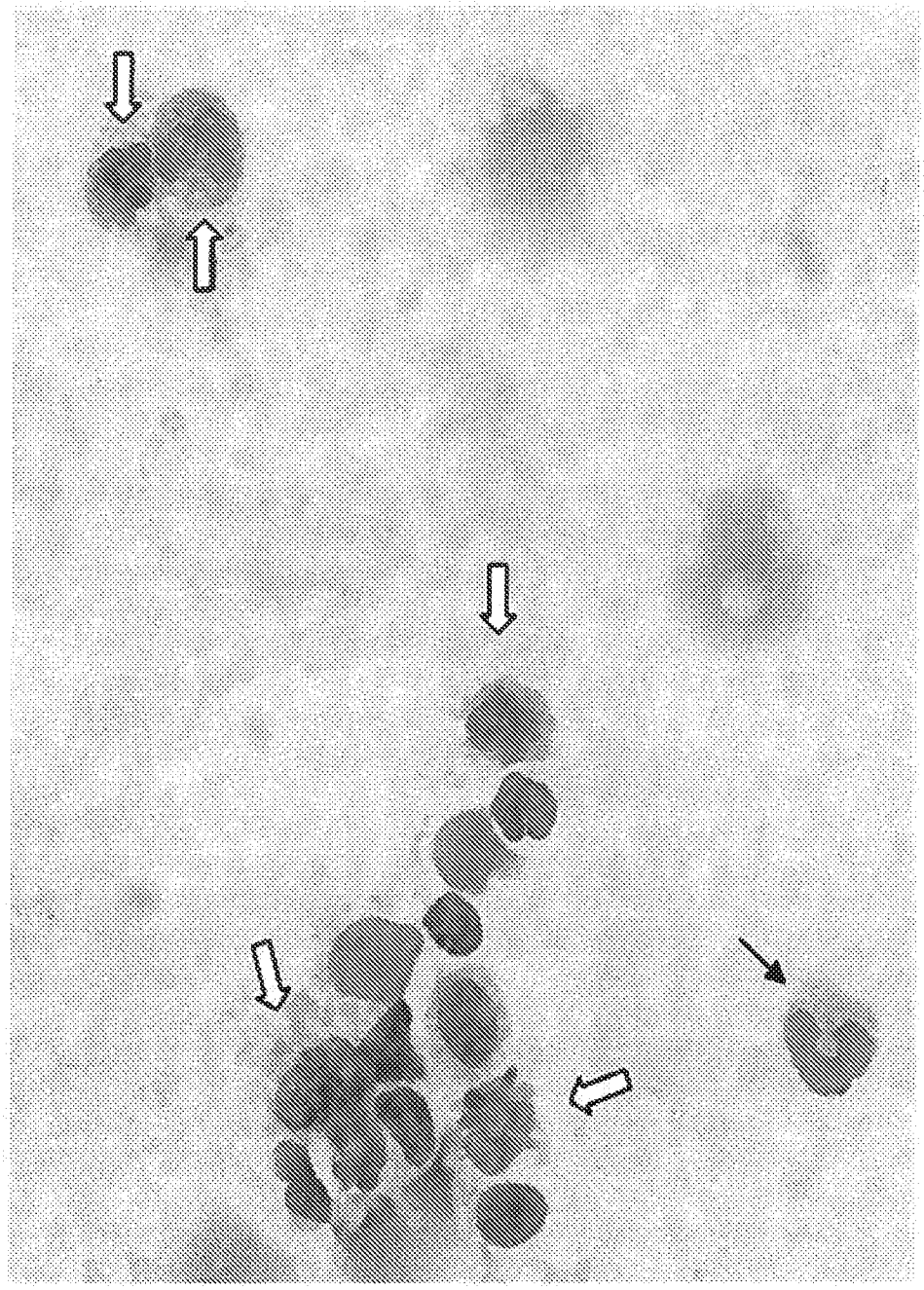


FIG. 58(C)

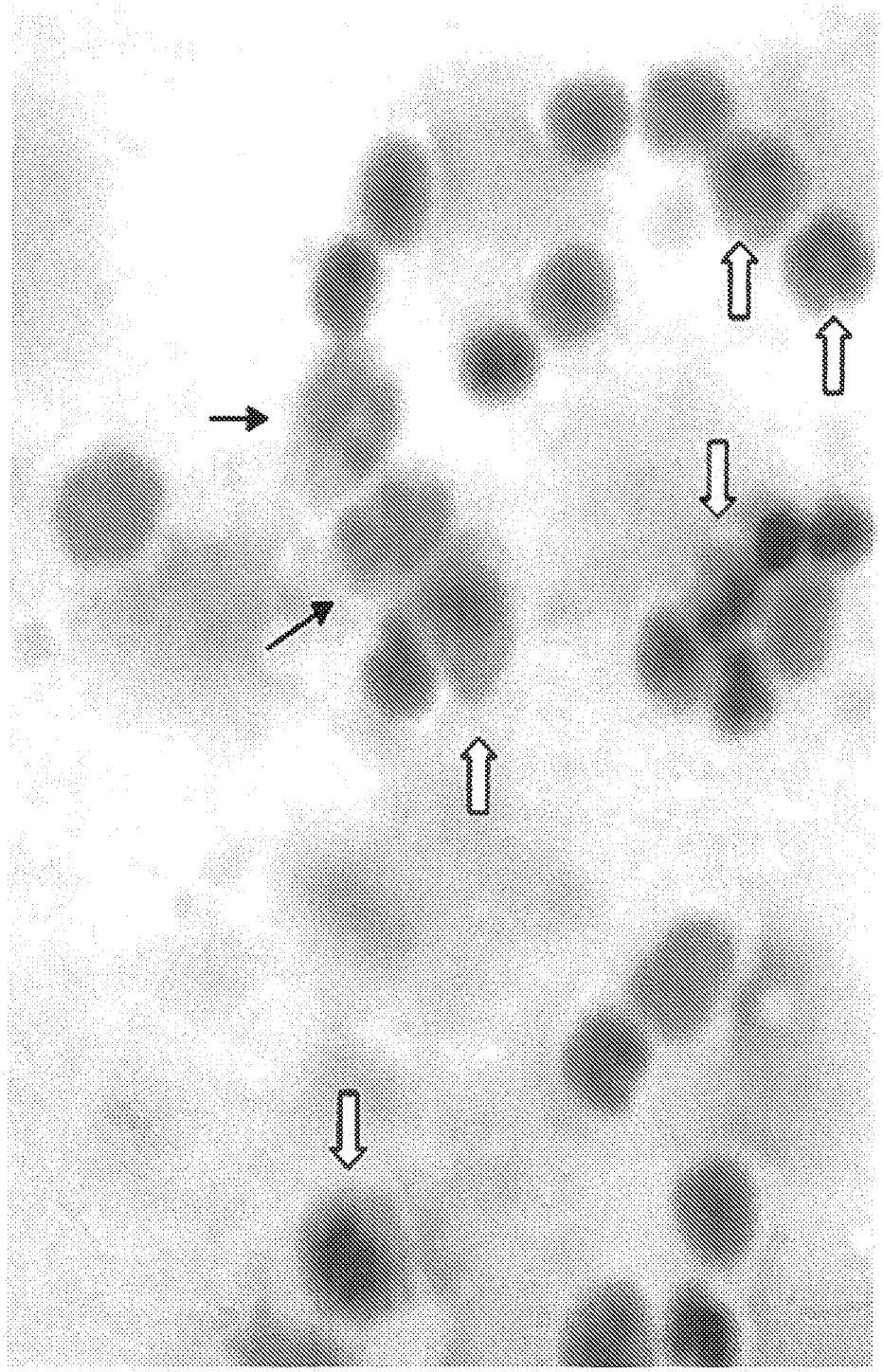


FIG. 59

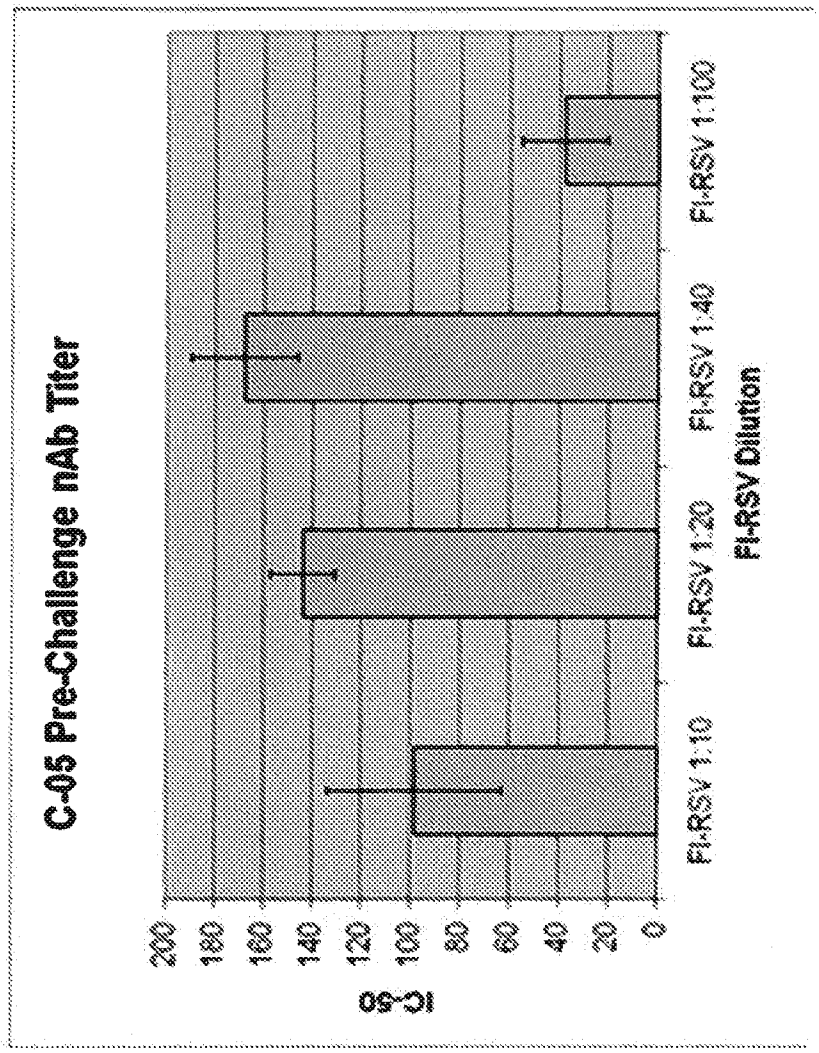

C-05 Pre-Challenge nAb Titer
IC-50
200
180
160
140
120
100
80
60
40
20
0
FI-RSV 1:10
FI-RSV 1:20
FI-RSV 1:40
FI-RSV 1:100
FI-RSV Dilution

FIG. 60

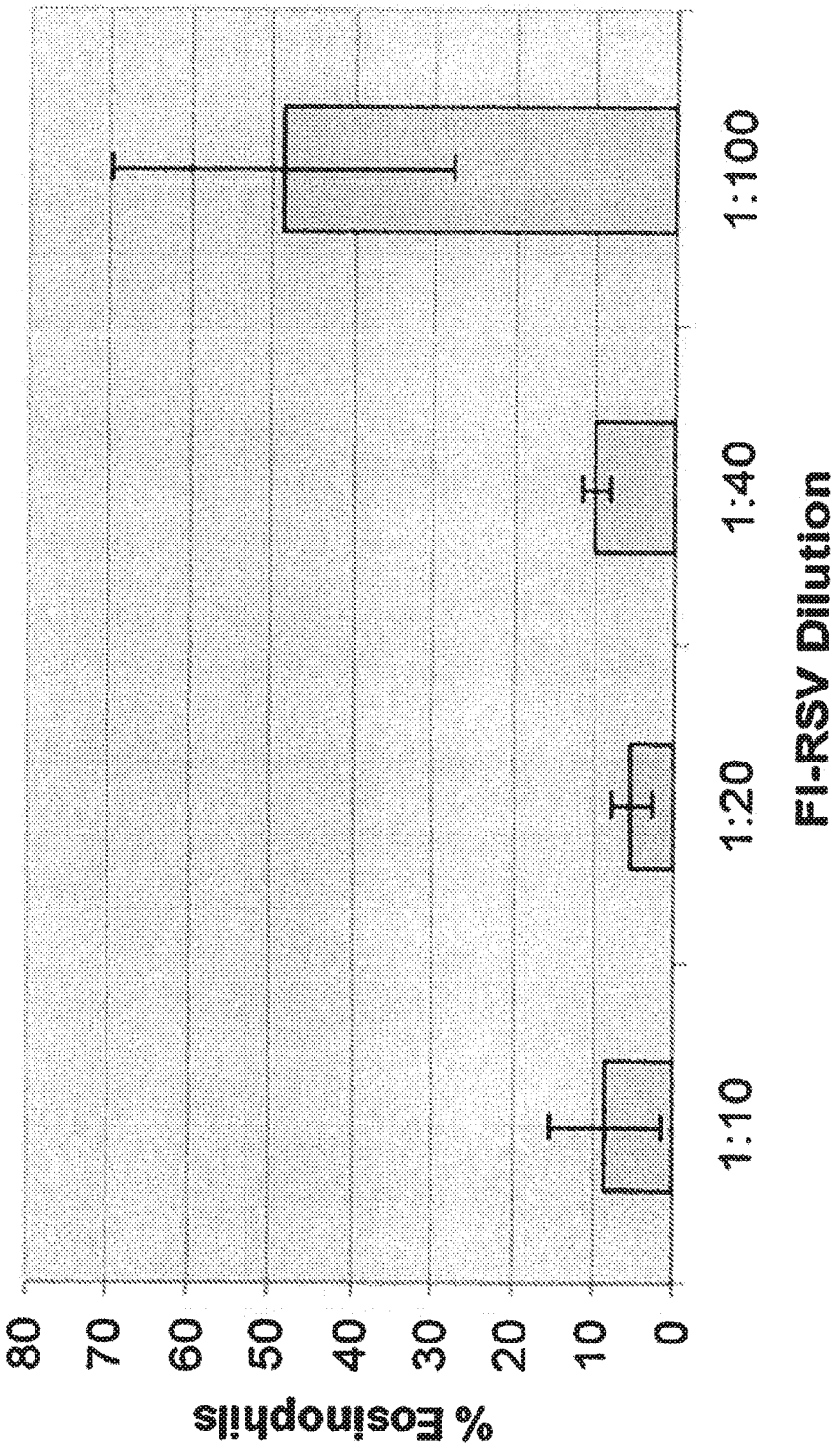

C-05 % eosinophils from BAL
% Eosinophils
80
70
60
50
40
30
20
10
0
1:10
1:20
1:40
1:100
FI-RSV Dilution

FIG. 61

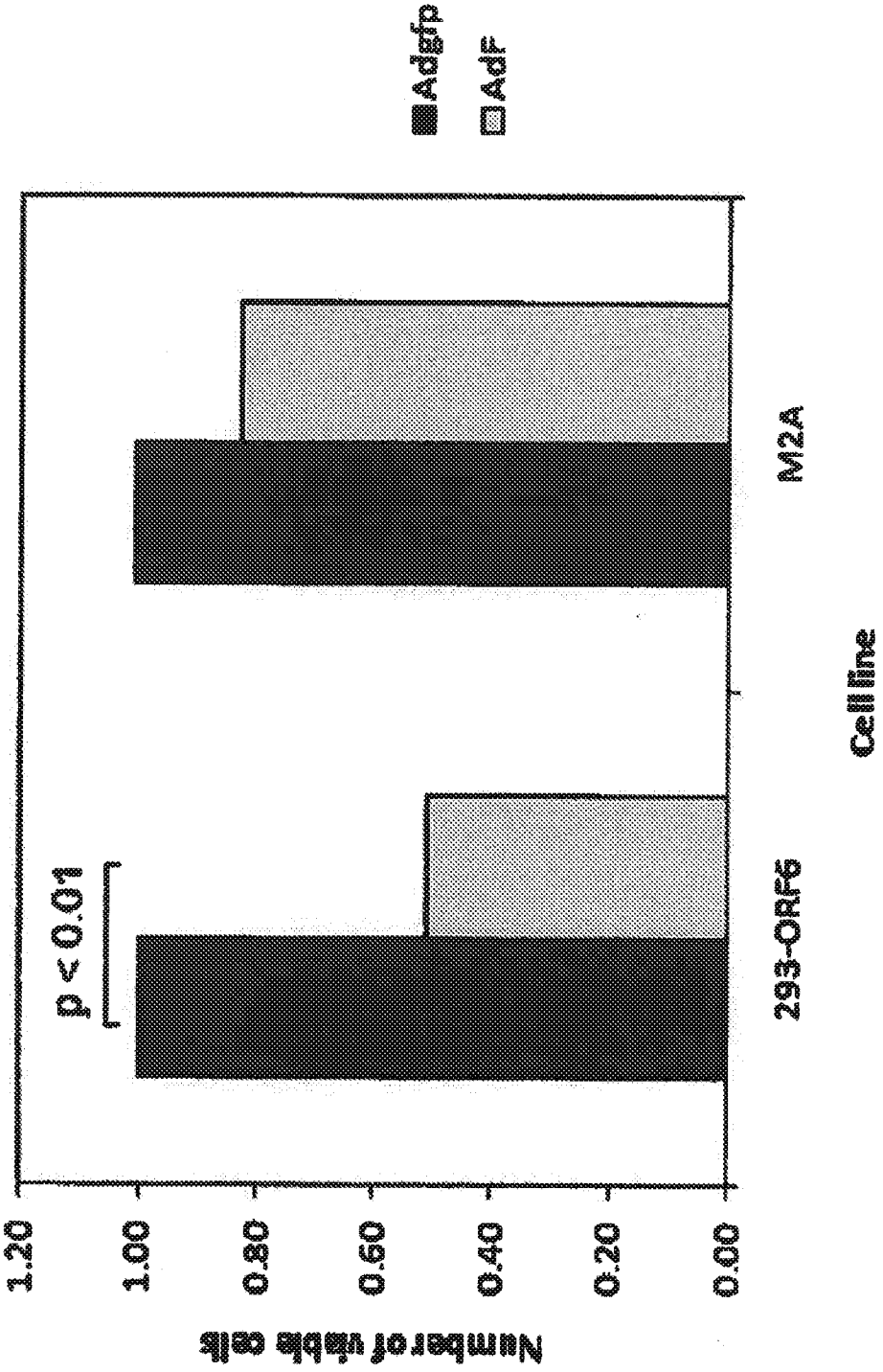

Adgfp
AdF
M2A
293-ORF6
Cell line
p < 0.01
1.20
1.00
0.80
0.60
0.40
0.20
0.00

FIG. 62

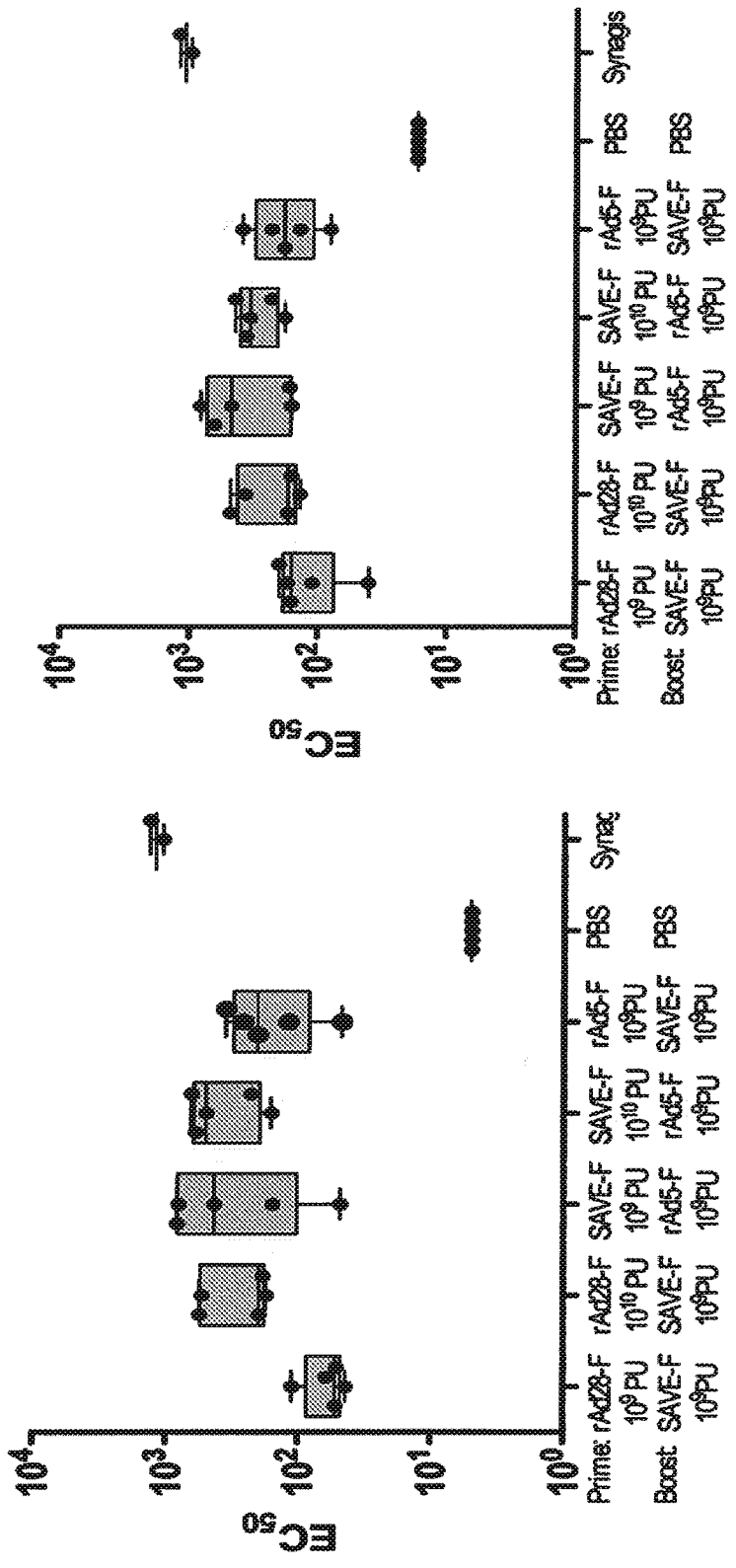

Week 7
week 5
EC50
Prime: rAd28-F SAVE-F rAd28-F SAVE-F rAd5-F PBS Synagis
10^9 PU 10^9 PU 10^10 PU 10^10 PU 10^9PU
Boost: SAVE-F rAd5-F SAVE-F rAd5-F SAVE-F PBS
10^9PU 10^9PU 10^9PU 10^9PU 10^9PU

FIG. 63

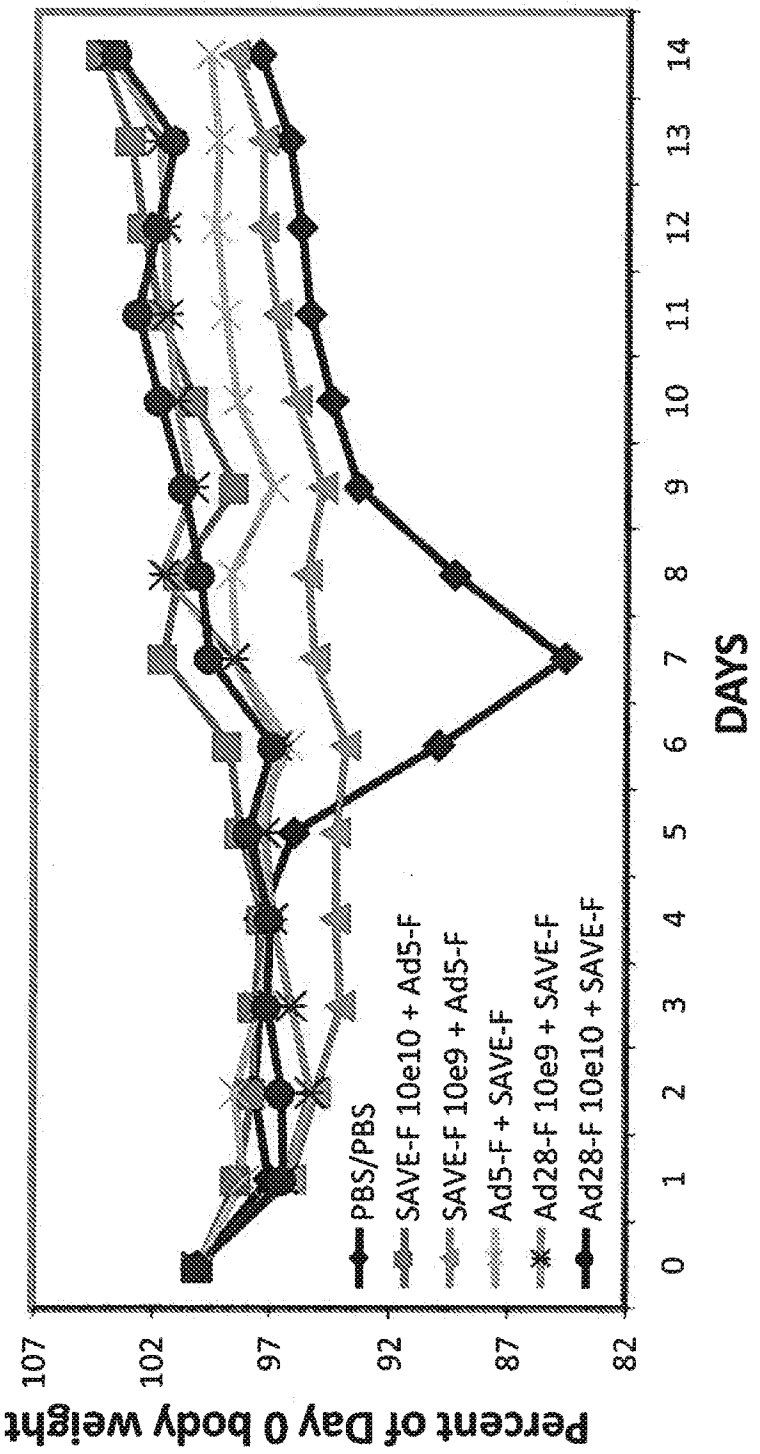

Percent of Day 0 body weight
107
102
97
92
87
82
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
DAYS
PBS/PBS
SAVE-F 10e10 + Ad5-F
SAVE-F 10e9 + Ad5-F
Ad5-F + SAVE-F
Ad28-F 10e9 + SAVE-F
Ad28-F 10e10 + SAVE-F

FIG. 64

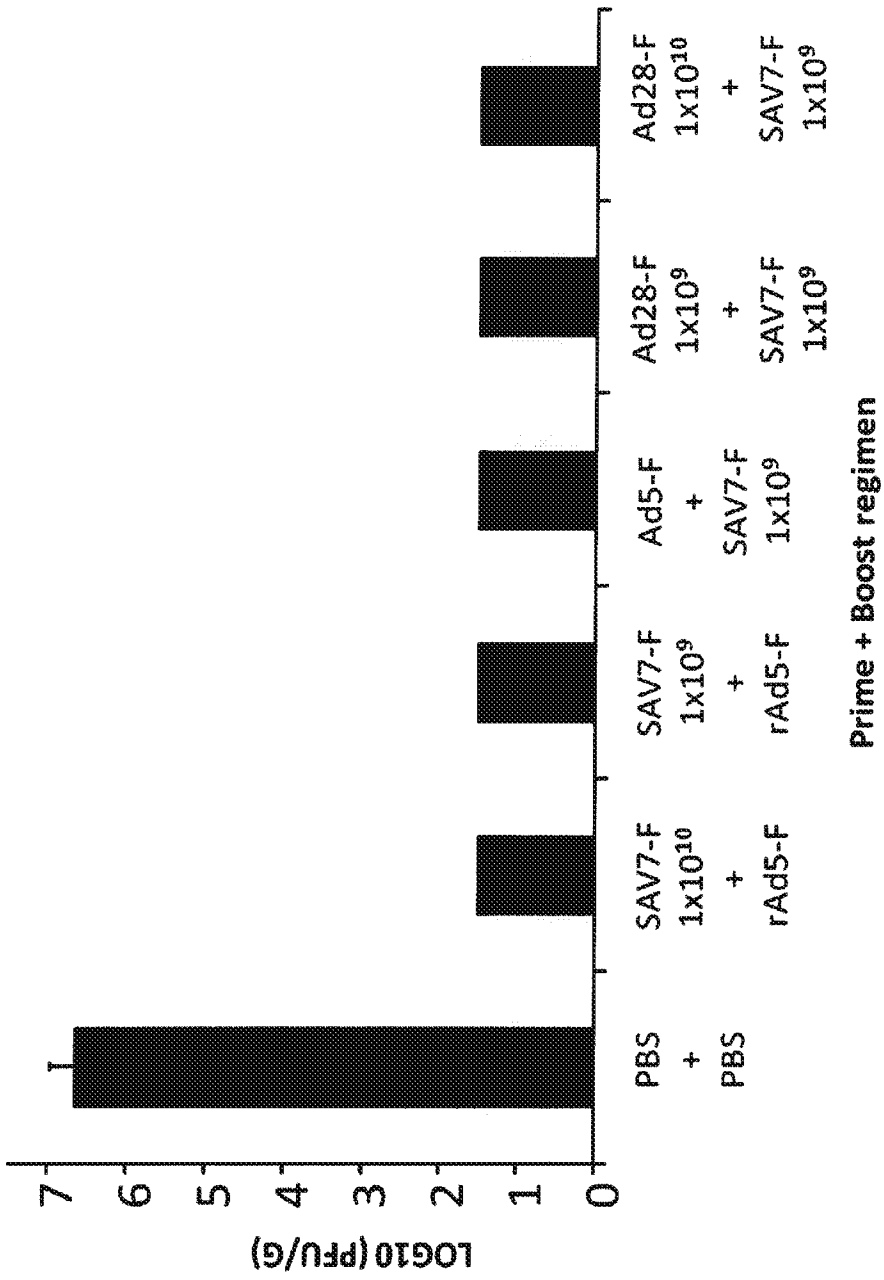

LOG10 (PFU/G)
0
1
2
3
4
5
6
7
PBS + PBS
SAV7-F 1x10^10 + rAd5-F
SAV7-F 1x10^9 + rAd5-F
Ad5-F + SAV7-F 1x10^9
Ad28-F 1x10^9 + SAV7-F 1x10^9
Ad28-F 1x10^10 + SAV7-F 1x10^9
Prime + Boost regimen

FIG. 65

Table 6. Representative plaque count with determination of mean count per dilution.

		Absolute plaque numbers														
				32	64	128	256	512	1024	2048	4096	8192	16384	32768	65536
RSV 1e6	group1	a	G1-1	4.0	11.0	13.0	26.0	31.3	33.3	52.0	49.0	42.3	41.7	52.0	#DIV/0!
			rep1	2.0	7.0	14.0	33.0	35.0	38.0	57.0	62.0	46.0	43.0		
			rep2	4.0	15.0	9.0	19.0	34.0	35.0	47.0	51.0	42.0	39.0	61.0	
			rep3	6.0	11.0	16.0	26.0	25.0	27.0	52.0	34.0	39.0	43.0	43.0	

Table 7. Example of determination of percentage of maximum plaque count for graphing.

Percent of maximum plaque number														
	Maximum plaque number													
														IC-50
		32	64	128	256	512	1024	2048	4096	8192	16384	32768	65536	
G1-1	52		0.21	0.25	0.50	0.60	0.64	1.00						308.65
rep1		0.04	0.13	0.27	0.63	0.67	0.73	1.10	1.19	0.88	0.83	0.00	0.00	
rep2		0.08	0.29	0.17	0.37	0.65	0.67	0.90	0.98	0.81	0.75	1.17	0.00	
rep3		0.12	0.21	0.31	0.50	0.48	0.52	1.00	0.65	0.75	0.83	0.83	0.00	