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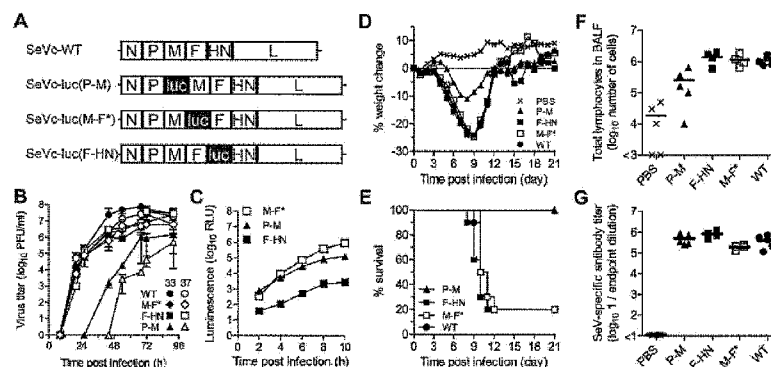


FIG. 1

(57) **Abstract:** The present invention relates to a Sendai virus or recombinant Sendai virus vector. In particular the present invention provides methods, vectors, formulations, compositions, and kits for a modified Enders strain Sendai viral vector. An immunogenic vector can be used in any in vitro or in vivo system. Moreover, some embodiments include vectors for imaging virus growth, location and transmission.

MODIFIED SENDAI VIRUS VACCINE AND IMAGING VECTOR

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application Serial No. 61/480,008
5 filed April 28, 2011. The above-identified application is incorporated herein by reference in its entirety.

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10 of Health (NIH) NIAID grant numbers P01 AI054955, R01 AI088729, R01 AI083370, R56 AI083370, R01 AI056974, R01 AI038956 and R01 AI11949, and NCI P30-CA21765. The U.S. Government has certain rights in this invention. The invention was also made in part with support by American Lebanese Syrian Associated Charities (ALSAC).

FIELD OF THE INVENTION

The present invention relates to recombinant virus vectors, methods for constructing
vectors, and use of such vectors. In particular the present invention provides methods,
recombinant virus constructions and compositions, and kits for a modified Enders strain Sendai
viral vector for protection against pathogens. Moreover, some embodiments include vectors for
20 imaging or tracing viral spread, clearance, and transmission.

BACKGROUND OF THE INVENTION

Vaccination is the single most effective mechanism for the control of infectious disease,
yet there remain numerous pathogens for which no vaccines exist. For example, the
25 paramyxoviruses include a number of important human pathogens transmitted via the respiratory route such as human respiratory syncytial virus (RSV), the parainfluenza viruses (PIVs), human metapneumovirus, measles virus, and mumps virus (Brown et al. 2007 J. Virol 81:12535; Hall et al. 2009 N. Engl. J. Med. 360:588). The human PIVs (hPIVs) consist of four serotypes (hPIV1-4) and, along with RSV and metapneumovirus, are the most common causes of respiratory tract
30 viral infections in children. The PIVs, RSV, and metapneumovirus are efficiently transmitted by direct contact and exposure to nasopharyngeal secretions (Hall et al. 2009 N. Engl. J. Med.

360:588; Hall et al., 1981 J. Pediatr. 99:100). Nearly all children become infected with RSV by age 1, with hPIV3 by age 2, and with hPIV1 and hPIV2 by age 5 (Schickli et. al. 2009 Hum.Vaccin. 5:582; Graham et al. 2011 Immunol. Rev. 239:146). In the United States, RSV can account for up to 20% hospitalizations of young children in an RSV season with annual costs as high as 0.4 billion dollars (Hall et al. 2009 N. Engl. J. Med. 360:588; Schickli et. al. 2009 Hum.Vaccin. 5:582; Graham et al. 2011 Immunol. Rev. 239:146; Shay et al.2001 J. Infect. Dis. 183:16; Shay et al. 1999 JAMA 282:1440; Paramore et. al. 2010 Pediatr. Pulmonol. 45:578; Hall et al. 2001 N. Engl. J. Med. 344: 1917; Bourgeois et al. 2009 Pediatrics 124: e1072; Boyce et al. 2000 J. Pediatr. 137: 865). For patients with bronchiolitis and pneumonia, RSV has been identified as the etiologic agent in as many as 90% and 50% cases, respectively (Paramore et. al. 2010 Pediatr. Pulmonol. 45:578; Hall et al. 2001 N. Engl. J. Med. 344: 1917). No licensed vaccines exist for any of these human pathogens.

Sendai virus (SeV) comprises an attractive vaccine and vaccine vector. It can act as a Jennerian vaccine for hPIV-1, the leading cause of laryngotracheobronchitis (pediatric croup), based on amino-acid sequence and antigenic similarities between the two viruses (Gorman et al. 1990 Virology 175: 211; Dave et al. 1994 Virology 199:376; Smith et al. 1994 Virology 205: 453). SeV can also be manipulated by reverse genetics to produce recombinant vaccines that could to vaccinate against virtually any other pathogen(s) of choice. The desirability of a Sendai virus-based vector depends on the following: (i) capacity for facile rescue by reverse genetics, (ii) capacity to carry a marker gene for virus tracking *in vivo* and *in vitro*, (iii) support of expression and immunogenicity of foreign proteins when respective genes are introduced into different positions within the SeV genome, (iv) limited growth in primates, (v) sufficient replication-competence to support vaccination and immunogenicity in primates. Because the hPIVs and RSV cause most of the respiratory viral disease infections in the most vulnerable population of children, infants, and elderly, novel methods and compositions are needed to protect humans from parainfluenza virus and respiratory syncytial virus infections.

SUMMARY OF THE INVENTION

The present invention relates to recombinant virus vectors, methods for constructing vectors, and use of such vectors. In one embodiment, the present invention provides methods, recombinant virus constructions and compositions, and kits for a modified Enders strain Sendai

viral vector for protection against pathogens. In one embodiment, the modified Enders strain Sendai viral vector is a chimera wherein a portion of the L gene of the Enders strain is replaced with the corresponding portion from the Z-strain of Sendai virus. Some embodiments include vectors for imaging or tracing viral spread, clearance, and transmission. In one embodiment, the present invention relates to a novel recombinant Sendai virus vaccine vector with the following attributes: (i) capacity for facile rescue of recombinant vectors by reverse genetics, (ii) capacity to carry a marker gene for virus tracking *in vivo* and *in vitro*, (iii) support of expression and immunogenicity of a foreign protein(s) when respective gene(s) are introduced into different positions within the SeV genome, (iv) limited growth in primates, (v) sufficient replication-competence to support immunogenicity in primates. Further, some embodiments also provide an attribute of (vi) limited growth at 33 °C and even less growth at 37 °C. Embodiments of the present invention exhibit an unexpected balance of virus vector attenuation, virus vector growth, capacity for foreign gene expression, and immunogenicity to support each of these desired attributes.

In one embodiment, the present invention provides a vector that is unexpectedly superior to other SeV vectors including unmodified Enders or Z strains in that it can be easily rescued and exhibits both attenuation and immunogenicity in primates. In addition to use as a vaccine, the present invention contemplates in one embodiment that methods, recombinant virus constructions and formulations, and kits will facilitate the use of this Sendai virus vector as a laboratory tool or in a pre-clinical/clinical research setting.

While it is not the intention that the present invention be limited to protection against the paramyxoviruses, it is contemplated that any foreign gene (or portion thereof) encoding an immunogen of interest may be inserted into the vaccine vector of the embodiments of the invention. For example, and not meant to be limiting, the present invention contemplates embodiments where the foreign gene is selected from genes from RSV, PIV, and HIV, including fragments, homologs, analogs, and any other gene of interest for targeting a pathogen/disease of interest. Further in one embodiment, the present invention contemplates a recombinant Sendai viral vector comprising a foreign gene encoding at least one of a respiratory syncytial virus (RSV) protein, a human parainfluenza (hPIV) protein, an antigenic fragment thereof, and combinations thereof. In some embodiments the method further comprises the recombinant Sendai viral vector, wherein the RSV protein is selected from the group of a type A protein G, a

type A protein F, a type B protein G, and a type B protein F. In other embodiments the method further comprises the recombinant Sendai viral vector, wherein the hPIV protein is selected from the group of a type 1 protein HN, a type 1 protein F, a type 2 protein HN, a type 2 protein F, a type 3 protein HN, and a type 3 protein F.

5 In still another embodiment, the present invention contemplates a recombinant Sendai viral vector comprising a modified Enders strain Sendai genome with a foreign gene encoding at least one of a respiratory syncytial virus (RSV) protein. In some embodiments, the recombinant Sendai viral vector includes said foreign gene is inserted between a Sendai virus F gene and a Sendai virus HN gene.

10 In still another embodiment, the foreign gene or genes may be any other foreign antigen from any pathogen.

In still other embodiments, the foreign gene is inserted between a Sendai virus P gene and a Sendai virus M gene. In still other embodiments, the foreign gene is inserted between a Sendai virus M gene and a Sendai virus F gene.

15 In further embodiments, the recombinant Sendai viral vector includes methods for the creation of a modified Enders vaccine comprising an Enders/Z strain chimera to facilitate the rescue of an infectious virus vector from cDNA and to ensure the virus is attenuated, but immunogenic in primates. In one embodiment, the Enders/Z strain chimera is a vector wherein a portion of the L gene of the Enders strain is replaced with the corresponding portion from the Z-strain of Sendai virus. In one embodiment, the Sendai virus L gene is modified such that it contains nucleic acid encoding the following amino acid changes from the Z strain: S to G at position 155, R to K at position 258, G to E at position 466, G to E at position 482, S to R at position 581, Q to R at position 717, T to I at position 800, and R to K at position 852.

20 In another embodiment, the present invention contemplates a composition comprising the recombinant Sendai viral vector and a pharmaceutically acceptable carrier or diluent or any carrier, adjuvant or diluent.

30 In one embodiment, the present invention contemplates a method, comprising: a) providing: i. a subject in need of vaccination against RSV; ii. one of the novel Sendai vectors described herein wherein the vector comprises an RSV gene or portion thereof; and b) administering said composition to said subject in an amount effective to elicit an immune response.

In yet another embodiment, the present invention contemplates a recombinant Sendai viral vector comprising a modified Enders strain Sendai genome with a foreign gene (or portion thereof) inserted between an intergenic junction selected from the group consisting of a N-P, P-M, an M-F, F-HN an HN-L gene junctions or any other position(s) within the genome. In one
5 embodiment, the foreign gene is a reporter gene. In one embodiment, the reporter gene may be luciferase but may (in other embodiment) be any other reporter gene (e.g. a gene encoding a fluorescent protein).

In further embodiments, the transcription start or stop sequences in the Sendai virus vector genome may be altered to increase or decrease transcription of downstream genes. For
10 example, the Sendai virus transcription start sequence upstream of the Sendai virus F gene may be mutated from AGGGATAAAG (SEQ. ID. NO.:19) to AGGGTGAAAG (SEQ. ID. NO.:20) to increase downstream transcription of an inserted foreign gene inserted between the M and F genes of the Sendai virus genome.

In still other embodiments, the present invention contemplates a kit comprising:

15 a) providing: i) a Sendai virus vector (i.e. one of the novel vectors described herein) with or without adjuvant and ii) instructions for use to vaccinate against a targeted pathogen.

In other embodiments, the present invention contemplates a kit comprising: a) providing:
i) a Sendai virus vector with or without adjuvant and and/in combination with another vaccine;
and ii) instructions for use to vaccinate against a targeted pathogen.

20 In yet another embodiment, the present invention contemplates a kit comprising:
a) providing: i) a vector carrying a marker gene; and ii) instructions for use to visualize the virus.

In one embodiment, the present invention contemplates a recombinant Sendai viral vector comprising a modified Sendai viral vector in which a portion of Z strain genome is added to the
Enders Sendai virus strain genome to create an Enders/Z chimera comprising a modified L gene.

25 In some embodiments the recombinant Sendai viral vector further comprises, wherein said modified L gene comprises nucleic acid encoding amino acid changes selected from the group comprising S to G at position 155, R to K at position 258, G to E at position 466, G to E at position 482, S to R at position 581, Q to R at position 717, T to I at position 800, R to K at position 852, and combinations thereof.

30 In yet further embodiments a recombinant Sendai viral vector disclosed herein further comprises a vector with immunogenic properties.

In other embodiments the recombinant Sendai viral vectors disclosed above further comprise having a foreign gene(s) inserted in any position(s) including but not limited to one or more than one intergenic junction selected from the group consisting of a N-P, a P-M, a M-F, a F-HN, a HN-L, and combinations thereof.

5 In yet other embodiments the recombinant Sendai viral vector with a foreign gene wherein said foreign gene facilitates virus tracking *in vitro*, *in vivo*, or combinations thereof.

In another embodiment the recombinant Sendai viral vector wherein said foreign gene for tracking is selected from the group of a luciferase, a green fluorescent protein, and combinations thereof.

10 In further embodiments the recombinant Sendai viral vectors described herein wherein at least a gene start/stop site is manipulated to alter gene transcription.

In another embodiment the recombinant Sendai viral vectors described herein wherein said foreign gene is a respiratory syncytial virus (RSV) F protein.

15 In other embodiments the recombinant Sendai viral vectors described herein wherein said foreign gene is a respiratory syncytial virus (RSV) G protein.

In other embodiments the recombinant Sendai viral vectors described herein wherein said foreign gene is a parainfluenza virus type 1 (PIV-1) protein.

In still further embodiments the recombinant Sendai viral vectors described herein wherein said foreign gene is a parainfluenza virus type 2 (PIV-2) protein.

20 In yet other embodiments the recombinant Sendai viral vectors described herein wherein said foreign gene is a parainfluenza virus type 3 (PIV-3) protein.

In yet other embodiments the recombinant Sendai viral vectors described herein wherein said foreign gene is a parainfluenza virus type 4 (PIV-4) protein.

25 In one embodiment the recombinant Sendai virus vectors described herein wherein said foreign gene is a reporter gene.

In one embodiment, the present invention contemplates a method of immunizing an animal or a model tissue culture system against infection comprising use of an effective amount of a Sendai viral vector or recombinant Sendai viral vector as described herein.

30 In one embodiment, the present invention contemplates a composition comprising the Sendai viral vector as described herein and a pharmaceutically acceptable carrier or diluent or any carrier, adjuvant or diluent.

In another embodiment, the present invention contemplates a method, comprising: a) providing: i. a subject in which vaccination is desired wherein said subject includes *in vitro*, *in vivo*, and combinations thereof; ii. a Sendai virus vector (i.e. one of the novel vectors described herein) or the composition described herein; and b) administering said vector or composition to
5 said subject in an amount effective to elicit an immune response.

In one embodiment the method further comprises the recombinant Sendai viral vector (i.e. one of the novel vectors described herein) or the composition described herein, wherein said foreign gene is inserted between a Sendai virus P gene and a Sendai virus M gene.

10 In one embodiment the method further comprises the recombinant Sendai viral vector (i.e. one of the novel vectors described herein) or the composition described herein, wherein said foreign gene is inserted between a Sendai virus M gene and a Sendai virus F gene.

In one embodiment the method further comprises the recombinant Sendai viral vector (i.e. one of the novel vectors described herein) or the composition described herein, wherein said foreign gene is inserted between a Sendai virus F gene and a Sendai virus HN gene.

15 In yet another embodiment, the present invention contemplates a recombinant Sendai viral vector comprising a modified Enders strain Sendai genome with a foreign gene or portion thereof inserted at an intergenic junction(s) selected from the group consisting of a N-P, a P-M, a M-F, a F-HN, a HN-L, and combinations thereof.

20 In a further embodiment the recombinant Sendai viral vector further comprises, wherein said modified Enders strain Sendai genome comprises a modified L gene.

In other embodiments the recombinant Sendai viral vector further comprises, wherein a portion of the L gene of the Enders strain is replaced with the corresponding portion from the Z-strain of Sendai virus.

25 In still other embodiments the recombinant Sendai viral vector further comprises, wherein said foreign gene is a reporter gene.

In one embodiment, the present invention contemplates a kit comprising: a) providing: i) the vector (i.e. one of the novel vectors described herein); and ii) instructions for use to vaccinate against a targeted pathogen.

30 In one embodiment the present invention contemplates a kit comprising: a) providing: i) the composition (i.e. one of the novel compositions described herein); and ii) instructions for use to vaccinate against a targeted pathogen.

In one embodiment the present invention contemplates a kit comprising: a) providing:

i) the vector (i.e. one of the novel vectors described herein); and ii) instructions for use to visualize the vector.

In one embodiment the present invention contemplates the recombinant Sendai viral vector
5 (i.e. one of the novel vectors described herein) wherein said vector is mixed with at least one other antigen or immunogen.

In yet another embodiment the present invention contemplates a method, comprising:

a) providing: i) a vector comprising an Enders Sendai virus strain genome; and ii) a Z
Sendai virus strain genome; and b) replacing at least a portion of a gene of the Enders strain
10 genome with the corresponding portion from the Z-strain of Sendai virus genome so as to generate a modified Sendai viral vector.

In one embodiment the method further comprises, wherein a portion of the L gene of the Enders strain is replaced with the corresponding portion from the Z-strain of Sendai virus so as to generate a modified Sendai viral vector comprising a modified L gene.

15 In one embodiment the method further comprises, wherein said modified L gene comprises nucleic acid encoding amino acid changes selected from the group comprising S to G at position 155, R to K at position 258, G to E at position 466, G to E at position 482, S to R at position 581, Q to R at position 717, T to I at position 800, R to K at position 852, and combinations thereof.

20 In one embodiment the method as described herein further comprising, step c) inserting a foreign gene or a portion thereof into said vector wherein said insertion is at an intergenic junction selected from the group consisting of a N-P, a P-M, a M-F, a F-HN, a HN-L, and combinations thereof.

In yet other embodiments the recombinant Sendai viral vectors described herein wherein
25 said foreign gene is a metapneumovirus protein.

In yet other embodiments said nucleic acid encoding amino acid changes selected from the group comprising S to G at position 155, R to K at position 258, G to E at position 466, G to E at position 482, S to R at position 581, Q to R at position 717, T to I at position 800, R to K at position 852 and combinations thereof.

30 Furthermore, descriptions of embodiments presented are not meant to be limiting and include all equivalent, comparable technologies, reagents, sources, diluents, uses etc. as known

by one skilled in the art. For example only and not meant to be limiting, specific sequences are presented but include the related sense, antisense, complementary, homologs, portions, fragments, 5' to 3' and 3' to 5', and analogs as known by one in the related arts such as molecular biology, biotechnology, along with any and all related arts. Moreover, while specific
 5 mention of treating humans for respiratory viral infection is presented it is contemplated that the Jennerian vaccine vector might be used as a backbone for development of other vaccines or procedures used in vitro or in vivo to diagnose or treat generally mammals, and more particularly humans. For example only, and not meant to be limiting the vaccine vector contemplates use in non-human mammals such as dogs, cats, horses, cattle, and primates. Moreover, vaccines and/or
 10 compositions optionally include pharmaceutically acceptable diluents and/or adjuvants but also include use of research type diluents/adjuvants and/or no diluents/adjuvants.

BRIEF DESCRIPTION OF THE FIGURES

The patent or application file contains at least one drawing executed in color. Copies of
 15 this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of necessary fees.

Within the sequence presented in Figure 7 you can find the different Sendai virus genes by doing a search with the sequences. The sequences presented in Figure 7B-7H were taken directly from the vector sequence in 7A and are presented individually. Related protein
 20 sequences are provided within the detailed description.

Figure 1. *In vitro* and *in vivo* phenotypes of luciferase-expressing Sendai viruses. (A)

Recombinant Sendai viruses were generated that contain the firefly luciferase gene (luc) inserted into the P-M, M-F, and F-HN gene junctions. **(B)** Multiple-step replication kinetics of wild-type
 25 (WT) and luciferase-expressing SeVs in LLC-MK2 cell cultures infected at a multiplicity of infection (MOI) of 0.01 PFU/cell at 33 °C (closed symbols) and 37 °C (open symbols). **(C)** Kinetics of luciferase reporter gene expression in LLC-MK2 cells infected with recombinant SeVs at an MOI of 5 PFU/cell, as measured by luminescence. **(D)** Changes in body weights of mice after intranasal inoculation of SeVs. **(E)** Percent survival of mice after intranasal
 30 inoculation of SeVs. **(F)** Total numbers of lymphocytes recovered from bronchoalveolar lavage fluid (BALF) of mice 10 days after infection, as measured by flow cytometry. **(G)** SeV-specific

binding antibody titers in sera of mice collected 10 days after infection, as measured by reciprocal endpoint dilutions in ELISA assays. For panels **D – G**, groups of five 8-week-old 129/Sv-strain mice were intranasally inoculated with 7,000 PFU of recombinant SeV or phosphate buffered saline (PBS) and the experiments were performed twice. Cumulative data are shown in panels **D** and **E**, and representative data are shown in panels **F** and **G**.

Figure 2. Non-invasive bioluminescence imaging of SeV infection in the respiratory tracts of living mice. Eight-week-old mice were intranasally inoculated with 7,000 PFU of SeVc-luc(P-M), SeVc-luc(F-HN), or SeVc-luc(M-F*). Every 24 hours the mice were intraperitoneally injected with luciferin substrate, anesthetized with isoflurane, imaged with a Xenogen Lumina device, and then allowed to recover. In one experiment, bioluminescence is shown on day 2 (**A**) or day 7; (**B**) post-infection (p.i.) for 129/SvJ mice infected with SeVc-luc(P-M)SeVc-luc(P-M), SeVc-luc(F-HN), or SeVc-luc(M-F*). In a second experiment, bioluminescence is shown on day 2 (**C**) or day 7 (**D**) for either 129/SvJ, DBA/2, BALB/c, or C57BL/6 mice infected with SeVc-luc(M-F*). The data are displayed as radiance, a measurement of bioluminescence intensity, on a rainbow log scale. Radiance values range from 1×10^6 (blue) to 1×10^9 (red) photons/s/cm²/steradian. Red circles show the regions of interest (ROI) for calculating the total flux (photons/s) in the nasopharynx, and red rectangles show the ROI areas for the trachea and lungs.

Figure 3. Kinetics of SeV spread and clearance in the respiratory tracts of 129/Sv mice. (A) The extents of infection were determined by non-invasive bioluminescence imaging of living, anesthetized mice every 24 h. Each data point represents the average bioluminescence of 6 mice. The total flux (photons/s) of bioluminescence intensity is calculated as the sum of radiance in the region of interest. (**B - D**) The extents of virus replication in the nasal turbinates, trachea, and lungs were determined by sacrificing groups of 3 mice at the reported days and measuring the titers of infectious viruses in LLC-MK2 cells. Both experiments were repeated and representative data is shown.

Figure 4. Virus replication and pathogenesis as a function of virus dose and mouse strain.

After intranasal inoculation of 129-strain mice with SeVc-luc(M-F*) at doses ranging from 70 to 7,000 PFU, the total flux of bioluminescence intensities (A) and viral titers (B) were measured as described in Figure 3. (C) The percentages of body weight change were measured for groups of ten mice after infection of 70 to 7,000 PFU of SeVc-luc(M-F*) in 129-strain mice. The experiment was repeated and representative data is shown. (D) SeV-specific binding antibody titers in sera of 129-strain mice were collected 10 days after inoculation with 70 to 7,000 PFU of SeVc-luc(M-F*) and are reported as the reciprocal endpoint dilutions in ELISA assays. Five infected and two control mice were used in the experiment, which was performed twice. Representative data is shown. (E) The total flux of bioluminescence intensities present in the nasopharynx, trachea, and lungs after 7,000 PFU intranasal inoculation of 129-Sv, DBA/2, BALB/c, or C57BL/6-strain mice with SeVc-luc(M-F*). The averages are for six animals, the experiment was repeated and the results from a representative experiment are shown. (F) The percentages of body weight change were measured for groups of 10 mice after infection with 7,000 PFU of SeVc-luc(M-F*). The experiment was repeated and representative data is shown.

Figure 5. SeV infection and immunity after contact transmission. One mouse per cage was directly inoculated with either 70 PFU (A,C,E) or 7,000 PFU (B,D,F) of SeVc-luc(M-F*) and then introduced into a cage with 3 naïve animals after one day. The total flux of bioluminescence intensities in the nasopharyngeal cavities of individual 129-strain (A – B) and BALB/c-strain (C – D) mice are shown. Serum was collected on day 60 and the contact mice were challenged with 7,000 PFU of SeVc-luc(M-F*) on day 63 so that potential re-infection could be monitored by bioluminescence. SeV-specific binding antibody titers were measured as reciprocal endpoint dilutions of sera collected on day 60 from mice co-housed with animals inoculated with 70 PFU (E) or 7,000 PFU (F). Open bars correspond to mice directly inoculated on day 0 and solid bars correspond to the contact mice. The experiment was performed in triplicate for 129-strain mice (3 donor animals and 9 transmitted) and duplicate for BALB/c-strain mice (2 donor animals and 6 transmitted).

Figure 6. Timing and tissue-tropic spread of SeV infection after contact transmission. The co-housing of contact mice with mice inoculated with SeVc-luc(M-F*) is described in Figure 5. The total flux of bioluminescence intensities in individual, representative 129-strain (A – B) and

BALB/c-strain (C – D) mice are shown for the nasopharynx (triangles), trachea (circles), and lungs (squares). Time until detection of bioluminescence in the nasopharynx (limit of detection: $> 6 \log_{10}$ photons/s) after inoculation of donors with either 70 PFU (E) or 7,000 PFU (F). Average percent weight change in BALB/c (G) and 129/SvJ mice (H). The contact transmission experiment was performed in triplicate for 129/SvJ mice and in duplicate for BALB/c mice. Open symbols and bars correspond to directly inoculated mice and solid symbols and bars correspond to the contact mice. In panels g and h, the symbol X corresponds to uninfected, PBS-inoculated control mice.

- 10 **Figure 7. Sequence Listings. FIG. 7A-H are as follows:** A, The sequence of a modified SeV construct (pSeVc) (Also see Table 1), and sequences for the individual Sendai virus genes are provided; B, NP cDNA: (SEQ. ID. NO.:4); C, P cDNA: (SEQ. ID. NO.:6); D, C cDNA (SEQ. ID. NO.:8); E, M cDNA: (SEQ. ID. NO.:10); F, F cDNA (SEQ. ID. NO.:12); G, HN cDNA (SEQ. ID. NO.:14); and H, L cDNA (SEQ. ID. NO.:16). Associated translations are shown in the detailed description. The sequences are listed 5' to 3' (left to right). The key to the figure is as follows:

Sequence Key for genes within the pSeVc plasmid

- 19  = Start codon
 = Stop Codon
20 **Violet**= NP gene sequence
Purple= P gene sequence
PURPLE ALL CAPS = C gene sequence (internal start site within the P gene)
orange = M gene sequence
Dark blue= F gene sequence
25 **Pink** = HN gene sequence
Bold black = L gene sequence
 = Transcription start signal
 = NotI (for cloning in gene of interest e.g. hPIV-2)

- 30 **Figure 8 Examples of sites in which foreign genes may be inserted into the Sendai virus vector genome.** Insertion sites may include, but are not limited to, positions upstream of the P,

M, F, and HN genes. The foreign gene cassettes are flanked by *NotI* restriction endonuclease sites, and Sendai virus vector cDNA plasmids have been engineered to have unique *NotI* restriction endonuclease sites upstream of the various Sendai virus genes.

- 5 **Figure 9. Construction of luciferase-expressing Sendai viruses.** (A) Nucleotide sequence of the firefly luciferase gene cassette. A pGEM3 cloning plasmid was engineered to contain flanking *NotI* restriction sites, the firefly luciferase reporter gene, gene end and gene start sequences. (B) To insert the luciferase reporter gene cassette into three gene junctions, three pSeV genome plasmids were cloned to contain a unique *NotI* restriction site in each of the P-M, M-F, and F-HN gene junctions. For the pSeV-luc(M-F*) genome plasmid, the naturally occurring suboptimal start signal AGGGATAAAG (SEQ. ID. NO.: 19) was also mutated to the more efficient start signal AGGGTGAAAG (SEQ. ID. NO.: 20) to compensate for expected attenuation due to the addition of the foreign gene and additional gene junction. The firefly luciferase gene cassette (panel A) was subcloned from the pGEM3 plasmid into the pSeV genome plasmids using the *NotI* restriction sites. (C) Design of pSeV cDNA plasmids for the rescue of WT and recombinant SeVs containing the luciferase reporter gene (luc). The locations of the SeV genes nucleoprotein (N), polymerase (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and large (L) protein are shown, as well as the T7 RNA polymerase promoter (T7) and hepatitis delta virus ribozyme sequence (ribo). Gene start sequences are shown in green and the naturally occurring, suboptimal AGGGATAAAG (SEQ. ID. NO.: 19) gene start sequence between the M and F genes of WT SeV is shown in yellow. Gene end sequences are shown in red. The 3' leader sequence upstream of the N gene and the 5' trailer sequence downstream of the L gene are not shown for simplicity.
- 25 **Figure 10. SeV protein expression and incorporation into virions.** (A) SeV protein expression in LLC-MK2 cells. Confluent monolayers of LLC-MK2 cells were infected with recombinant SeVs at an MOI of 5 PFU/cell and incubated for 16 h. Following radiolabeling and immunoprecipitation, viral proteins in the lysates were resolved by SDS-PAGE and visualized with a phosphorimager. (B) Ratios of SeV protein expression. Protein expression was quantified with ImageQuant® 5.2 software and normalized to the expression level of the N protein. The data represent the averages (+/- standard deviation) from three experiments. (C) SeV
- 30

composition. Recombinant SeVs were harvested from the allantoic cavities of embryonated chicken eggs, purified by centrifugation through a sucrose gradient, separated by SDS-PAGE, and visualized with Coomassie Blue.

5 **Figure 11. Immunological responses to infection by recombinant SeVs in mice.** Groups of five 8-week-old 129/SvJ mice were intranasally inoculated with 30 μ l containing 7,000 PFU of recombinant SeV or PBS. On day 10 p.i., serum was collected and the mice were euthanized to recover bronchoalveolar lavage fluid (BALF). Experiments were performed twice with representative data shown. Each data point represents an individual animal and horizontal bars show group averages. The numbers of CD4+ (A) and CD8+ (B) T cells recovered from BALF were determined by flow cytometry. (C) Luciferase-specific binding antibody titers in sera were determined by ELISA assays and are expressed as reciprocal endpoint dilutions.

15 **Figure 12. Bioluminescence and Sendai virus titers in the respiratory tracts of 129/SvJ mice.** Groups of three 8-week-old mice were intranasally inoculated with 7,000 PFU of recombinant SeV. (A) *In vivo* bioluminescence was measured for all three luciferase-expressing viruses on days 4 and 6 p.i., after which lungs were immediately harvested and homogenized so that *ex vivo* luciferase activity could be measured. A fit of the data with a least squares linear regression model yielded an R^2 value of 0.878. RLU denotes relative light units. (B) Comparison between light detected by the camera and viral titers of homogenates from the nasopharynx (triangles), trachea (circles), and lungs (squares). Each point represents data from a single mouse infected with SeVc-luc(M-F*) and studied on day 2, 3, 5 or 7 p.i.. Least squares linear regression yielded R^2 values of 0.864, 0.915 and 0.961 for the nasopharynx, trachea and lungs, respectively.

25 **Figure 13. Bioluminescence and Sendai virus titers in the respiratory tracts of BALB/c and 129/SvJ mice.** Groups of three 8-week-old mice were intranasally inoculated with either 70 or 7,000 PFU of SeVc-luc(M-F*). (A) *In vivo* bioluminescence was measured in BALB/c mice infected with 7,000 PFU of virus on days 2, 3, 5 and 7 p.i. after which the animals were euthanized and tissues were harvested so that virus titers from tissue homogenates could be measured by plaque titration in LLC-MK2 cells. Correlations between virus titers in tissue homogenates and light detected by the camera were found with R^2 values of 0.928, 0.656, and

0.846 for the nasopharynx, trachea, and lungs, respectively. Virus titers in homogenates from the nasopharynx (**B**) and lungs (**C**) of both BALB/c- and 129-strain mice infected with either 70 or 7,000 PFU of SeVc-luc(M-F*) were measured by plaque titration in LLC-MK2 cells. The data represent the average virus titers of six mice (+/- standard deviation).

5 **Figure 14. Tissue-specific timing and magnitude of Sendai virus spread in the respiratory tracts of intact mice after inoculation and contact transmission.** In each group, one BALB/c or 129/SvJ mouse was inoculated intranasally with either 70 or 7,000 PFU of SeVc-luc(M-F*) and three contact animals were co-housed one day later as described in Figure 5. (**A-D**) Time
 10 until detection of bioluminescence in the nasopharynx (nasop.), trachea, and lungs (limit of detection: $> 6 \log_{10}$ photons/s) after direct inoculation (open bars) and after contact transmission (solid bars). (**E-H**) Overall magnitude of infection after direct inoculation (open bars) and after contact transmission (solid bars) as determined by integration of daily measurements of total flux with respect to time. The areas under the curve (AUC) of bioluminescence are expressed as the total amount of photons on a $\log_{10}$ scale. The experiment was performed in triplicate for 129-
 15 strain mice (3 donor animals and 9 transmitted) and duplicate for BALB/c-strain mice (2 donor animals and 6 transmitted).

Figure 15. Diagram of PSEV construction. Sendai virus Enders strain cDNA was cloned into the pUC vector containing a T7 promoter upstream and a RNA self-cleaving HDV sequence
 20 downstream.

Figure 16. Persistence of Z strain vaccine and Enders-based vaccines in primates after intranasal and intratracheal inoculation. African green monkeys were inoculated by the intranasal and intratracheal routes with either Sendai virus Z vaccine (**A** and **B** (Skiadopoulos et.
 25 al. 2002 Virology 297:153)) or Sendai virus Enders-based vaccines (**C** and **D**). Virus loads were monitored in the URT or LRT following infections.

**Figure 17. Immunogenicity of unmodified Enders Sendai virus and a recombinant modified Sendai virus Enders-based vaccine expressing RSV F (SeVc-RSVF(F-HN)) in
 30 African green monkeys.** Sera were taken 25 days after vaccinations and tested for antibody responses to RSV F (A) and Sendai virus (B) by ELISA.

Figure 18. Modified Sendai virus Enders-based vaccine expressing RSV F protects

primates from RSV. Control (left, **A**) and test (right, **B**) animals were challenged with RSV approximately 1 month after vaccination. Bronchoalveolar lavage (BAL) samples were collected for 10 days after challenge and tested for RSV growth. All animals that received the modified Sendai virus Enders-based vaccine expressing RSV F (SeVc-RSVF(F-HN)) were protected from RSV infection of the lower respiratory tract.

Figure 19. Modified Sendai virus Enders-based vaccine expressing RSV F (SeVc-RSVF(F-

HN)) is protective against RSV at low dose. The modified Sendai virus Enders-based vaccine expressing RSV F was used to vaccinate animals at doses of 10e6, 10e4 and 10e2. All vaccinated animals were protected from RSV challenge as demonstrated by measuring virus in the lung after challenge.

DEFINITIONS

To facilitate the understanding of this invention a number of terms (set off in quotation marks in this Definitions section) are defined below. Terms defined herein (unless otherwise specified) have meanings as commonly understood by a person of ordinary skill in the areas relevant to the present invention.

As used herein, the term "subject" or "patient" refers to any organism to which compositions in accordance with the invention may be administered, e.g., for experimental, diagnostic, prophylactic, and/or therapeutic purposes. Typical subjects include animals (e.g., mammals such as mice, rats, rabbits, non-human primates, and humans; insects; worms; etc.). *In vitro* systems may also be used (e.g. to express SeV or other proteins for study within the target cell and/or for isolation). For example only and not meant to be limiting LLC-MK2 cells in culture are contemplated (See Figure 1).

As used herein, the term "immune response" refers to the alteration in the reactivity of an organism's immune system upon exposure to an antigen. The term "immune response" encompasses but is not limited to one or both of the following responses: antibody production (e.g., humoral immunity), and induction of cell-mediated immunity (e.g., cellular immunity including helper T cell and/or cytotoxic T cell responses).

As used herein, the term "primer" refers to an oligonucleotide, whether occurring naturally (e.g., as in a purified restriction digest) or produced synthetically, capable of acting as a point of initiation of nucleic acid synthesis when placed under conditions in which synthesis of a primer extension product complementary to a nucleic acid strand is induced (i.e., in the presence of nucleotides, an inducing agent such as DNA polymerase, and under suitable conditions of temperature and pH). The primer is preferably single-stranded for maximum efficiency in amplification, but may alternatively be double-stranded. If double-stranded, the primer is first treated to separate its strands before being used to prepare extension products. In preferred embodiments, the primer is attached to the end of a nucleic acid such that a hairpin forms from self-hybridization. Preferably, the primer is an oligodeoxyribonucleotide. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the inducing agent. The exact lengths of the primers will depend on many factors, including temperature, source of primer and use of the method. It is also contemplated that primers can be used in PCR (see below) to artificially insert desired nucleotide sequences at the ends of nucleic acid sequences.

As used herein, the term "polymerase chain reaction" ("PCR") refers to the method of K. B. Mullis U.S. Pat. Nos. 4,683,195, 4,683,202, and 4,965,188, hereby incorporated by reference, that describe a method for increasing the concentration of a segment of a target sequence in a DNA mixture without cloning or purification. Because the desired amplified segments of the target sequence become the predominant sequences (in terms of concentration) in the mixture, they are said to be "PCR amplified." Similarly, the term "modified PCR" as used herein refers to amplification methods in which a RNA sequence is amplified from a DNA template in the presence of RNA polymerase or in which a DNA sequence is amplified from an RNA template the presence of reverse transcriptase.

As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes, each of which cut double-stranded DNA at or near a specific nucleotide sequence.

The terms "antigen," "antigenic," and "antigenically active," refer to any substance that can be recognized by a specific humoral and/or cell-mediated immune response. The terms "immunogen," "immunogenic" and "immunologically active" refer to any substance that is capable of inducing a specific humoral and/or cell-mediated immune response. An antigen or

immunogen generally contains at least one epitope. Antigens and immunogens are exemplified by, but not restricted to molecules, which contain a peptide, polysaccharide, nucleic acid sequence, and/or lipid. Complexes of peptides with lipids, polysaccharides, or with nucleic acid sequences are also contemplated, including (without limitation) glycopeptide, lipopeptide, glycolipid, etc. These complexes are particularly useful immunogens where smaller molecules with few epitopes do not stimulate a satisfactory immune response by themselves.

As used herein the term "nucleic acid sequence" refers to an oligonucleotide, a nucleotide or a polynucleotide, and fragments or portions thereof, and vice versus, and to DNA or RNA of genomic or synthetic origin, which may be single or double-stranded, and represent the sense or antisense strand. Similarly, "amino acid sequence" as used herein refers to peptide or protein sequence.

As used herein the term "antisense" when used in reference to DNA refers to a sequence that is complementary to a sense strand of a DNA duplex. A "sense strand" of a DNA duplex refers to a strand in a DNA duplex that is transcribed by a cell in its natural state into a "sense mRNA." Thus an "antisense" sequence is a sequence having the same sequence as the non-coding strand in a DNA duplex.

As used herein, the terms "complementary" or "complementarity" are used in reference to polynucleotides (i.e., a sequence of nucleotides) related by the base-pairing rules. For example, for the sequence "5'-A-G-T-3'," is complementary to the sequence "3'-T-C-A-5'."

Complementarity may be "partial," in which only some of the nucleic acids' bases are matched according to the base pairing rules. Or, there may be "complete" or "total" complementarity between the nucleic acids. The degree of complementarity between nucleic acid strands has significant effects on the efficiency and strength of hybridization between nucleic acid strands. This is of particular importance in amplification reactions, as well as detection methods that depend upon binding between nucleic acids.

The term "homology" refers to a degree of complementarity. There may be partial homology or complete homology (i.e., identity). A partially complementary sequence is a nucleic acid molecule that at least partially inhibits a completely complementary nucleic acid molecule from hybridizing to a target nucleic acid is "substantially homologous." The inhibition of hybridization of the completely complementary sequence to the target sequence may be examined using a hybridization assay (Southern or Northern blot, solution hybridization and the

like) under conditions of low stringency. A substantially homologous sequence or probe will compete for and inhibit the binding (i.e., the hybridization) of a completely homologous nucleic acid molecule to a target under conditions of low stringency. This is not to say that conditions of low stringency are such that non-specific binding is permitted; low stringency conditions require that the binding of two sequences to one another be a specific (i.e., selective) interaction. The absence of non-specific binding may be tested by the use of a second target that is substantially non-complementary (e.g., less than about 30% identity); in the absence of non-specific binding the probe will not hybridize to the second non-complementary target.

As used herein the term “vaccine” refers to an immunogenic composition that is administered to a host to provide some degree of protection from an infection and/or disease from a target virus or pathogen. Moreover, some degree of protection includes but is not limited to decreasing, reducing, modifying, and/or ameliorating one or more symptoms of an infection and/or disease. Generally, some symptoms of respiratory diseases include common cold symptoms and more particularly for example only and not meant to be limiting, breathing difficulty or labored breathing, cough, fever, croupy cough (often described as a “seal bark” cough), cyanosis (bluish skin color due to lack of oxygen), nasal flaring, stuffy nose, wheezing congested and/or runny nose. Moreover, respiratory diseases can affect their lungs, causing bronchiolitis or pneumonia. Such a composition might include a “pharmaceutically acceptable” diluent and/or carrier or any carrier, adjuvant or diluent. For example only, and not meant to be limiting acceptable diluents and/or carriers can be found in Remingtons “The Science and Practice of Pharmacy,” 21st Ed. 2005 (herein incorporated by reference in its entirety). The phrase “pharmaceutically acceptable” is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio. Numerous vaccine formulations are known to those skilled in the art.

Vaccines can be administered alone or in combination with various adjuvants/carriers. Pharmaceutical carriers are known to those skilled in the art. These most typically would be standard carriers for administration of vaccines to humans, including solutions such as sterile

water, saline, and buffered solutions at physiological pH. Other ingredients include excipients, carriers, thickeners, diluents, buffers, preservatives, and surface active.

As used herein the term(s) "administering" and "administer" are used interchangeably and include for example only and not meant to be limiting, administering by aerosol, droplet, parenteral injection (such as intraperitoneal, subcutaneous, or intramuscular), intranasal via droplet, inhalation et al. See Remingtons "The Science and Practice of Pharmacy," 21st Ed. 2005.

As used herein the term "Sendai virus" is a mouse parainfluenza virus that is the murine homologue of hPIV-1.

As used herein the term "reporter gene" includes a means of facilitating virus tracking. For example only and not meant to be limiting, the reporter gene as described herein includes luciferase, green fluorescent protein, red fluorescent protein, along with other means of visually tracking (e.g. with marked probes or antibodies) as known to those skilled in the art. Further, while specific examples are given any other means of fluorescent, bioluminescent, luminescent, and related reporter proteins useful for tracking are contemplated by the present invention.

As used herein the term "host cell" refers to any cell capable of replicating and/or transcribing and/or translating a heterologous gene. Thus, a "host cell" refers to any eukaryotic or prokaryotic cell (e.g., such as LLC-MK2 cells (See Figure 1), bacterial cells, E. coli, yeast cells, mammalian cells, avian cells, amphibian cells, plant cells, fish cells, and insect cells), whether located *in vitro* or *in vivo*.

As used herein the term "wild-type" when made in reference to a gene refers to a gene that has the characteristics of a gene isolated from a naturally occurring source. The term "wild-type" when made in reference to a gene product refers to a gene product that has the characteristics of a gene product isolated from a naturally occurring source. The term "naturally-occurring" as used herein as applied to an object refers to the fact that an object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory is naturally-occurring. A wild-type gene is that which is most frequently observed in a population and is thus arbitrarily designated the "normal" or "wild-type" form of the gene.

In contrast, the term "modified" or "mutant" when made in reference to a gene or to a gene product refers, respectively, to a gene or to a gene product which displays modifications in

sequence and/or functional properties (i.e., altered characteristics) when compared to the wild-type gene or gene product. It is noted that naturally-occurring mutants can be isolated; these are identified by the fact that they have altered characteristics when compared to the wild-type gene or gene product.

5 As used herein the term "a modified Enders" means a Enders Sendai virus strain genome comprising a portion of a Z Sendai virus strain genome. For example only and not meant to be limiting, a Enders Sendai virus strain genome might contain one or more Z Sendai virus strain genes (or portions thereof) such as a Z strain NP gene, a Z strain P gene, a Z strain M gene, a Z strain F gene, a Z strain HN gene, and a Z strain L gene. Additionally, while specific
10 combinations of Enders Sendai strain genome and Z Sendai strain genome have been provided they are not meant to be limiting and encompass use of other equivalent Sendai virus strain genomes.

 As used herein a "chimera" means a Enders Sendai virus strain genome containing one or more portions of a different Sendai virus strain genome in operable combination. More
15 particularly, for example only (and not meant to be limiting) in one embodiment, the present invention contemplates a Enders Sendai virus strain genome comprising one or more portions of a Z Sendai virus strain genome. Additionally, while specific combinations of Enders Sendai strain genome and Z Sendai strain genome have been provided they are not meant to be limiting and encompass use of other equivalent Sendai virus strain genomes.

20 As used herein the term "purified" refers to molecules, either nucleic acid or amino acid sequences that are removed from their natural environment, isolated or separated. An "isolated nucleic acid sequence" is therefore a purified nucleic acid sequence. "Substantially purified" molecules are at least 60% free, preferably at least 75% free, and more preferably at least 90% free from other components with which they are naturally associated.

25 As used herein the term "purified" or "to purify" also refers to the removal of contaminants from a sample. The removal of contaminating proteins results in an increase in the percent of polypeptide of interest in the sample. In another example, recombinant polypeptides are expressed in plant, bacterial, yeast, or mammalian host cells and the polypeptides are purified by the removal of host cell proteins; the percent of recombinant polypeptides is thereby increased
30 in the sample.

As used herein, the term "*in vitro*" refers to events that occur in an artificial environment, e.g., in a test tube or reaction vessel, in cell culture, etc., rather than within a multi-cellular organism.

As used herein, the term "*in vivo*" refers to events that occur within a multi-cellular organism. For example only and not meant to be limiting, such as a mammal more particularly a human and/or non-human animal.

As used herein the term "gene" refers to a nucleic acid (e.g., DNA) sequence that comprises coding sequences necessary for the production of a polypeptide, precursor, or RNA (e.g., rRNA, tRNA). The polypeptide can be encoded by a full length coding sequence or by any portion of the coding sequence so long as the desired activity or functional properties (e.g., enzymatic activity, ligand binding, signal transduction, immunogenicity, etc.) of the full-length or fragment are retained. The term also encompasses the coding region of a structural gene and the sequences located adjacent to the coding region on both the 5' and 3' ends on either end such that the gene corresponds to the length of the full-length mRNA. Sequences located 5' of the coding region and present on the mRNA are referred to as 5' non-translated sequences. Sequences located 3' or downstream of the coding region and present on the mRNA are referred to as 3' non-translated sequences. The term "gene" encompasses both cDNA and genomic forms of a gene. A genomic form or clone of a gene contains the coding region interrupted with non-coding sequences termed "introns" or "intervening regions" or "intervening sequences." Introns are segments of a gene that are transcribed into nuclear RNA (hnRNA); introns may contain regulatory elements such as enhancers. Introns are removed or "spliced out" from the nuclear or primary transcript; introns therefore are absent in the messenger RNA (mRNA) transcript. The mRNA functions during translation to specify the sequence or order of amino acids in a nascent polypeptide.

As used herein, the term "heterologous gene" refers to a gene that is not in its natural environment. For example, a heterologous gene includes a gene from one species introduced into another species. A heterologous gene also includes a gene native to an organism that has been altered in some way (e.g., mutated, added in multiple copies, linked to non-native regulatory sequences, etc). Heterologous genes are distinguished from endogenous genes in that the heterologous gene sequences are typically joined to DNA sequences that are not found naturally associated with the gene sequences in the chromosome or are associated with portions of the

chromosome not found in nature (e.g., genes expressed in loci where the gene is not normally expressed).

As used herein, the terms "in operable combination," "in operable order," and "operably linked" as used herein refer to the linkage of nucleic acid sequences in such a manner that a nucleic acid molecule capable of directing the transcription of a given gene and/or the synthesis of a desired protein molecule is produced. The terms also refer to the linkage of amino acid sequences in such a manner so that a functional protein is produced.

As used herein, the term "cell culture" refers to any *in vitro* culture of cells. Included within this term are continuous cell lines (e.g., with an immortal phenotype), primary cell cultures, transformed cell lines, finite cell lines (e.g., non-transformed cells), and any other cell population maintained *in vitro*.

As used herein, the term "Multiplicity of Infection (MOI)" refers to the ratio of infectious virus particles to target cells (i.e. the ratio of infectious virus particles deposited in a well, relative to the number of target cells in that well).

As used herein, the term "Plaque Forming Units (PFU)" refers to a measure of the number of virus particles capable of infecting cells and consequently forming plaques in a target cell monolayer.

As used herein the term "therapeutically effective amount" includes within its meaning a non-toxic but sufficient amount of an agent or compound to provide the desired therapeutic effect. The exact amount required will vary from subject to subject depending on factors such as the species being treated, the age and general condition of the subject, the severity of the condition being treated, the particular agent being administered and the mode of administration and so forth. Thus, it is not possible to specify an exact "effective amount". However, for any given case, an appropriate "effective amount" may be determined by one of ordinary skill in the art using only routine experimentation.

As used herein, the term "kit" refers to any delivery system for delivering materials. In the context of reaction assays, such delivery systems include systems that allow for the storage, transport, or delivery of reaction reagents (e.g., oligonucleotides, enzymes, etc. in the appropriate containers) and/or supporting materials (e.g., buffers, written instructions for performing the assay etc.) from one location to another. For example, kits include one or more enclosures (e.g., boxes) containing the relevant reaction reagents and/or supporting materials. As used herein, the

term "fragmented kit" refers to delivery systems comprising two or more separate containers that each contain a subportion of the total kit components. The containers may be delivered to the intended recipient together or separately. For example, a first container may contain an enzyme for use in an assay, while a second container contains oligonucleotides. The term "fragmented kit" is intended to encompass kits containing Analyte specific reagents (ASR's) regulated under section 520(e) of the Federal Food, Drug, and Cosmetic Act, but are not limited thereto. Indeed, any delivery system comprising two or more separate containers that each contains a subportion of the total kit components are included in the term "fragmented kit." In contrast, a "combined kit" refers to a delivery system containing all of the components of a reaction assay in a single container (e.g., in a single box housing each of the desired components). The term "kit" includes both fragmented and combined kits.

As used herein, the term SeVc refers to any construct that is based on the SeVc backbone described below. For example and not meant to be limiting, one embodiment is a SeVc-luc(M-F*) that denotes a modified SeVc construct containing a luciferase reporter gene cloned into the M-F gene junction. See Table 1.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to recombinant virus vectors, methods for constructing vectors, and use of such vectors. In one embodiment, the present invention provides methods, recombinant virus constructions and compositions, and kits for a modified Enders strain Sendai viral vector for protection against pathogens. Moreover, some embodiments include vectors for imaging or tracing viral spread, clearance, and transmission. In one embodiment, the present invention contemplates a novel recombinant Sendai virus vaccine vector with the following attributes: (i) capacity for facile rescue of recombinant vectors by reverse genetics, (ii) capacity to carry a marker gene for virus tracking *in vivo* and *in vitro*, (iii) support of expression and immunogenicity of a foreign protein(s) when respective gene(s) are introduced into different positions within the SeV genome, (iv) limited growth in primates, (v) sufficient replication-competence to support immunogenicity in primates. Further, some embodiments also provide an attribute of (vi) limited growth at 33 °C and even less growth at 37 °C. The present invention exhibits an unexpected balance of virus vector attenuation, virus vector growth, foreign gene expression, and immunogenicity to support each of these desired attributes.

In one embodiment, the present invention provides a vector that is unexpectedly superior to other SeV vectors including unmodified Enders or Z strains in that it can be easily rescued and exhibits both attenuation and immunogenicity in primates. Methods, recombinant virus constructions and formulations, and kits will facilitate the use of this Sendai virus vector as a laboratory tool or in a pre-clinical/clinical research setting.

More particularly the present invention relates to a modified Sendai virus, which can be used as a vaccine. In one embodiment of a method to create recombinant viruses, a plasmid can be used, which contains the entire Sendai virus genome, flanked by a T7 promoter and a hepatitis delta virus ribozyme sequence. See Figure 8 for examples of four intergenic positions in which foreign genes can be placed prior to virus rescue (Brown et. al. J. Virology, 2007, 81: 12535).

Below are sequences of a RSV F gene, protein sequences for the different Sendai virus genes as presented in a modified Sendai virus construct (pSeVc), and a sequence for the pSeVc recombinant carrying the RSV F gene insert in the F-HN position in the Sendai virus genome (pSeVc-RSVF(F-HN)). Associated translations are also shown. All sequences are listed 5' to 3' (left to right). (Also see Figure 7 for sequences).

RSV F gene sequence (insert from genomic construct) (SEQ. ID NO.: 1)

atggagttgctaactcctcaagcaaatgcaattaccacaatcctcactgcagtcacatttggcttctggcctcaaaacatcactgaagaatttt
atcaatcaacatgcagtcagtttagcaaggctatcttagtgctctgagaactgggtggatataccagtggtataactatagaattaagtaatatca
20 agaaaaataagtgtaatggaacagatgccaaggcaaaattgataaaacaagaattagataaatataaaaatgctgtaacagaattgcagttgc
tcatgcaagcacacaagcaacaacaatcgagccagaagagaactaccaaggtttatgaattatacactcaacaatgccaaaaaacaa
tgaacattaagcaagaaaaggaaaagaagatttcttggttttgttaggtgttggatctgcaatcgccagtgccgttctgtatctaaggctct
gcacctagaaggggaagtgaacaagatcaaaagtgtctactatccacaacaaggctgtagtcagcttatcaaatggagttagtgtcttaac
cagcaaaagtgttagacctcaaaaactatagataaacaattgttacctattgtgaacaagcaagctgcagcatatcaaatatagaactgtg
25 atagagttccaacaaaagaacaacagactactagagattaccaggggaatttagtgtaatgcaggtgtaactacacctgtaagcacttacatgt
taactaatagtgattattgtcattaatcaatgatatgcctatacaaatgatcagaaaaagttaatgtccaacaatgttcaaatagttagacagca
aagttactctatcatgtccataataaaagaggaagtcttagcatatgtagtacaattaccactatattggtgttatggatacacacctgttggaact
acacacatccccctctatgtacaaccaacacaaaagaagggtccaacatctgtttaacaagaactgacagaggatggtagtgcacatgcag
gatcagtatcttctcccaagctgaacatgtaaagtcaatcaaatcgagtatttggacacaatgaacagtttaacattaccaagtgaag
30 taaatctctgcaatgttgacatattcaaccccaaatatgattgtaaaattatgacctcaaaaacagatgtaagcagctccgttatcacatctctag
gagccattgtgtcatgtatggcaaaaactaaatgtacagcatccaataaaaaatcggtgaatcataaagacattttctaacgggtgcgattatgta
tcaataaaaggggtggacactgtgtctgttaggtaacacattatattgtaaataagcaagaaggtaaaagtctctatgtaaaagggtgaacaa
taataaattctatgaccattagttatccccctctgatgaattgatgcataatctcaagtcaacgagaagattaaccagagcctagcatttatt
cgtaaatccgatgaattattacataatgaattgtgtgtaaatccaccacaaatATCATGATACTACTATAATTatagtgat
35 tatagtaattgttatcattaattgctgttgactgctcttactgtgaaggccagaagcacaccagtcacactaagcaaatgaactgagtg
gtataaataattgtcatttagtaactaa

RSV F Translation sequence (SEQ. ID NO.: 2)

5 MELLILKANAIITILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIELSNIKKKNCNGTDAKAKLIKQELD
 KYKNAVTELQLLMQSTQATNNRARRRELPRFMNYTLNNAKKTNVTLSKKRRRFLGFLGVGSAIASGVAVSKVLHLEGE
 VNKIKSALLSTNKAVVSLNGVSVLTSKVLDLKNYIDKQLLPIVNKQSCSISNIETVIEFQQKNNRLEITREFSVNAGVTP
 VSTYMLTNSELLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEVLAYVVQLPLYGVMDTPCWKLHTSP
 10 LCTTNTKEGSNICLTRDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDTMNSLTPSEVNLCNVDIFNPKYDCKIMTSK
 TDVSSSVITSLGAIVSCYGKTKCTASNKNRGIKTFSGNCDYVSNKGVDTVSGNTLYYVKNQEGKSLYVKGEPIINFYDP
 LVFSPDEFDASISQVNEKINQSLAFIRKSDHELLHNVIAGKSTTNIMITTHIIVILLSLIAVGLLLYCKARSTPVTLS
 KDQLSGINNIAFSN.

15 **NP protein sequence: (SEQ. ID NO.:5)**

MAGLLSTFDTFSSRRSEINKSGGGAVIPGQRSTVSFVLGPSVTDDADKLFIAITFLAHS�DTDKQHSQRGGFLVSLLAM
 AYSSPELYLTNGVNADVKYVIYNIEKDPKRTKTGFIKTRDMEYERTTEWLFGPMVNKSPLFQQQRDAADPDLLQTYG
 20 YPACLGAIIVQVWIVLVKAITSSAGLRKGFFNRLEAFRQDGTVKGALVFTGETVEGIGSVMRSQQSLVSLMVETLVTMNTA
 RSDLTITLEKNIQIVGNIRYDAGLASFMNTIKYGVETKMAALTLSNLRPDINKIRSLIDTYLSKGPAPFICILKDPVHGEF
 APGNYPALWSYAMGVAVVQNKAMQQYVTGRTYLDMEMFLLGQAVAKDAESKISSALEDELGVTDAKERLRHHLANLSGGD
 GAYHKPTGGGAIEVALDNADIDLETEAHADQDARGWGGESGERWARQVSGGHFVTLHGAERLEEETNDEDVSDIERRIAMR
 LAERRQEDSATHGDEGRNNGVDHDEDDTAAGIGGI

25

P protein sequence: (SEQ. ID NO.:7)

MDQDAFILKEDSEVEREAPGGRESLSDVIGFLDAVLSSEPTDIGGDRSWLHNTINTPQGPSSAHRAKSEGEVSTPSTQD
 30 NRSGEESRVSGRTSKPEAEAHAGNLDKQNIHRAFGGRTGTNSVSQDLGDGGDSGILENPPNERGYPRSGIEDENREMAAHP
 DKRGEDQAEGLPEEVRGGTSLPDEGEGGASNNGRSMEPGSSHSARVTGVLVIPSPLEEAVLRRNKRPTNSGSKPLTPAT
 VPGTRSPPLNRYNSTGSPGKPPSTQDEHINSGDTPAVRVKDRKPPIGTRSVSDCPANGRPPIHPGLETDSTKKGIGENTSS
 MKEMATLLTSLGVIQSAQEFESSRDASYVFARRALKSANYAEMTFNVCGLILSAEKSSARKVDENKQLLKQIQESVESFRD
 IYKRFSEYQKEQNSLLMSNLSTLHIITDRGGKTDNTDSLTRSPSVFAKSKENKTKATRFDPMETLEDKMKYKPDIREDEF
 35 RDEIRNPVYQERDTEPRASNASRLPSKEKPTMHSRLRVISSPLSRAEKAAYVKSLSKCKTDQEVKAVMELVEEDIESLT
 N

C protein sequence (SEQ. ID NO.:9)

40 MPSFLKKILKLRGRRQEEESRSM LSDSSMLSCRVNQLTSEGTEAGSTTPSTLPKDQALPIEPKVRAKEKSQHRRPKIIDQ
 VRRVESLGEQASQRQKHMLETLINKIYTGPLGEEVLVQTLYLRIWAMEETPESLKILQMREDIRDQVLKMKTERWLRTLIRG
 EKTKLKDFQKRYEEVHPYLMKEKVEQVIMEEAWSLAAHIVQE

45 **M protein sequence: (SEQ. ID NO.:11)**

MADIYRFPKFSYEDNGTVEPLPLRTGPDKKAIPYIRIIKVGDPKPHGVRYLDLLLLGFFETPKQTTLNLSVSDLTEPTSYS
 ICGSGSLPIGVAKYYGTDQELLKACTDLRITVRRTRVRAGEMIVYMVDSIGAPLLPWSGRLRQGMIFNANKVALAPQCLPVD
 KDIRFRVVFVNGTSLGAITIAKIPKTLADLALPNSISVNLLVTLKTGISTEQKGVLPVDDQGEKKLNFMVHLGLIRRKVG

KIYSVEYCKSKIERMRLIFSLGLIGGISFHVQVTGTLSTKTFMSQLAWKRAVCFPLMDVNPMMNLVIWAASVEITGVDAVFQ
PAIPRDFRYYPNVVAKNIGIRKRL

5 F protein sequence (SEQ. ID NO.:13)

MTAYIQRSCISTSLLVLTTLVSCQIPDRLSNIGVIVDEGKSLKIAGSHESRYIVLSLVPGVDLENGCGTAQVIQYKSL
LNRLLIPLRDALDLQEALITVTNDTTQAGVPQSRFFGAVIGTIALGVATSAQITAGIALAEAREAKRDIALIKESMTKTH
KSIELLQNAVGEQILALKTLQDFVNDEIKPAISELGCETAALRLGIKLTQHYSGLLTAFGSNFGTIGEKSLTLQALSSLYS
10 ANITEIMTTIRTGQSNIDYVYTEQIKGTVIDVDLERYMVTLSVKIPILSEVPGVLIHKASSISYNIDGEEWYVTPSHIL
SRASFLGGADITDCVESRLTYICPRDPAQLIPDSQQKCILGDTTRCPVTKVVDLIPKFAFVNGGVVANCIASTCTCGTGR
RPISQDRSKGVVFLTHDNCGLIGVNGVELYANRRGHDATWGVQNLTVGPAIAIRPVDISLNLADATNFLQDSKAELEKARK
ILSEVGRWYNSRETIVITIIIVMVILVVIIVIVIVLYRLRRSMLMGNPDDRIIPRDTYTLPEKIRHMYTNGGFDAMAEKR

15

HN protein sequence (SEQ. ID NO.:15)

GKRDSYWSTSPSGSTTKLASGWERSSKVDTWLLILSFTQWALSIAITVIICIIISARQGYSMKEYSMTVEALNMSSREVKE
LTSLIRQEVIAVAVNIQSSVQTGIPVLLNKNSRDVIQIMDKSCSRQELTQLCESTIAVHHAEGIAPLEPHSFWRCPVGE
20 LSSDPKISLLPGPSLLSGSTTISGCVRLPSLSIGEAIYAYSSNLITQGCADIGKSYQVLQLGYSISLNSDMFDPDLPVVSHT
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EGRLKLGDRVYIYTRSSGWHSQLQIGVLDVSHPLTINWTPHEALSRPGNEECNWNTPKCEISGVYTDAYPLSPDAANV
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25 AES

L protein sequence (SEQ. ID NO.:17)

SSQNPSDILYPECHLNSPIVRGKIAQLHVLLDVNQPYRLKDDSIINITKHKIRNGGLSPRQI
30 KIRSLGKALQRTIKDLDRYTFEPYPTYSQELLRLDIPEICDKIRSVFAVSDRLTRELSSGFQ
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35 AVTAADKVRHMYAQKAIKLTLYECHAVFCTIINGYRERHGGQWPPCDFPDHVCLE
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40 YCLNWRFEALFGQRCNEIFGKFTFFNWMHPVLERCTIYVGDYCPVADRMHRQLQD
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GMTINPTISPTVRDQYFKGKNWLRCVLPANVGGFNYSMTSRFCFVRNIGDPAVAALAD
45 LKRFIRADLLDKQVLYRVMNQEPGDSSFLDWASDPYSCNLPHSQSITTIKNTARSVLQE
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 IAQTRANLSLENLKLTPVSTSTNLSHRLKDTATQMKFSSATLVRASRFITISNDNMALK
 EAGESKDTNLVYQQIMLTGLSLFEFNMRYKKGSLGKPLILHLHLNNGCCIMESPQEANIP
 PRSTLDLEITQENNKLIYDPDPLKDVDLELFSKVRDVVHTVDMTYWSDDEVIRATSICTA
 5 MTIADTMSQLDRDNLKEMIALVNDDDVNSLITEFMVIDVPLFCSTFGGILVNQFAYS
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 IYPAEVALVGKKLNNVTSLGQRVKVLFNGNPGSTWIGNDECEALIWNELQNSSIGL
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20

pSeVc-RSVF(F-HN) (SEQ. ID NO.:18)

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In particular, the present invention provides methods, compositions, recombinant virus
 constructions and formulations, and kits for a modified Enders strain Sendai viral vector. Viruses
 can be used to protect humans or other animals from infection or for use *in vitro*. Moreover,
 some embodiments include vectors for imaging *in vitro* or *in vivo* viral spread, clearance, and
 transmission. Furthermore, the disclosed embodiments contemplate an Enders Sendai virus
 vector with insertion of a foreign gene. More particularly, for example only and not meant to be
 limiting, the foreign gene might be an RSV F gene and/or G gene, an hPIV gene from Type 1-4,
 for use as a vaccine either alone or in combination with other vectors. Insertion of a foreign gene
 into any of the intergenic junctions is acceptable; however, it is believed that it might be
 preferable to insert the foreign gene between the Sendai virus F and HN genes because it
 provides sufficient virulence for infection of a primate host while providing sufficient RSV F
 gene expression to confer protective immunity. Further, studies show that a small amount of
 inoculum and small dose of Sendai virus might enhance the ratio of upper respiratory tract as
 compared to lower respiratory tract infection such that there is an increased margin of safety.

While it is not necessary to understand the mechanism of action, it is believed that use of
 the modified Enders strain (a E/Z chimera) instead of the Z strain, of the Sendai virus, provides
 greater viral attenuation in primates, yet preserves sufficient replication-competence to support
 facile virus rescue and the induction of an immune response. Furthermore, embodiments of the
 present invention contemplate some advantages, for example only and not meant to be limiting,
 including the following: (i) an unmodified Sendai virus vaccine is well tolerated in humans based
 on Phase I clinical trials, (ii) humans are not a natural host of Sendai virus and no confirmed
 cases of Sendai viral infection in humans have been reported, (iii) animal studies have shown
 production of antibodies and stimulation of cellular immunity upon intranasal inoculation of
 Sendai virus vaccine along with long term immunity (protection is observed when animals are
 challenged with pathogen months after inoculation (Jones et al. 2009, Vaccine 27:1848). The
 modified Sendai viral vector comprises a partial replacement of the Enders L region with the Z

strain L portion effectively resulting in eight amino acid changes to the encoded L protein of the modified Enders strain. The modified amino acids are as follows: S155G, R258K, G466E, G482E, S581R, Q717R, T800I, and R852K. While it is not necessary to understand the mechanism of action, it is believed these modifications have been demonstrated to enable efficient generation of an infectious clone of an Enders-based Sendai virus from cDNA relative to the unmodified Enders strain genome, and enable an attenuated virus to elicit an immune response in primates.

I. Development of Modified Enders Strain

While it is not necessary to understand the mechanism of action, it is believed that the recombinant Sendai viral vector comprised of a foreign gene and a modified L gene provides an efficient and safe vector for use as a vaccine. Various pSeV gene chimeras were constructed containing Z strain and Enders strain genes in order to be able to identify the region that affects virus rescue and growth. The results of virus rescue and growth indicate that a chimeric Enders/Z viral vector is desired. For example only and not meant to be limiting, it is believed an AscI/NheI fragment containing the N-terminal half of the L gene is important for efficient growth. Viruses containing this part from the Z strain grew better than other test constructs. The construction and virus growth data are summarized in Figure 15 and Table 1 below.

Summary of Rescue Results for Various Enders Strain/Z Strain Sendai Viruses:

Table 1. Comparison of Virus Growth by examination of HA titer from allantoic fluid from injected eggs

Virus	Portion from Z strain	Portion from pSeVE(3)	Origin of genes						Maximum HA titer
			NP	P	M	F	HN	L	
SeV(E)3			E	E	E	E	E	E	370
SeVa	KpnI/NotI (15,281-6,670)	KpnI/NotI (6670-15281)	Z	Z	Z	Z	E	E	730
SeVb	NotI/AscI (6,670-8,441)	NotI/AscI (8,441-6,670)	E	E	E	E	Z	E	240
SeVc	AscI/NheI (8,441-	AscI/NheI (11,960-	E	E	E	E	E	*Z/E(1-1,135	6,600

	11,960)	8,441)						Z; 1,136- 2,228E)	
SeVd	Sall/NotI (6,670- 2,074)	Sall/NotI (2,074- 6,670)	Z	E	E	E	Z	Z	2,200

Table 1. Rescue of various Enders/Z chimeric SeVs.

Method: 293T cells in 6 well plates were infected with recombinant vaccinia virus vTF7.3 (10 ul/well) for 1 hour and transfected with full genome Sendai virus cDNAs together with Sendai virus NP, P and L genes in pTF1 vector. The cells were cultured for 2 days, and then, collected cells were injected into embryonated eggs. After three-days culture at 35 C, virus titers in allantoic fluids were measured. Z: Strain Z, E: Strain Enders.

Four chimeras were made to create a full Sendai virus genome, combining genes from Enders and Z origin. Rescued viruses were then tested for growth and measured by maximum HA titer. *In the case of SeVc, the L gene was derived partially from Enders and partially from Z, effectively resulting in a modified Enders strain with eight amino acid changes to the Enders L protein. This modification unexpectedly yielded a virus with capacity for facile rescue by reverse genetics, attenuation in primates, and sufficient replication-competence to support immunogenicity in primates. Sendai viruses SeVb, SeVc and SeVd were also diluted at various concentrations and injected into eggs to see their growth. The data also show that SeVc and SeVd grew much better than SeVb. Thus, while it is not necessary to understand the mechanism of action, based on this data, pSeVc was used for further construction of recombinant viruses. The following describes the behavior of the modified Enders-based vaccine carrying the RSV F gene.

Modified Enders-based vaccine is attenuated in primates

Figure 16 shows the difference between growth of the Sendai virus Z, the Sendai virus Enders and a modified Sendai virus Enders-based vaccine SeVc-RSVF(F-HN) in African green monkeys. The Sendai virus Enders vaccine and the Sendai virus Enders-based SeVc-RSVF(F-HN) vaccine grew to a lesser titer in both the upper and lower respiratory tract (URT and LRT) of African green monkeys compared to the SeV Z strain (MPIV1 below).

Referring to Figure 16, 16A and B show the mean daily virus titers of a Z strain Sendai virus (MPIV1) and the human parainfluenza virus type 1 (HPIV1) in a nasopharyngeal swab (URT) or tracheal lavage (LRT) after intranasal and intratracheal vaccination (Skiadopoulos et. al. 2002 Virology 297:153) respectively. As demonstrated in this panel, the peak MPIV1 titer after a 10^6 dose (administered IT and IN) exceeded 10^4 in the URT and exceeded 10^6 in the LRT.

Referring to Figure 16, 16C and D show daily titers following vaccination with the Sendai virus Enders vaccine (SeV) or the recombinant modified Sendai virus Enders-based vaccine expressing RSV F (SeVc-RSVF(F-HN)). When the vaccine doses were 10^6 (administered IT and IN) peak viral titers were lower than with Z. They were approximately 10^3 in the URT (16C, top, 1 log reduced compared to Z) and approximately 10^4 in the LRT (16D, bottom, 2 logs reduced compared to Z). The Enders based Sendai virus vector was clearly attenuated compared to Sendai virus Z, yet it maintained immunogenicity.

The Sendai virus Enders-based vaccines were also considerably lower in titers compared to a b/hPIV-3-based RSV F vaccine after administration to African green monkeys. The b/hPIV-3-based RSV F vaccine is already in clinical trials in infants. The b/hPIV-3-based RSV F vaccine grew to peak titers of $>10^5$ and 10^7 in the URT and LRT respectively in African green monkeys, even when the vaccine was administered at a dose of only 2×10^5 (Tang et. al. 2004 J. Virol. 78:11198).

Enders based Sendai virus vaccine elicits an immune response in African green monkeys.

Figure 17 shows that the modified Sendai virus Enders-based vaccine carrying the RSV F gene in the F-HN position (SeVc-RSVF(F-HN)) elicits an immune response against RSV F (A) and against the Sendai virus components (B) in African green monkeys. The antibody response against RSV F was demonstrated by testing animal sera in an enzyme-linked immunosorbant assay (ELISA) approximately 3 weeks after vaccination. Control animals were African green monkeys that received PBS by the intranasal and intratracheal routes approximately 3 weeks previously. The second group of animals received an unmodified Sendai virus (SeV) by the same routes and the third group of animals received the modified SeV Enders-based vaccine carrying the RSV F gene in the F-HN position (SeVc-RSVF(F-HN)).

The recombinant modified Sendai virus Enders-based vaccine expressing RSV F (SeVc-RSVF(F-HN)) protects African green monkeys from RSV infection

Figure 18 shows that the modified Sendai virus Enders-based vaccine carrying the RSV F gene in the F-HN position completely protected African green monkeys from RSV infection of the lower respiratory tract. Results show an analysis of BAL samples from three to ten days after RSV challenge. The test animals that received the modified Sendai virus Enders-based vaccine carrying the RSV F gene (SeVc-RSVF(F-HN)) exhibited no challenge virus in the BAL (green, right panel B) as compared to control animals on the left (blue and red, A).

Low dose modified Sendai virus Enders-based vaccine expressing RSV F is protective

Figure 19 shows that when the RSV F gene is placed in the F-HN position of the Enders-based SeV (SeVc-RSVF(F-HN)), it also confers complete protection against RSV in cotton rats, even when administered at a dose as low as 10^2 . Vaccine activity at such a low dose is attractive, both in terms of vaccine efficacy and in terms of vaccine manufacturing. Results show titers of the RSV challenge virus in the lungs three days after challenge in control and vaccinated cotton rats.

Thus, the SeV Enders-based RSV vaccine with RSV in the F-HN position (possibly also in P-M and M-F or other positions) has sufficient growth to protect both African green monkeys and cotton rats from RSV challenge. Vaccine doses can be as low as 10^2 . The virus is attenuated in African green monkeys compared to the SeV Z strain and compared to the b/hPIV3-RSV F vaccine that is currently in clinical trials in infants. It is believed that the SeV Enders-based vaccine appears to be an extremely attractive candidate as a non-recombinant vaccine for hPIV-1 and as a recombinant vaccine for any other pathogen.

II. PIV Model System Utilizing Luciferase

Experimental studies on hPIV infection in tissue culture and animal models have helped reveal basic replication mechanisms and evaluate preclinical vaccine candidates [Murphy et al. 2002, J Clin Invest 110: 21-27; Moscona, A. 2005, J Clin Invest 115: 1688-1698; and Schaap-Nutt et al. 2010, Vaccine 28: 2788-2798]. However, being able to visualize the spread of PIV

infection in individual, living animals that are fully susceptible to PIV-associated disease would enable more thorough investigations of PIV pathogenesis, virus-host interactions, and virus transmission. Placement of a marker gene in the Sendai virus backbone can assist the study of virus growth, virus localization and virus transmission both *in vivo* and *in vitro*.

5 As demonstration of the marking system, the luciferase gene was placed in various positions within the modified Sendai virus vector. Figure 9 demonstrates the strategy. Referring to Figure 9, in panel A is shown sequences within a pGEM3 cloning plasmid engineered to contain flanking *NotI* restriction sites, the firefly luciferase reporter gene, gene end and gene start sequences. To insert the luciferase reporter gene cassette into three gene junctions (panel B),
 10 three pSeV genome plasmids were cloned to contain a unique *NotI* restriction site in each of the P-M, M-F, and F-HN gene junctions. For the pSeV-luc(M-F*) genome plasmid, the naturally occurring suboptimal start signal AGGGATAAAG (SEQ. ID NO.: 19) was also mutated to the more efficient start signal AGGGTGAAAG (SEQ. ID NO.: 20) to compensate for expected attenuation due to the addition of the foreign gene and additional gene junction. The firefly
 15 luciferase gene cassette (panel a) was subcloned from the pGEM3 plasmid into the pSeV genome plasmids using the *NotI* restriction sites. Panel C shows the design of pSeV cDNA plasmids for the rescue of WT and recombinant SeVs containing the luciferase reporter gene (luc). The locations of the Sendai virus genes nucleoprotein (N), polymerase (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and large (L) protein are shown, as well as the
 20 T7 RNA polymerase promoter (T7) and hepatitis delta virus ribozyme sequence (ribo). Gene start sequences are shown in green and the naturally occurring, suboptimal AGGGATAAAG (SEQ. ID NO.: 19) gene start sequence between the M and F genes of WT Sendai virus is shown in yellow. Gene end sequences are shown in red. The 3' leader sequence upstream of the N gene and the 5' trailer sequence downstream of the L gene are not shown for simplicity.

25 The rescued viruses expressing the luciferase gene can be administered to mice and tracked over an extended time course. The upper and lower respiratory tract can be monitored in this way. The non-invasive bioluminescence imaging of Sendai virus infection in the respiratory tracts of living mice is shown in Figure 2. Eight-week-old mice were intranasally inoculated with 7,000 PFU of SeVc-luc(P-M), SeVc-luc(F-HN), or SeVc-luc(M-F*). Every 24
 30 hours the mice were intraperitoneally injected with luciferin substrate, anesthetized with isoflurane, imaged with a Xenogen Lumina device, and then allowed to recover. Referring to

Figure 2, in one experiment, bioluminescence is shown on day 2 (panel a) or day 7 (panel b) post-infection (p.i.) for 129/SvJ mice infected with SeVc-luc(P-M), SeVc-luc(F-HN), or SeVc-luc(M-F*). In a second experiment, bioluminescence is shown on day 2 (panel c) or day 7 (panel d) for either 129/SvJ, DBA/2, BALB/c, or C57BL/6 mice infected with SeVc-luc(M-F*). The data are displayed as radiance, a measurement of bioluminescence intensity, on a rainbow log scale. Radiance values range from 1×10^6 (blue) to 1×10^9 (red) photons/s/cm²/steradian. Red circles show the regions of interest (ROI) for calculating the total flux (photons/s) in the nasopharynx, and red rectangles show the ROI areas for the trachea and lungs. While it is not necessary to understand the mechanism of action, it is believed the methodology can also support (i) studies of virus growth *in vitro*, (ii) studies of virus in multiple mouse strains (iii) studies of virus transmission between animals, (iv) studies of virus dosing, (v) studies of vaccine volumes and (vi) studies of adjuvants (examples are provided below). The text below describes the luciferase system in greater detail, emphasizing its numerous applications.

A. INTRODUCTION

Mice are poorly permissive to infection by the hPIVs, and hPIV infection in cotton rats, hamsters, guinea pigs, and ferrets is usually asymptomatic with minimal or undetectable pathology in the lungs [Karron et al. 2007, Parainfluenza Viruses. 5th Ed. pp. 1497-1526]. As a result, a number of studies have used Sendai virus (SeV) infection in mice as a model to investigate PIV pathogenesis in an experimental setting [Nagai, Y. 1999, Rev Med Virol 9: 83-99 and Faisca et al. 2007, Res Vet Sci 82: 115-125]. Sendai virus is the murine counterpart of hPIV1, the leading cause of laryngotracheobronchitis (pediatric croup) [Denny et al. 1983, Pediatrics 71: 871-876]. Sendai virus and hPIV1 have 78% amino-acid sequence identity [Takimoto et al. 2005, Viral Immunol 18: 255-266], elicit cross-protective immunity [Dave et al. 1994, Virology 199: 376-383; Hurwitz et al. 1997, Vaccine 15: 533-540; and Sangster et al. 1995, Virology 207: 287-291] and share tissue-tropic and epidemiological similarities [Karron et al. 2007, Parainfluenza Viruses. 5th Ed. pp. 1497-1526 and Faisca et al. 2007, Res Vet Sci 82: 115-125]. Moreover, while it is not necessary to understand the mechanism of action, it is believed that Sendai virus shows promise as a Jennerian vaccine for hPIV1 [Slobod et al. 2004, Vaccine 22: 3182-3186] and a vaccine vector for hRSV, hPIV3, and hPIV2 [Jones et al. 2009,

Vaccine 27: 1848-1857; Zhan et al. 2007, Vaccine 25: 8782-8793; and Zhan et al. 2008, Vaccine 26: 3480-3488].

Despite Sendai virus and the hPIVs being first isolated in the 1950s and having been studied for over 50 years [Karron et al. 2007, Parainfluenza Viruses. 5th Ed. pp. 1497-1526],
5 fundamental aspects of PIV infection and immunity remain unknown yet would directly bear upon our understanding of PIV pathogenesis and transmission as well the development of control measures. For example, the spatial and temporal spread of natural infection in the respiratory tract after Sendai virus transmission remains unknown because classical experiments measuring virus titers from sacrificed mice were limited by large inter-animal variability and error, resulting
10 in ambiguous results [Iida, T. 1972, J Gen Virol 14: 69-75 and van der Veen et al. 1970, Arch Gesamte Virusforsch 31: 237-246]. It is also unknown how hPIV and Sendai virus transmission often results in immunity without causing severe pathology in their natural host. The contribution of LRT infection to transmission is unknown. Finally, while infection in the lungs and the concomitant host response are clearly associated with disease severity [Karron et al. 2007,
15 Parainfluenza Viruses. 5th Ed. pp. 1497-1526; Faisca et al. 2007, Res Vet Sci 82: 115-125; Hall, CB 2001, N Engl J Med 344: 1917-1928; and Henrickson, KJ 2003, Clin Microbiol Rev 16: 242-264], many questions remain about the contribution of infection in the URT and trachea to clinical outcome and protective immunity [Sealy et al. 2010, Vaccine 28: 6749-6756 and Rudraraju et al. 2011, Virology 410: 429-436]. While it is not necessary to understand the
20 mechanism of action, it is believed that there are no published studies investigating how the dose of virus inoculum, replicative fitness of the virus, or genetic susceptibility of the host influences the growth and clearance of Sendai virus in the URT and trachea.

Thus, the present invention contemplates embodiments to measure the *in vivo* dynamics of PIV infection and immunity in living animals. Therefore, three luciferase-expressing SeVs
25 were generated for non-invasive bioluminescence imaging in mice. Analogous systems have been previously reported for DNA and positive-strand RNA viruses [Luker et al. 2008, Antiviral Res 78: 179-187] but have been elusive for negative-strand RNA viruses until now, largely due to virus attenuation [Hasan et al. 1997, J Gen Virol 78 (Pt 11): 2813-2820] or genetic instability resulting from reporter gene insertion [Manicassamy et al. 2010, Proc Natl Acad Sci U S A 107:
30 11531-11536]. Sendai virus is believed to be an ideal candidate for non-invasive imaging because (i) foreign-gene expression by paramyxovirus vectors is usually stable genetically

[Bukreyev et al. 2006, J Virol 80: 10293-10306], (ii) *in vivo* imaging of a non-replicating Sendai virus in intact mice has been successfully demonstrated [Griesenbach et al. 2008, Biomaterials 29: 1533-1540] and (iii) the match of Sendai virus and the murine host would enable pathogenesis studies [Faisca et al. 2007, Res Vet Sci 82: 115-125]. For the pathogenesis and transmission studies described here, the reporter virus SeVc-luc(M-F*) was engineered, which expresses high levels of luciferase yet replicates and causes disease in mice similar to wild-type (WT) virus. The *in vivo* dynamics of Sendai virus infection was imaged in living, intact mice after direct inoculation and after contact transmission as a function of virus dose and mouse strain. Unexpectedly, a dichotomous tissue tropism was discovered in which the URT and trachea supported robust virus growth, efficient transmission, and protective immunity even under conditions resulting in little infection in the lungs. Overall, the bioluminescence imaging system and tissue-tropic differences in PIV infection reported here provide a model for understanding *in vivo* infection and transmission by respiratory paramyxoviruses and a means for targeting antiviral therapies and directing live vaccines on a tissue-specific basis.

B. MATERIALS AND METHODS

I. Cell culture. Monolayer cultures of LLC-MK2 cells were grown in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% penicillin, and 1% streptomycin at 37°C + 5% CO₂.

II. Recombinant Sendai viruses. Unique *NotI* recognition sites were cloned into the P-M, M-F and F-HN intergenic junctions of an Enders-based pSeV viral genome plasmid, using cloning sites described previously [Tokusumi et al. 2002, Virus Res 86: 33-38]. The firefly luciferase gene was amplified by PCR using the pGL3 Basic vector (Promega) and a pair of *AscI* tagged primers, subcloned into a shuttle plasmid containing a Sendai virus intergenic junction and flanking *NotI* restriction sites [Tokusumi et al. 2002, Virus Res 86: 33-38] and then subcloned into the unique *NotI* site of each of the pSeV viral genome plasmids. Within the pSeV-luc(M-F) plasmid, the start signal upstream of the F protein was changed from AGGGATAAAG (SEQ. ID. NO.: 19) to AGGGTGAAAG (SEQ. ID. NO.: 20) using QuikChange™ Site-Directed Mutagenesis Kit (Stratagene Corp). The recombinant SeVs were rescued from the pSeV genome plasmids as described previously [Zhan et al. 2008, Vaccine 26: 3480-3488]. The modified

Enders strain Sendai genome consists of a modified Sendai virus L gene that contains the following amino acid changes: S to G at position 155, R to K at position 258, G to E at position 466, G to E at position 482, S to R at position 581, Q to R at position 717, T to I at position 800, and R to K at position 852.

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III. Luciferase Expression *in vitro*. SeV-infected LLC-MK2 cells (MOI 5 PFU/cell) were incubated at 33°C + 5% CO₂ and lysates collected at various times p.i.. Luciferase assays were performed using the Luciferase Assay System (Promega) and the levels of expression measured using an automated luminometer (Turner Biosystems, Inc.) as described previously [Luque et al. 2007, J Virol 81: 3130-3141].

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IV. Viral Titers and Bioluminescence Imaging. Virus titers from multistep growth curves (MOI of 0.01 PFU/cell) and homogenized tissues were determined by plaque titration in LLC-MK2 cells as described previously [Luque et al. 2010, J Virol 84: 810-821]. Eight week-old female 129x1/SvJ mice or BALB/c mice (Jackson Laboratories) were anesthetized using isoflurane (Baxter Health Care Corporation) and inoculated intranasally (i.n.) with 30 µl of PBS or virus. For Figures 1D, 1E, 2, 3, 4E, 4F, and 12, at 3 d before inoculation with PBS or virus, mice were anesthetized by IP injection of 300 µl avertin (300 mg/kg concentration) and chest hair was removed by shaving and application of a depilatory cream. Animals were monitored daily for weight loss, morbidity and mortality. Prior to imaging, mice were injected intraperitoneally with luciferin (Xenogen Corp) at a dose of 150 mg/kg of body weight and anesthetized with isoflurane for 5 min. *In vivo* images were acquired with the IVIS CCD camera system (Caliper Life Sciences) and analyzed with Living Image 3.2 software (Caliper Life Sciences) using an exposure of 60s, 30s, or 5s (binning of 4 and an f/stop of 1). Pseudocolor images (representative of bioluminescence) of mice are displayed using a binning of 4 on a colorimetric scale ranging from 1×10^6 to 1×10^9 surface radiance (photons/s/cm²/steradian), which is defined as the number of photons that leave a cm² of tissue and radiate into a solid angle of one steradian. To quantify bioluminescence, regions of interest (ROI) were defined manually and graphed data are expressed as total flux (photons/s), which is defined as the radiance in each pixel summed over the ROI area (cm²) x 4π. All animal studies were approved by the Animal Care and Use Committee of St. Jude Children's Research Hospital and were performed in

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compliance with relevant institutional policies, the Association for the Accreditation of Laboratory Animal Care guidelines, the National Institutes of Health regulations and local, state and federal laws.

5 **V. Immunology.** Sera and BALF were collected from euthanized animals on day 10 or day 60 p.i.. BALF samples (3 ml) were centrifuged to collect cellular material and plated in a tissue culture dish for 1 h at 37 °C to remove adherent cells. Suspension cells were harvested, total lymphocytes were counted microscopically, and red blood cells were lysed. For flow cytometric analyses, cells were stained with FITC-conjugated anti-CD4 (RM4-4) and PE-conjugated anti-
10 CD8b (53-5.8) antibodies (BD Biosciences Pharmingen). Lymphocytes were gated based on forward and side scatter, and the percentages of CD4⁺ and CD8⁺ T cell populations were measured within this gate. ELISAs were used to measure the levels of Sendai virus-specific or luciferase-specific antibodies present in the sera. Briefly, 96-well plates were coated overnight with disrupted, purified Sendai virus (10 µg/ml) or firefly luciferase (1 µg/ml, Abcam). Plates
15 were blocked with PBS containing 1% BSA and then incubated with 10-fold serially diluted serum samples. After incubation, plates are washed, incubated with HRP-Goat anti mouse IgG (Southern Biotechnologies) and then washed further. To quantify levels of antibodies, TMB substrate (Kirkegaard and Perry Laboratories) was added to the wells followed by stop solution and absorbance was read at a wavelength of 450 nm. GraphPad Prism non-linear regression
20 software was used to calculate antibody titers.

VI. Contact Transmission. Donor animals were inoculated intranasally with 30 µL of SeVc-luc(M-F*) and were individually placed into cages containing 3 naïve contact mice at 24 h p.i.. Bioluminescence was monitored daily until levels of luminescence were consistently at
25 background levels (~15 days). Sera were collected on day 60 so that Sendai virus-specific antibody levels could be measured as described above. On day 63, mice were challenged with 7000 PFU SeVc-luc(M-F*) administered intranasally and bioluminescence was measured daily.

C. Supplementary Material and Methods

30 **I. *In vitro* Expression of Sendai Virus Proteins.** Viral protein expression levels were analyzed by radioimmunoprecipitation as previously reported [Luque et al. 2007, J Virol 81: 3130-3141

and Luque et al. 2010, J Virol 84: 810-821]. Briefly, LLC-MK2 cells were infected at an MOI of 5 PFU/cell, labeled with 50 μ Ci [³⁵S]Promix (Amersham Pharmacia Biotech), lysed with ice-cold RIPA buffer and clarified by centrifugation. Supernatant was incubated overnight at 4°C with mouse anti- NP, P, M, F, and HN monoclonal antibodies, and immune complexes were adsorbed to protein G-Sepharose (GE Healthcare) before fractionation on 12% NuPAGE bis-Tris SDS-PAGE gels (Invitrogen) and visualization as described previously [Luque et al. 2010, J Virol 84: 810-821].

II. Sendai Virus Composition. The allantoic cavities of 10-day-old embryonated hen eggs were inoculated with viruses. Allantoic fluid was harvested 72 hpi and centrifuged 45 min at 3000 rpm to remove cellular debris. Supernatants were layered over a 60-20% sucrose gradient and centrifuged at 24,000 rpm for 3.5 hrs to isolate virions. Isolated virions were diluted in TNE buffer and further purified over a 20% sucrose cushion by centrifugation at 24,000 rpm for 15 hrs. Virus pellets were resuspended in RIPA buffer and total protein concentrations were determined using the BCA protein assay kit (Thermo Sci.). Equal protein levels were run on a 4-12% SDS-PAGE gel, the gel was stained using the Blue BANDit™ protein stain (Amresco), and then dried with a BioRad gel dryer at 60 °C for 45 minutes.

III. *In vivo* Infection and Transmission. The measurement of T-lymphocyte influx in BALF for CD4+ and CD8+ T-cells is described in the main text. Luciferase-specific ELISAs were performed essentially as Sendai virus-specific ELISAs as described in the main text except using firefly luciferase protein (Abcam) was used to coat 96-well plates. Bioluminescence imaging and viral titer determinations from dissected tissues are also described in the main text. In contact transmission experiments, the time until detection was measured as the first day bioluminescence > 10⁶ log₁₀ photons/s was recorded. Bioluminescence areas under the curve (AUC) were calculated by integrating bioluminescence intensities with respect to time using IgorPro software (Wavemetrics).

D. RESULTS

I. *In vitro* Properties of Luciferase-expressing Viruses

To develop a model in which PIV infection could be visualized non-invasively in intact
5 mice, three recombinant Sendai viruses (SeVc viruses) were generated in which firefly luciferase
was inserted into the P-M, M-F and F-HN gene junctions of Sendai virus (Figure 1a, Figure 9).

Insertion of an additional gene and gene junction into the Sendai virus genome was
expected to decrease downstream viral gene expression and, consequently, reduce virus
replication [Tokusumi et al. 2002, Virus Res 86: 33-38]. To generate a luciferase-expressing
10 Sendai virus expected to suffer little or no attenuation, the SeVc-luc(M-F*) virus was
constructed to contain both the luciferase reporter gene and a more efficient transcription start
sequence AGGGTGAAAG (SEQ. ID. NO.: 20) upstream of the F gene (Figure 9). Thus, the
attenuating effects of reporter gene insertion could be counteracted by optimization of the
naturally inefficient gene start sequence upstream of the F gene [Kato et al. 1999, J Virol 73:
15 9237-9246]. For the SeVc-luc(P-M) and SeVc-luc(F-HN) constructs in which the luciferase gene
was inserted into the P-M and F-HN gene junctions, respectively, the naturally occurring
suboptimal transcription start sequence upstream of the F gene was left intact (Figure 9).

To determine if the viruses were attenuated or temperature restricted, multiple-step
growth curves at a multiplicity of infection (MOI) of 0.01 PFU/cell were measured in LLC-MK2
20 cells at 33 and 37 °C (Figure 1b). Titers of SeVc-luc(M-F*), SeVc-luc(F-HN) and WT were
similar at both temperatures and similar to each other, showing these two luciferase-expressing
viruses were not substantially attenuated or temperature restricted. In contrast, the SeVc-luc(P-
M) virus had reduced growth kinetics at 33 °C and grew even slower at 37 °C. To determine how
efficiently the SeVc viruses expressed the reporter gene, *in vitro* luciferase expression in LLC-
25 MK2 cell lysates (MOI 5 PFU/cell) was measured with a luminometer (Figure 1c). Upstream
insertion of the reporter gene in SeVc-luc(P-M) resulted in higher reporter-gene expression than
downstream insertion in SeVc-luc(F-HN), as has been described previously for insertions of
secreted alkaline phosphatase [Tokusumi et al. 2002, Virus Res 86: 33-38]. Luciferase
expression by SeVc-luc(M-F*) exceeded that of SeVc-luc(P-M) within 6 h p.i. (post-infection),
30 showing the enhanced gene start sequence engineered into the M-F* virus (Figure 9) increases
reporter-gene transcription at later time points, perhaps due to greater downstream transcription

of the L polymerase gene. To determine how the reporter gene insertions may have altered expression of the Sendai virus genes, Sendai virus protein expression in LLC-MK2 cells (MOI 5 PFU/cell) was measured by radioimmunoprecipitation. Low levels of expression of the M, F, HN and presumably L proteins by the SeVc-luc(P-M) virus (Figure 10a) most likely caused the high level of attenuation of this virus construct. Viral protein expression by SeVc-luc(M-F*) and SeVc-luc(F-HN) was sufficient to generate virions with WT-like compositions (Figure 10b,c), and these two reporter viruses grew to levels similar to wild-type virus *in vitro*.

II. Virulence of Luciferase-expressing Viruses

An ideal luciferase-reporter virus for non-invasive bioluminescence imaging and pathogenesis studies would express high levels of luciferase without altering virus replication and disease severity in the natural murine host compared to WT virus. To determine if the three luciferase-expressing SeVc viruses generated here retained the virulence of WT Sendai virus *in vivo*, 129/SvJ mice were inoculated intranasally with 7,000 PFU of virus, a dose known to induce substantial levels of morbidity and mortality in this mouse strain [Faisca et al. 2005, Am J Physiol Lung Cell Mol Physiol 289: L777-787]. In this experiment the mice were anesthetized with isoflurane and intranasally inoculated with virus in a 30 μ l volume, a method of inoculation that delivers $\sim 1/3$ of the volume to the nasopharynx and $\sim 1/2$ of the volume to the lungs [Southam et al. 2002, Am J Physiol Lung Cell Mol Physiol 282: L833-839]. Infection with WT, SeVc-luc(M-F*), and SeVc-luc(F-HN) resulted in average weight losses of $\sim 25\%$ and mortality rates of 80% (Figure 1d,e), showing these two luciferase-expressing viruses remained fully virulent at this dose. In contrast, the attenuated SeVc-luc(P-M) virus induced only 12% weight loss and no mortality. Infection of 129/SvJ mice with 70,000 or 700,000 PFU of SeVc-luc(P-M) also resulted in 100% survival (data not shown), further demonstrating that the attenuated SeVc-luc(P-M) virus is avirulent.

Acute viral pneumonia by Sendai virus induces high levels of lymphocyte infiltration in bronchoalveolar lavage fluid (BALF) with a peak at ~ 10 dpi [Mo et al. 1995, J Virol 69: 1288-1291]. To determine if the luciferase-expressing viruses promoted lymphocyte influx comparable to WT, 129/SvJ mice infected with 7,000 PFU were sacrificed at 10 dpi for recovery of BALF. Similarly high numbers of total lymphocytes, CD4⁺ T-lymphocytes, and CD8⁺ T-lymphocytes were detected in BALF after infection with WT, SeVc-luc(M-F*), and SeVc-luc(F-HN), while

lymphocyte influx after infection with attenuated SeVc-luc(P-M) was decreased ~10-fold (Figure 1f; Figure 11a-b). To determine the extents to which the reporter viruses elicited antibodies that bind to Sendai virus or luciferase, sera was also collected 10 dpi. All three SeVc viruses elicited anti-Sendai virus antibody titers similar to WT (Figure 1g). The titers of anti-luciferase antibodies were also similar to each other for the three reporter viruses (Figure 11c). Thus despite being attenuated and avirulent in 129/SvJ mice, SeVc-luc(P-M) elicited a robust antibody response. SeVc-luc(M-F*) induced WT-like levels of morbidity and mortality while expressing high levels of luciferase, making it best suited as a surrogate for WT virus in bioluminescence imaging experiments on pathogenesis and transmission.

III. Dynamics of Infection in Living Animals

To determine if non-invasive bioluminescence accurately reflected *in vivo* infection, 129/SvJ mice were intranasally inoculated with 7,000 PFU, imaged with a Xenogen IVIS instrument, and immediately euthanized so respiratory tissues could be collected for *ex vivo* measurement of luminescence and viral titers. Consistent with previous studies in immunocompetent mice [Tashiro et al. 1988, Virology 165: 577-583 and Miyamae et al. 2005, J Vet Med Sci 67: 369-377], viral titers and bioluminescence were limited to the respiratory tract and in these studies were distinctly visualized in the nasopharynx, trachea, and lungs. As shown in Figure 12, *in vivo* bioluminescence intensities in living animals correlated well with *ex vivo* luminescence (R^2 0.878) and viral titers in the nasopharynx (R^2 0.864), trachea (R^2 0.915), and lungs (R^2 0.961), validating the technique as a means to measure *in vivo* infection non-invasively. To determine if the luciferase-reporter genes were genetically stable in the three SeVc viruses, lung tissues were recovered from 7,000-PFU-inoculated 129/SvJ mice at 7 dpi, homogenized, and plaqued in LLC-MK2 cells. Five plaques for each of the three luciferase-expressing viruses were picked, RT-PCR transcribed, and sequenced. All of the individual plaques contained the luciferase insert, had no mutations, and expressed luciferase after infection in LLC-MK2 cells. While it is not necessary to understand the mechanism of action, it is believed this shows that the luciferase reporter gene was genetically stable in all three of the SeVc viruses after 7 days of replication *in vivo*.

Using the bioluminescence imaging system presented herein, the kinetics and tropism of infection were measured in intact 129/SvJ mice and compared our results to the conventional

method of virus titer determination from dissected tissues (Figures 2 and 3). Just as SeVc-luc(M-F*) and SeVc-luc(F-HN) had *in vitro* replication rates and *in vivo* pathogenicities similar to WT, these SeVc viruses also had WT-like titers in the nasal turbinates, trachea, and lungs. In the nasal turbinates, high virus titers ($>10^5$ PFU) were detected by 2 dpi and were maintained until 9 dpi, after which rapid clearance occurred (Figure 3b). High levels of bioluminescence from the nasopharynx ($>10^8$ photons/s) were similarly observed for 129/SvJ mice infected with SeVc-luc(M-F*) between 2 and 9 dpi with a peak around 5 dpi (Figure 3a). In the lungs, virus titers peaked by 5 dpi and were cleared to low levels by 9 dpi. Infection with the attenuated SeVc-luc(P-M) resulted in peak lung titers of $\sim 10^4$ PFU at 5 dpi, nearly 100-fold lower than WT (Figure 3d), and similarly low levels of bioluminescence were observed in the lungs (Figure 3a), consistent with its attenuated and avirulent phenotype. However, SeVc-luc(P-M) grew to high peak titers ($\sim 10^5$ PFU) in the nasal turbinates, a level similar to WT at 7 dpi (Figure 3c), and had high levels of bioluminescence in the nasopharynx between 3 and 6 dpi (Figure 3a).

IV. Tissue Tropism and Viral Dose

While lower inoculating doses of Sendai virus are known to reduce infection and pathology in the lungs, we are unaware of any published studies on the dose dependence of infection in the URT or trachea. Preliminary studies showed that the mouse infectious dose 50 (MID₅₀) for SeVc-luc(M-F*) was 9 PFU and that a 70-PFU dose resulted in 100% infection, similar to results obtained for WT Sendai virus in mice [Kiyotani et al. 1993, J Virol 67: 7618-7622] and hPIV1 in humans [Reichelderfer et al. 1958, Science 128: 779-780]. 129/SvJ mice were inoculated intranasally with 70, 700 or 7,000 PFU of SeVc-luc(M-F*) in equal 30 μ l volumes and then measured bioluminescence and viral titers. Compared to a 7,000-PFU dose, 70 PFU-inoculation resulted in ~ 10 -fold lower viral titers and bioluminescence in the lungs (Figure 4a,b) and lower weight loss (Figure 4c). In contrast, infection in the nasopharynx and trachea after 70-PFU inoculation was only delayed ~ 1 d compared to 7,000-PFU, reaching a similar level by ~ 5 dpi (Figure 4a,b) and inducing relatively high titers of Sendai virus-specific antibodies ($> 10^5$) (Figure 4d). Thus, while it is not necessary to understand the mechanism of action, it is believed that low-dose inoculation of WT-like SeVc-luc(M-F*) resulted in infection biased to the URT and trachea, inducing a robust antibody response without causing severe pathogenicity.

V. Tissue Tropism and Host Genetics

Various strains of recombinant inbred mice differ in their susceptibilities to lung infection by Sendai virus [Faisca et al. 2005, Am J Physiol Lung Cell Mol Physiol 289: L777-787; Brownstein, DG 1987, J Virol 61: 1670-1671; Brownstein et al. 1981, Am J Pathol 105: 156-163; and Brownstein et al. 1986, Lab Anim Sci 36: 126-129]. For example, 129/SvJ and DBA/2 mice are highly susceptible to lung infection and its resulting pathogenesis while BALB/c and C57BL/6 mice are highly resistant. How host genetics affects Sendai virus replication in the URT and trachea has not been previously reported. Therefore, the *in vivo* dynamics of Sendai virus infection was measured in 129/SvJ, DBA/2, C57BL/6, and BALB/c strains of mice intranasally inoculated with 7,000 PFU of SeVc-luc(M-F*). As expected from previous studies, the extent of infection in the lungs and weight loss correlated with each other and followed the trend C57BL/6 < BALB/c << DBA/2 < 129/SvJ (Figures 2 and 4). In contrast, the URT and trachea were highly permissive to Sendai virus infection, having similarly high levels of bioluminescence for all four strains of mice. Thus, the URT and trachea of BALB/c and C57BL/6 mice were highly permissive to Sendai virus infection despite genetic resistance in the lungs. While it is not necessary to understand the mechanism of action, it is believed that these results show that genetic susceptibility to Sendai virus infection is tissue specific and that reduced infection in the lungs is not due to lower infection in the URT or trachea. In subsequent experiments on transmission, light-coated BALB/c and 129/SvJ strains of mice were used. Therefore, Sendai virus titers in groups of sacrificed BALB/c mice were measured and found that the *ex vivo* titers correlate with bioluminescence in intact mice (Figure 13a) just as they had for 129/SvJ mice. Compared to 129/SvJ mice, infection in the lungs of BALB/c mice was decreased at least 10-fold as measured by both bioluminescence (Figure 4e) and viral titers (Figure 13b-c). Consequently, the BALB/c mice had only very mild clinical symptoms, including very little weight loss (Figure 4f). In contrast, nasopharyngeal infection in BALB/c mice reached a level similar to that in 129/SvJ mice by 3 dpi, as measured by both bioluminescence (Figure 4e) and viral titer (Figure 13b-c). Overall, it is believed that the bioluminescence imaging studies revealed three conditions in which robust infection in the URT and trachea was observed despite reduced infection in the lungs and little apparent weight loss: an attenuated virus, a low virus dose, and a resistant strain of mouse.

VI. Dynamics of Infection During Contact Transmission

Infection control requires an understanding of how pathogens are transmitted. Sendai virus, the hPIVs, and hRSV are known to transmit primarily via contact with respiratory secretions as opposed to long-range transmission of small-particle aerosols [Iida, T. 1972, J Gen Virol 14: 69-75; van der Veen et al. 1970, Arch Gesamte Virusforsch 31: 237-246; Henrickson, K.J. 2003, Clin Microbiol Rev 16: 242-264; Hall et al. 1981, J Pediatr 99: 100-103; and McLean et al. 1967, Can Med Assoc J 96: 1449-1453]. It is also known that growth of Sendai virus [Iida, T. 1972, J Gen Virol 14: 69-75] and influenza virus [Lowen et al. 2007, PLoS Pathog 3: 1470-1476] in the URT promotes transmission. Two longstanding, fundamental questions about PIV transmission that remained unknown were (i) how growth of virus in the lungs of donors influences transmission and (ii) what factors determine the timing of transmission and the tissue-specific spread of infection after transmission. To address these fundamental questions about PIV transmission, BALB/c or 129/SvJ donor mice were inoculated with 70 or 7,000 PFU of SeVc-luc(M-F*) and then placed 3 naïve contact mice in a cage with 1 donor mouse at 1 dpi. Bioluminescence was measured daily in inoculated and contact mice until primary infection cleared, collected sera on day 60, challenged the mice with 7,000 PFU of SeVc-luc(M-F*) on day 63, and then imaged the mice daily for reinfection (Figure 5). It is believed that transmission to every naïve contact mouse was observed by nasopharyngeal bioluminescence and seroconversion, even for resistant BALB/c mice exposed to donor animals inoculated at the lower dose. The timing of transmission was not influenced by the extent of lung infection in donors as lung titers were ~10-fold lower in BALB/c versus 129/SvJ donor mice after 7,000-PFU inoculation (Figure-13c) yet the transmission times (difference in time until detection in inoculated versus transmitted animals) were a similar 3.3 and 3.4 days, respectively (Figure 6f). LRT infection occurred in both strains of mice and may contribute to transmission. Regardless, the primary determinant of transmission appeared to be virus shedding in the URT and trachea. For example, high-titer ($> 10^5$ PFU) shedding in the nasal cavities and trachea of 129/SvJ donor mice (Fig 4a,b) and contact transmission (Figure 6e,f) both occurred ~ 1 day earlier after 7,000-PFU inoculation compared to 70-PFU. Overall, while it is not necessary to understand the mechanism of action, it is believed these results demonstrate how animals that suffer little apparent weight loss are able to promote efficient transmission of Sendai virus Enders strain.

In order to investigate the magnitude of Sendai virus infection after transmission, previous studies measured *ex vivo* titers in groups of contact mice sacrificed different times after exposure to infected cagemates [Iida, T. 1972, J Gen Virol 14: 69-75 and van der Veen et al. 1970, Arch Gesamte Virusforsch 31: 237-246]. These classical studies yielded highly ambiguous results in which titers varied 100-fold from day to day and the progression of infection in the respiratory tract after transmission was not clear. Therefore, non-invasive bioluminescence imaging was used to measure for the first time the temporal and spatial spread of PIV infection throughout the respiratory tract in individual, living mice after transmission. The inoculated dose was varied in donors and the mouse strain so that viral and host determinants of transmission could be investigated. Under all four conditions tested (129/SvJ or BALB/c mice infected at 70 or 7,000 PFU), the tropism and magnitude of infection in contact animals after transmission was similar to that observed after direct inoculation with a 70-PFU dose of SeVc-luc(M-F*) delivered intranasally. After transmission, bioluminescence was first observed in the nasopharynx and then spread to the trachea and lungs an average of 0.8 and 1.0 days later, respectively (Figure 14a-d). Robust infection was observed in the nasopharynx and trachea (Fig 6a-d, Fig 14e-h), and low levels of lung infection were consistent with little weight loss after transmission (Figure 6g-h). For all four groups of mice, Sendai virus-specific antibody titers on day 60 were similarly high ($\sim 10^6$) and the animals were universally protected during challenge on day 63 (Figure 5). After challenge a low level of bioluminescence ($< 10^6$ photons/s), but no weight loss, was detected in only 1 contact mouse out of 30, the animal with the lowest level of bioluminescence after primary infection on days 5-12 (Figure 5b, solid black circles). As this individual animal also had the lowest level of Sendai virus-specific antibodies at day 60 before challenge, a threshold level of infection may be required for protective immunity. Overall, while it is not necessary to understand the mechanism of action, it is believed that Sendai virus infection after transmission was observed to be robust enough in the URT and trachea, yet limited enough in the lungs, to induce protective immunity without causing significant weight loss in the matched murine host that is susceptible to Sendai virus infection.

E. DISCUSSION

The current embodiments provide the generation and use of luciferase-reporter viruses to study for the first time the kinetics of PIV infection in living mice after direct inoculation and

after contact transmission. Compared to WT Sendai virus, the luciferase-expressing virus SeVc-luc(M-F*) had a similar replication rate *in vivo* and elicited similar levels of weight loss, mortality, lymphocyte influx in BALF, and serum antibody titers. Both susceptible (129/Sv) and resistant (BALB/c) strains of mice were intranasally infected with 70- and 7,000 PFU doses of SeVc-luc(M-F*), and the spread of infection was measured by both bioluminescence in intact mice and *ex vivo* virus titers from sacrificed animals. The consequences of infection in the URT and trachea were found to be distinct from infection in the lungs. Unexpectedly, under all conditions tested including 70 PFU inoculation in resistant BALB/c mice, the URT and trachea supported robust Sendai virus growth, efficient contact transmission, and protective immunity independent of the extents of infection in the lungs. In contrast, the extent of infection in the lungs varied by virus dose and mouse strain and also correlated highly with weight loss and mortality. Overall, the results reported here reveal a tissue-specific dichotomy in the respiratory tract in which asymptomatic infection in the URT and trachea supports efficient transmission while the extent of infection and host response in the lungs determines clinical outcome.

While it is not necessary to understand the mechanism of action, the present invention contemplates for the first time the development of a non-invasive, bioluminescence imaging system to visualize infection throughout living animals by a negative-strand RNA virus, using the prototypic respiratory paramyxovirus Sendai virus. The development of a non-attenuated paramyxovirus that expresses high enough levels of a reporter gene for non-invasive imaging in small animals has been a challenge because these non-segmented negative-strand RNA viruses have a polarized transcription mechanism [Lamb et al. 2007, Paramyxoviridae: The Viruses and Their Replication. 5th Ed. pp. 1449-1496]. A significant advance described here is the generation of the SeVc-luc(M-F*) virus in which the expected attenuating effects of reporter-gene insertion [Tokusumi et al. 2002, Virus Res 86: 33-38] are counteracted by enhancement of the naturally occurring, suboptimal gene-start sequence upstream of the F gene [Kato et al. 1999, J Virol 73: 9237-9246]. Expression of the F gene, a virulence factor [Anderson et al. 2008, J Virol 82: 10510-10518 and Luque et al. 2010, J Virol 84: 810-821], is also downregulated by hPIV1 [Bousse et al. 2002, J Virol 76: 8244-8251], hPIV3 [Spriggs et al. 1986, J Virol 59: 646-654], PIV5 [Rassa et al. 1998, Virology 247: 274-286], measles virus [Cattaneo et al. 1987, Virology 160: 523-526] and canine distemper virus (CDV) [Anderson et al. 2008, J Virol 82: 10510-10518] by readthrough transcription or long untranslated regions.

Thus, for example only and not meant to be limiting, the present invention embodiments contemplate that other WT-like reporter paramyxoviruses that express high levels of luciferase could be engineered by inserting the reporter gene into the M-F junction and maintaining F gene expression through compensating mutations. Reporter gene expression without attenuation of Sendai virus has also been achieved by construction of a bicistronic gene that contains an internal ribosome entry site [Touzelet et al. 2009, Virus Res 140: 40-48], although it is not yet clear if this alternative approach yields sufficient luciferase expression for non-invasive imaging of *in vivo* infection. Insertion of an enhanced green fluorescent protein (eGFP) reporter gene downstream in the H-L junction of a non-attenuated CDV has enabled *ex vivo* imaging of paramyxovirus dissemination in dissected ferret tissues [Rudd et al. 2006, J Virol 80: 9361-9370 and von Messling et al. 2004, Proc Natl Acad Sci U S A 101: 14216-14221]. However, insertion of a luciferase reporter gene near the 5' end of the genome to avoid attenuation is expected to result in relatively low levels of reporter gene expression, limiting the sensitivity of non-invasive imaging techniques as was observed here with the SeVc-luc(F-HN) reporter virus.

While it is not necessary to understand the mechanism of action, it is believed that the use of the luciferase reporter gene in the present work enabled the measurement of infection throughout the entire respiratory tracts of intact animals such that the spread and clearance of infection could be measured after direct inoculation or transmission. Thus it is expected that an alternate form of this Sendai virus vector could be constructed in which a different reporter gene is used including, but not limited to, a fluorescent protein such as eGFP. eGFP-expressing reporter viruses have been also used to study the dynamics of CDV infection in ferrets [Rudd et al. 2006, J Virol 80: 9361-9370 and von Messling et al. 2004, Proc Natl Acad Sci U S A 101: 14216-14221] and measles virus infection in monkeys [Lemon et al. 2011, PLoS Pathog 7: e1001263 and de Swart et al. 2007, PLoS Pathog 3: e178]. It is contemplated that an advantage of the eGFP reporter gene is that the tropism of infection in dissected tissues can be studied on a cellular level. Moreover, eGFP-expressing viruses can also be used to quantify and type infected cells in peripheral blood, the skin, and mouths of living animals. eGFP-expressing hPIV3 and SeVs have been used to study the cellular tropism of PIV infection in well differentiated, primary epithelial cultures. In the case of hPIV3, infection was found to be restricted to ciliated epithelial cells and cause little cytopathology [Zhang et al. 2005, J Virol 79: 1113-1124]. In contrast, Sendai virus was found to infect ciliated and non-ciliated cells, but not goblet cells, and was

observed to induce ciliostasis, cell sloughing, apoptosis, and cellular degeneration [Villénave et al. 2010, J Virol 84: 11718-11728]. It is unknown if cell-free virus or cell-associated virus is associated with Sendai virus transmission.

Surprisingly, the URT was found here to be highly permissive to Sendai virus infection even under conditions known to limit infection in the lungs: after a low virus dose, for an attenuated virus, and in resistant mouse strains. Intranasal inoculation of RSV in human subjects has also recently been shown to result in equally high peak nasal titers for viral doses that span a 100-fold range [Devincenzo et al. 2010, Am J Respir Crit Care Med 182: 1305-1314]. Of course, after natural transmission of RSV or hPIVs in humans, high inoculating doses of virus in the lungs may play a role in the development of severe disease, as was observed here for high-dose inoculation of Sendai virus in mice.

Therefore, while it is not necessary to understand the mechanism of action, the present invention embodiments contemplate that Sendai virus is a promising Jennerian vaccine against hPIV1 [Karron et al. 2007, Parainfluenza Viruses. 5th Ed. pp 1497-1526 and Takimoto et al. 2005, Viral Immunol 18: 255-266], and recombinant Sendai virus vaccine vectors containing an envelope gene from RSV, hPIV3 or hPIV2 inserted into the F-HN gene junction have been shown to elicit both B- and T-cell responses that lead to protection from challenge in small-animal models [Jones et al. 2009, Vaccine 27: 1848-1857; Zhan et al. 2007, Vaccine 25: 8782-8793; and Zhan et al. 2008, Vaccine 26: 3480-3488]. While Sendai virus is pathogenic in mice, an ongoing clinical trial has demonstrated Sendai virus to be well tolerated in humans [Slobod et al. 2004, Vaccine 22: 3182-3186]. In non-human primates, Sendai virus has been shown to protect against hPIV1 challenge with no associated adverse events [Hurwitz et al. 1997, Vaccine 15: 533-540 and Skiadopoulos et al. 2002, Virology 297: 153-160]. The results are likely due in part to the sensitivity of Sendai virus to human IFN-mediated innate immunity [Bousse et al. 2006, Virus Res 121: 23-32]. Moreover, embodiments of the present invention also contemplate that as Sendai virus is developed further as a vaccine vector, the luciferase-expressing SeVs and imaging system developed here will be useful in investigating how the vaccine dose, volume, and position of foreign antigen insertion in the Sendai virus genome influence tissue-specific vector growth and the immune response in small animal models. Replacing the luciferase reporter gene in Sendai virus with a vaccine antigen could alter *in vivo* replication of the vector. For example, three different recombinant hPIV3 vectors expressing hPIV1 HN, hPIV2 HN, or

measles virus HA inserted into the P-M gene junction were found to replicate to different levels in hamsters [Skiadopoulos et al. 2002, *Virology* 297: 136-152].

While it is not necessary to understand the mechanism of action, it is believed that another novel finding here was that the efficiency and timing of Sendai virus transmission occurred independent of the extent of pulmonary infection, clinical symptoms, and host genetics. hPIV1 transmission from asymptomatic human donors has also been observed in an experimental setting [Reichelderfer et al. 1958, *Science* 128: 779-780] and is consistent with epidemiological observations for PIV outbreaks in general [Hall, CB 2001, *N Engl J Med* 344: 1917-1928 and Henrickson, KJ 2003, *Clin Microbiol Rev* 16: 242-264]. These observations suggest that LRT infection and the severity of clinical symptoms would be poor predictors of transmission potential for surveillance and infection control efforts. Consistent with previous work [Iida, T. 1972, *J Gen Virol* 14: 69-75 and Kiyotani et al. 1993, *J Virol* 67: 7618-7622], it was observed that Sendai virus transmission coincides with high-titer virus growth in the URT and is remarkably efficient because of the high infectivity of the virus (e.g., the MID₅₀ of Sendai virus is < 10 PFU). hPIV1, hPIV3 and hRSV are similarly highly infectious and also transmit predominantly by direct contact or indirect exposure to nasal secretions [Hall et al. 1981, *J Pediatr* 99: 100-103; McLean et al. 1967, *Can Med Assoc J* 96: 1449-1453; Hall et al. 1981, *Infect Immun* 33: 779-783; Parrott et al. 1975, *Dev Biol Stand* 28: 389-399; and Tyrrell et al. 1959, *Br Med J* 2: 909-911]. While it is not necessary to understand the mechanism of action, in the absence of an available prophylactic drug for uninfected individuals in high-risk groups (e.g., premature infants and the immunocompromised), it is believed that the results described here suggest that infection control of PIV would be best focused on reducing URT shedding from infected individuals, disinfecting contaminated surfaces, and hand washing. In contrast to infection control, which would be best served by limiting URT infection, therapeutic antivirals would be better targeted to the LRT to control clinical manifestations of PIV-associated disease.

Genetic factors have been identified that modulate viral susceptibility and disease severity in humans [Stephens, HA 2010, *Curr Top Microbiol Immunol* 338: 99-114; Zhang et al. 2009, *Infect Genet Evol* 9: 1148-1157; and Arkwright et al. 2008, *Curr Opin Infect Dis* 21: 217-222] and in the lungs of mice [Faisca et al. 2005, *Am J Physiol Lung Cell Mol Physiol* 289: L777-787; Brownstein, DG 1987, *J Virol* 61: 1670-1671; Brownstein et al. 1986, *Lab Anim Sci* 36: 126-129; Simon et al. 2009, *Infect Genet Evol* 9: 1253-1259; Boon et al. 2009, *J Virol* 83:

10417-10426; Anh et al. 2006, *Am J Physiol Lung Cell Mol Physiol* 291: L426-435; Itoh et al. 1991, *J Vet Med Sci* 53: 275-279; and Stark et al. 2002, *J Med Virol* 67: 92-100] . While it is not necessary to understand the mechanism of action, it is believed the present results show for the first time that genetic factors limiting virus growth in the lungs of resistant BALB/c mice, compared to susceptible 129/Sv mice, do not limit robust virus growth in the URT and trachea and, consequently, do not limit transmission. Furthermore, similarly high extents of infection in the URT and trachea and low levels of infection in the lungs were observed after transmission whether BALB/c or 129/Sv mice were exposed to cagemates inoculated at high or low viral doses. While it is not necessary to understand the mechanism of action, it is believed this shows host genetics do not play a major role in PIV transmission, at least for these strains of mice. These observations reinforce the notion presented here that transmission and pathogenesis are independent consequences of URT versus LRT infection, respectively, and may be most effectively countered by tissue-specific strategies. Additional experiments are needed to delineate mechanisms responsible for the high permissivity of the URT and trachea to Sendai virus infection compared to the lungs. While it is not necessary to understand the mechanism of action, it is contemplated that potential mechanisms include the site of inoculation in the nasal cavity, lower temperature in the URT, tissue-specific differences in virus replication and innate immunity, and antiviral mechanisms in the lungs such as the presence of surfactant proteins. One potential contributing factor to reduced replication in the lungs may be lower levels of secreted tryptase Clara, which is required for cleavage of the F protein from an inactivate precursor so that viral entry may occur [Kido et al. 1992, *J Biol Chem* 267: 13573-13579 and Tashiro et al. 1992, *J Virol* 66: 7211-7216].

Asymptomatic infection that promotes immunity and transmission represents a balanced relationship that benefits both virus and host. Such has been the case for several enzootic (clinically unapparent) epidemics of Sendai virus in which subclinical infections were maintained in mouse and hamster colonies for years without evolving increased pathogenicity and only occasionally causing apparent disease in suckling and old animals [Profeta et al. 1969, *Am J Epidemiol* 89: 316-324 and Zurcher et al. 1977, *Lab Anim Sci* 27: 955-962]. Such epidemiological observations are reminiscent of the low virulence yet high transmissibility of the reverse-genetics engineered Sendai virus described here, which was derived from the Enders strain that had been attenuated through numerous rounds of passage in embryonated chicken

eggs and contained modifications to the L gene. While it is not necessary to understand the mechanism of action, it is believed results reported here for the Enders-based strain show that increased shedding of virus in the lungs increases neither the transmission time nor the transmission efficiency, thus there may be no selective advantage for increased Sendai virus replication in the lungs. Instead, the following mechanism for symbiotic virus-host interplay in enzootic epidemics of Sendai virus is suggested: natural infection after transmission is limited enough in the lungs to avoid clinical signs of disease yet robust enough in the nasopharynx and trachea to promote efficient transmission and induce protective immunity.

Epizootic (clinically apparent) outbreaks of Sendai virus have also occurred that caused morbidity and high rates of mortality in mouse colonies [Bhatt et al. 1974, *Am J Epidemiol* 100: 222-229; Ishida et al. 1978, *Adv Virus Res* 23: 349-383; and Nakagawa et al. 1980, *Nippon Juigaku Zasshi* 42: 337-344]. Two closely related, highly pathogenic field isolates of Sendai virus are the Ohita and Hamamatsu strains [Sakaguchi et al. 1994, *Arch Virol* 135: 159-164 and Itoh et al. 1997, *J Gen Virol* 78 (Pt 12): 3207-3215]. While inoculation with only a few PFU of unpassaged Hamamatsu strain Sendai virus results in mortality in mice, after 50 passages in eggs the virus was attenuated by as much as 400-fold in MLD_{50} [Kiyotani et al. 2001, *Arch Virol* 146: 893-908]. Adaptations of the highly pathogenic Ohita and Hamamatsu strains to LLC-MK2 cells and chicken eggs were found to have selected for mutations in the C protein and untranslated leader region, respectively, that increase replication in culture cells but attenuate replication and pathogenesis in the lungs of mice [Garcin et al. 1997, *Virology* 238: 424-431; Fujii et al. 2002, *J Virol* 76: 8540-8547; and Sakaguchi et al. 2003, *Virology* 313: 581-587]. While it is not necessary to understand the mechanism of action, it is believed that, the bioluminescence imaging system described here would be useful in determining if the mutations that attenuate replication in the lungs also attenuate replication in the URT and trachea, thereby reducing transmission, or if the attenuating mutations actually promote sustained transmission by supporting nasal and tracheal shedding of virus while reducing pathogenesis in the lungs. Such experiments may also reveal if the observations on Sendai virus spread and transmission reported here for the attenuated, egg-adapted Enders strain extend to unpassaged, highly pathogenic field isolates.

In summary, while it is not necessary to understand the mechanism of action, it is believed that the development of the non-attenuated reporter virus SeVc-luc(M-F*) has been

described, which can be used to quantify tissue-specific infection in living mice, and a candidate vaccine vector SeVc-luc(P-M), which replicates preferentially in the URT. While it is not necessary to understand the mechanism of action, it is contemplated that the results reveal how infection by Sendai virus Enders strain spreads in individual, living animals after direct inoculation and after transmission. A major novel finding was an apparent phenotypic dichotomy of infection in the URT and trachea in comparison to the lungs that results in an observed decoupling of pathogenesis and transmission. While it is not necessary to understand the mechanism of action, it is believed the imaging tools developed here will provide a method to study how the dynamics of infection and transmission are determined by viral factors, host genetics, host age, immune status, environmental conditions, and inoculation mode. For example only and not meant to be limiting, infection can be tracked non-invasively in WT and knockout mice before *ex vivo* immune responses are measured and then understood in terms of the preceding infection. A similar strategy could also be developed to image infection by other paramyxoviruses in small-animal models. While it is not necessary to understand the mechanism of action, overall, the present invention embodiments contemplate the model system and results, that suggest tissue-targeted approaches to PIV infection control and vaccine development, and the non-invasive bioluminescence imaging technique is expected to assist in preclinical testing of vaccine candidates and antiviral therapeutics.

III. Utilities

Thus in one embodiment the invention contemplates a candidate Sendai virus vector comprising an Enders L gene with substituted amino acids, that can be used as a non-recombinant vector or as a recombinant vector to express any gene, or more than one gene, in any position. In another embodiment the invention contemplates a candidate Sendai virus vaccine vector that is an Enders/Z chimera such as that with a modified Enders/Z L gene, capable of expressing either the RSV F or G gene in the F-HN position of the Sendai virus genome.

In yet another embodiment the foreign gene might be placed in the P-M intergenic junction. Moreover, in other embodiments the foreign gene contemplated by the invention includes a gene or genes from hPIV type 1-4 wherein said gene(s) is inserted in between the P-M and/or F-HN genes. Further, in other embodiments, the invention also contemplates vaccine

candidates with one or more foreign genes from more than one source inserted in one or more intergenic positions such that one or more targeted diseases might be acted upon at one time to elicit a targeted immune response.

While it is not necessary to understand the mechanism of action, it is contemplated that a modified non-recombinant or recombinant SeV vaccine vector may encompass a modified L gene containing the following amino acid substitutions: S155G, R258K, G466E, G482E, S581R, Q717R, T800I, and R852K. Moreover, in a further embodiment the invention contemplates a visual means of tracking infection by use of bioluminescence. The instant disclosure contemplates a system comprising a luciferase vector that is capable of imaging the progression of virus and associated pathogenic disease within a living animal with the ability to investigate candidate vaccine vectors for utility in protecting against targeted diseases. Moreover, in some embodiments, the invention contemplates host cells for expression of viral proteins. In one example, and not meant to be limiting, the expressed viral proteins might be used as an immunogen for eliciting an immune response from a subject against a targeted pathogen or multiple targeted pathogens. For example only and not meant to be limiting, host cells include LLC-MK2 cells (See Figure 1).

In other embodiments, the present invention contemplates therapeutic and/or diagnostic uses. While it is not necessary to understand the mechanism of action, in some embodiments, the present invention contemplates generation of antibodies for use against the modified virus, modified viral proteins, including fragments, analogs, homologs, peptides, and/or combinations thereof. Such antibodies could be utilized for example within a diagnostic immunoassay such as an ELISA, RIA, and Immunoprecipitation among others for identification/diagnosis of infection and/or disease. Generation of antibodies is known by those of skill in the art. Further, lab procedures/guidance can be found in *Antibodies: A Laboratory Manual* by Harlow et al. (1988); *Using Antibodies: A Laboratory Manual* by Harlow et al. (1999); Köhler, G.; Milstein, C. (1975). "Continuous cultures of fused cells secreting antibody of predefined specificity". *Nature* 256 (5517): 495.

Moreover, in some embodiments, the present invention contemplates use of generated antibodies as a therapeutic for treatment against targeted pathogens including use of the antibodies for delivery of secondary drugs, toxins, among others. Furthermore, it is contemplated that viral proteins can be used for vaccine against virus. Numerous vaccine formulations are

known to those skilled in the art. Vaccines can be administered alone or in combination with various adjuvants/carriers. Pharmaceutical carriers are known to those skilled in the art. These most typically would be standard carriers for administration of vaccines to humans, including solutions such as sterile water, saline, and buffered solutions at physiological pH. Other ingredients include excipients, carriers, thickeners, diluents, buffers, preservatives, and surface active.

In other embodiments, while it is not necessary to understand the mechanism of action, the present invention contemplates use of the SeVc backbone alone or in combination with other genes of interest for use as a vaccine, a research tool, a diagnostic tool, a imaging tool, and includes any other similar, equivalent, related uses by one of skill in the art.

EXPERIMENTAL

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

EXAMPLE I

Cell culture

Monolayer cultures of LLC-MK2 cells were grown in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% penicillin, and 1% streptomycin at 37°C + 5% CO₂.

EXAMPLE II

Recombinant Sendai Viruses

Unique *NotI* recognition sites were cloned into the P-M, M-F and F-HN intergenic junctions of an Enders-based pSeV viral genome plasmid, using cloning sites described previously [Tokusumi et al. 2002, Virus Res 86: 33-38]. The firefly luciferase gene was amplified by PCR using the pGL3 Basic vector (Promega) and a pair of *AscI* tagged primers, subcloned into a shuttle plasmid containing a Sendai virus intergenic junction and flanking *NotI*

restriction sites [Tokusumi et al. 2002, Virus Res 86: 33-38] and then subcloned into the unique *NotI* site of each of the pSeV viral genome plasmids. Within the pSeV-luc(M-F) plasmid, the start signal upstream of the F protein was changed from AGGGATAAAG (SEQ. ID. NO.: 19) to AGGGTGAAAG (SEQ. ID. NO.: 20) using QuikChange™ Site-Directed Mutagenesis Kit (Stratagene Corp). The recombinant SeVs were rescued from the pSeV genome plasmids as described previously [Zhan et al. 2008, Vaccine 26: 3480-3488]. The modified Enders strain Sendai genome consists of a modified Sendai virus L gene that contains the following amino acid changes: S to G at position 155, R to K at position 258, G to E at position 466, G to E at position 482, S to R at position 581, Q to R at position 717, T to I at position 800, and R to K at position 852.

EXAMPLE III

Luciferase Expression *in vitro*

SeV-infected LLC-MK2 cells (MOI 5 PFU/cell) were incubated at 33°C + 5% CO₂ and lysates collected at various times p.i.. Luciferase assays were performed using the Luciferase Assay System (Promega) and the levels of expression measured using an automated luminometer (Turner Biosystems, Inc.) as described previously [Luque et al. 2007, J Virol 81: 3130-3141].

EXAMPLE IV

Viral Titers and Bioluminescence Imaging

Virus titers from multistep growth curves (MOI of 0.01 PFU/cell) and homogenized tissues were determined by plaque titration in LLC-MK2 cells as described previously [Luque et al. 2010, J Virol 84: 810-821]. Eight week-old female 129x1/SvJ mice or BALB/c mice (Jackson Laboratories) were anesthetized using isoflurane (Baxter Health Care Corporation) and inoculated intranasally (i.n.) with 30 µl of PBS or virus. For Figures 1D, 1E, 2, 3, 4E, 4F, and 12, at 3 d before inoculation with PBS or virus, mice were anesthetized by IP injection of 300 µl avertin (300 mg/kg concentration) and chest hair was removed by shaving and application of a depilatory cream. Animals were monitored daily for weight loss, morbidity and mortality. Prior to imaging, mice were injected intraperitoneally with luciferin (Xenogen Corp) at a dose of 150

mg/kg of body weight and anesthetized with isoflurane for 5 min. *In vivo* images were acquired with the IVIS CCD camera system (Caliper Life Sciences) and analyzed with Living Image 3.2 software (Caliper Life Sciences) using an exposure of 60s, 30s, or 5s (binning of 4 and an f/stop of 1). Pseudocolor images (representative of bioluminescence) of mice are displayed using a
5 binning of 4 on a colorimetric scale ranging from 1×10^6 to 1×10^9 surface radiance (photons/s/cm²/steradian), which is defined as the number of photons that leave a cm² of tissue and radiate into a solid angle of one steradian. To quantify bioluminescence, regions of interest (ROI) were defined manually and graphed data are expressed as total flux (photons/s), which is defined as the radiance in each pixel summed over the ROI area (cm²) $\times 4\pi$. All animal studies
10 were approved by the Animal Care and Use Committee of St. Jude Children's Research Hospital and were performed in compliance with relevant institutional policies, the Association for the Accreditation of Laboratory Animal Care guidelines, the National Institutes of Health regulations and local, state and federal laws.

15 EXAMPLE V

Immunology

Sera and BALF were collected from euthanized animals on day 10 or day 60 p.i.. BALF samples (3 ml) were centrifuged to collect cellular material and plated in a tissue culture dish for
20 1 h at 37 °C to remove adherent cells. Suspension cells were harvested, total lymphocytes were counted microscopically, and red blood cells were lysed. For flow cytometric analyses, cells were stained with FITC-conjugated anti-CD4 (RM4-4) and PE-conjugated anti-CD8b (53-5.8) antibodies (BD Biosciences Pharmingen). Lymphocytes were gated based on forward and side scatter, and the percentages of CD4+ and CD8+ T cell populations were measured within this
25 gate. ELISAs were used to measure the levels of Sendai virus-specific or luciferase-specific antibodies present in the sera. Briefly, 96-well plates were coated overnight with disrupted, purified Sendai virus (10 µg/ml) or firefly luciferase (1 µg/ml, Abcam). Plates were blocked with PBS containing 1% BSA and then incubated with 10-fold serially diluted serum samples. After incubation, plates are washed, incubated with HRP-Goat anti mouse IgG (Southern
30 Biotechnologies) and then washed further. To quantify levels of antibodies, TMB substrate (Kirkegaard and Perry Laboratories) was added to the wells followed by stop solution and

absorbance was read at a wavelength of 450 nm. GraphPad Prism non-linear regression software was used to calculate antibody titers.

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EXAMPLE VI

Contact Transmission

Donor animals were inoculated intranasally with 30 μ L of SeVc-luc(M-F*) and were individually placed into cages containing 3 naïve contact mice at 24 h p.i.. Bioluminescence was monitored daily until levels of luminescence were consistently at background levels (~15 days). Sera were collected on day 60 so that Sendai virus-specific antibody levels could be measured as described above. On day 63, mice were challenged with 7000 PFU SeVc-luc(M-F*) administered intranasally and bioluminescence was measured daily.

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EXAMPLE VII

In vitro Expression of Sendai Virus Proteins

Viral protein expression levels were analyzed by radioimmunoprecipitation as previously reported [Luque et al. 2007, J Virol 81: 3130-3141 and Luque et al. 2010, J Virol 84: 810-821]. Briefly, LLC-MK2 cells were infected at an MOI of 5 PFU/cell, labeled with 50 μ Ci [³⁵S]Promix (Amersham Pharmacia Biotech), lysed with ice-cold RIPA buffer and clarified by centrifugation. Supernatant was incubated overnight at 4°C with mouse anti- NP, P, M, F, and HN monoclonal antibodies, and immune complexes were adsorbed to protein G-Sepharose (GE Healthcare) before fractionation on 12% NuPAGE bis-Tris SDS-PAGE gels (Invitrogen) and visualization as described previously [Luque et al. 2010, J Virol 84: 810-821].

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EXAMPLE VIII

Sendai Virus Composition

The allantoic cavities of 10-day-old embryonated hen eggs were inoculated with viruses. Allantoic fluid was harvested 72 hpi and centrifuged 45 min at 3000 rpm to remove cellular

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debris. Supernatants were layered over a 60-20% sucrose gradient and centrifuged at 24,000 rpm for 3.5 hrs to isolate virions. Isolated virions were diluted in TNE buffer and further purified over a 20% sucrose cushion by centrifugation at 24,000 rpm for 15 hrs. Virus pellets were resuspended in RIPA buffer and total protein concentrations were determined using the BCA protein assay kit (Thermo Sci.). Equal protein levels were run on a 4-12% SDS-PAGE gel, the gel was stained using the Blue BANDit™ protein stain (Amresco), and then dried with a BioRad gel dryer at 60 °C for 45 minutes.

EXAMPLE IX

In vivo Infection and Transmission

The measurement of T-lymphocyte influx in BALF for CD4+ and CD8+ T-cells is described in the main text. Luciferase-specific ELISAs were performed essentially as Sendai virus-specific ELISAs as described in the main text except using firefly luciferase protein (Abcam) was used to coat 96-well plates. Bioluminescence imaging and viral titer determinations from dissected tissues are also described in the main text. In contact transmission experiments, the time until detection was measured as the first day bioluminescence $> 10^6 \log_{10}$ photons/s was recorded. Bioluminescence areas under the curve (AUC) were calculated by integrating bioluminescence intensities with respect to time using IgorPro software (Wavemetrics).

EXAMPLE X

In vitro Properties of Luciferase-expressing Viruses

To develop a model in which PIV infection could be visualized non-invasively in intact mice, three recombinant Sendai viruses (SeVc viruses) were generated in which firefly luciferase was inserted into the P-M, M-F and F-HN gene junctions of Sendai virus (Figure 1a, Figure 9).

Insertion of an additional gene and gene junction into the Sendai virus genome was expected to decrease downstream viral gene expression and, consequently, reduce virus replication [Tokusumi et al. 2002, Virus Res 86: 33-38]. To generate a luciferase-expressing Sendai virus expected to suffer little or no attenuation, the SeVc-luc(M-F*) virus was

constructed to contain both the luciferase reporter gene and a more efficient transcription start sequence AGGGTGAAAG (SEQ. ID. NO.: 20) upstream of the F gene (Figure 9). Thus, the attenuating effects of reporter gene insertion could be counteracted by optimization of the naturally inefficient gene start sequence upstream of the F gene [Kato et al. 1999, J Virol 73: 9237-9246]. For the SeVc-luc(P-M) and SeVc-luc(F-HN) constructs in which the luciferase gene was inserted into the P-M and F-HN gene junctions, respectively, the naturally occurring suboptimal transcription start sequence upstream of the F gene was left intact (Figure S1).

To determine if the viruses were attenuated or temperature restricted, multiple-step growth curves at a multiplicity of infection (MOI) of 0.01 PFU/cell were measured in LLC-MK2 cells at 33 and 37 °C (Figure 1b). Titers of SeVc-luc(M-F*), SeVc-luc(F-HN) and WT were similar at both temperatures and similar to each other, showing these two luciferase-expressing viruses were not substantially attenuated or temperature restricted. In contrast, the SeVc-luc(P-M) virus had reduced growth kinetics at 33 °C and grew even slower at 37 °C. To determine how efficiently the SeVc viruses expressed the reporter gene, *in vitro* luciferase expression in LLC-MK2 cell lysates (MOI 5 PFU/cell) was measured with a luminometer (Figure 1c). Upstream insertion of the reporter gene in SeVc-luc(P-M) resulted in higher reporter-gene expression than downstream insertion in SeVc-luc(F-HN), as has been described previously for insertions of secreted alkaline phosphatase [Tokusumi et al. 2002, Virus Res 86: 33-38]. Luciferase expression by SeVc-luc(M-F*) exceeded that of SeVc-luc(P-M) within 6 h p.i. (post-infection), showing the enhanced gene start sequence engineered into the M-F* virus (Figure 9) increases reporter-gene transcription at later time points, perhaps due to greater downstream transcription of the L polymerase gene. To determine how the reporter gene insertions may have altered expression of the Sendai virus genes, Sendai virus protein expression in LLC-MK2 cells (MOI 5 PFU/cell) was measured by radioimmunoprecipitation. Low levels of expression of the M, F, HN and presumably L proteins by the SeVc-luc(P-M) virus (Figure 10a) most likely caused the high level of attenuation of this virus construct. Viral protein expression by SeVc-luc(M-F*) and SeVc-luc(F-HN) was sufficient to generate virions with WT-like compositions (Figure 10b,c), and these two reporter viruses grew to levels similar to wild-type virus *in vitro*.

EXAMPLE XI

Virulence of Luciferase-expressing Viruses

An ideal luciferase-reporter virus for non-invasive bioluminescence imaging and pathogenesis studies would express high levels of luciferase without altering virus replication and disease severity in the natural murine host compared to WT virus. To determine if the three luciferase-expressing SeVc viruses generated here retained the virulence of WT Sendai virus *in vivo*, 129/SvJ mice were inoculated intranasally with 7,000 PFU of virus, a dose known to induce substantial levels of morbidity and mortality in this mouse strain [Faisca et al. 2005, Am J Physiol Lung Cell Mol Physiol 289: L777-787]. In this experiment the mice were anesthetized with isoflurane and intranasally inoculated with virus in a 30 μ l volume, a method of inoculation that delivers $\sim 1/3$ of the volume to the nasopharynx and $\sim 1/2$ of the volume to the lungs [Southam et al. 2002, Am J Physiol Lung Cell Mol Physiol 282: L833-839]. Infection with WT, SeVc-luc(M-F*), and SeVc-luc(F-HN) resulted in average weight losses of $\sim 25\%$ and mortality rates of 80% (Figure 1d,e), showing these two luciferase-expressing viruses remained fully virulent at this dose. In contrast, the attenuated SeVc-luc(P-M) virus induced only 12% weight loss and no mortality. Infection of 129/SvJ mice with 70,000 or 700,000 PFU of SeVc-luc(P-M) also resulted in 100% survival (data not shown), further demonstrating that the attenuated SeVc-luc(P-M) virus is avirulent.

Acute viral pneumonia by Sendai virus induces high levels of lymphocyte infiltration in bronchoalveolar lavage fluid (BALF) with a peak at ~ 10 dpi [Mo et al. 1995, J Virol 69: 1288-1291]. To determine if the luciferase-expressing viruses promoted lymphocyte influx comparable to WT, 129/SvJ mice infected with 7,000 PFU were sacrificed at 10 dpi for recovery of BALF. Similarly high numbers of total lymphocytes, CD4+ T-lymphocytes, and CD8+ T-lymphocytes were detected in BALF after infection with WT, SeVc-luc(M-F*), and SeVc-luc(F-HN), while lymphocyte influx after infection with attenuated SeVc-luc(P-M) was decreased ~ 10 -fold (Figure 1f; Figure 11a-b). To determine the extents to which the reporter viruses elicited antibodies that bind to Sendai virus or luciferase, sera was also collected 10 dpi. All three SeVc viruses elicited anti-Sendai virus antibody titers similar to WT (Figure 1g). The titers of anti-luciferase antibodies were also similar to each other for the three reporter viruses (Figure 11c). Thus despite being attenuated and avirulent in 129/SvJ mice, SeVc-luc(P-M) elicited a robust antibody

response. SeVc-luc(M-F*) induced WT-like levels of morbidity and mortality while expressing high levels of luciferase, making it best suited as a surrogate for WT virus in bioluminescence imaging experiments on pathogenesis and transmission.

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EXAMPLE XII

Dynamics of Infection in Living Animals

To determine if non-invasive bioluminescence accurately reflected *in vivo* infection, 129/SvJ mice were intranasally inoculated with 7,000 PFU, imaged with a Xenogen IVIS
10 instrument, and immediately euthanized so respiratory tissues could be collected for *ex vivo* measurement of luminescence and viral titers. Consistent with previous studies in immunocompetent mice [Tashiro et al. 1988, Virology 165: 577-583 and Miyamae et al. 2005, J Vet Med Sci 67: 369-377], viral titers and bioluminescence were limited to the respiratory tract and in these studies were distinctly visualized in the nasopharynx, trachea, and lungs. As shown
15 in Figure 12, *in vivo* bioluminescence intensities in living animals correlated well with *ex vivo* luminescence (R^2 0.878) and viral titers in the nasopharynx (R^2 0.864), trachea (R^2 0.915), and lungs (R^2 0.961), validating the technique as a means to measure *in vivo* infection non-invasively. To determine if the luciferase-reporter genes were genetically stable in the three SeVc viruses, lung tissues were recovered from 7,000-PFU-inoculated 129/SvJ mice at 7 dpi,
20 homogenized, and plaqued in LLC-MK2 cells. Five plaques for each of the three luciferase-expressing viruses were picked, RT-PCR transcribed, and sequenced. All of the individual plaques contained the luciferase insert, had no mutations, and expressed luciferase after infection in LLC-MK2 cells. While it is not necessary to understand the mechanism of action it is believed, this shows that the luciferase reporter gene was genetically stable in all three of the
25 SeVc viruses after 7 days of replication *in vivo*.

Using the bioluminescence imaging system presented herein, the kinetics and tropism of infection were measured in intact 129/SvJ mice and compared our results to the conventional method of virus titer determination from dissected tissues (Figures 2 and 3). Just as SeVc-luc(M-F*) and SeVc-luc(F-HN) had *in vitro* replication rates and *in vivo* pathogenicities similar to WT,
30 these SeVc viruses also had WT-like titers in the nasal turbinates, trachea, and lungs. In the nasal turbinates, high virus titers ($>10^5$ PFU) were detected by 2 dpi and were maintained until 9 dpi,

after which rapid clearance occurred (Figure 3b). High levels of bioluminescence from the nasopharynx ($>10^8$ photons/s) were similarly observed for 129/SvJ mice infected with SeVc-luc(M-F*) between 2 and 9 dpi with a peak around 5 dpi (Figure 3a). In the lungs, virus titers peaked by 5 dpi and were cleared to low levels by 9 dpi. Infection with the attenuated SeVc-luc(P-M) resulted in peak lung titers of $\sim 10^4$ PFU at 5 dpi, nearly 100-fold lower than WT (Figure 3d), and similarly low levels of bioluminescence were observed in the lungs (Figure 3a), consistent with its attenuated and avirulent phenotype. However, SeVc-luc(P-M) grew to high peak titers ($\sim 10^5$ PFU) in the nasal turbinates, a level similar to WT at 7 dpi (Figure 3c), and had high levels of bioluminescence in the nasopharynx between 3 and 6 dpi (Figure 3a).

EXAMPLE XIII

Tissue Tropism and Viral Dose

While lower inoculating doses of Sendai virus are known to reduce infection and pathology in the lungs, we are unaware of any published studies on the dose dependence of infection in the URT or trachea. Preliminary studies showed that the mouse infectious dose 50 (MID₅₀) for SeVc-luc(M-F*) was 9 PFU and that a 70-PFU dose resulted in 100% infection, similar to results obtained for WT Sendai virus in mice [Kiyotani et al. 1993, J Virol 67: 7618-7622] and hPIV1 in humans [Reichelderfer et al. 1958, Science 128: 779-780]. 129/SvJ mice were inoculated intranasally with 70, 700 or 7,000 PFU of SeVc-luc(M-F*) in equal 30 μ l volumes and then measured bioluminescence and viral titers. Compared to a 7,000-PFU dose, 70 PFU-inoculation resulted in ~ 10 -fold lower viral titers and bioluminescence in the lungs (Figure 4a,b) and lower weight loss (Figure 4c). In contrast, infection in the nasopharynx and trachea after 70-PFU inoculation was only delayed ~ 1 d compared to 7,000-PFU, reaching a similar level by ~ 5 dpi (Figure 4a,b) and inducing relatively high titers of Sendai virus-specific antibodies ($> 10^5$) (Figure 4d). Thus, while it is not necessary to understand the mechanism of action, it is believed that low-dose inoculation of WT-like SeVc-luc(M-F*) resulted in infection biased to the URT and trachea, inducing a robust antibody response without causing severe pathogenicity.

EXAMPLE XIV

Tissue Tropism and Host Genetics

Various strains of recombinant inbred mice differ in their susceptibilities to lung infection by Sendai virus [Faisca et al. 2005, Am J Physiol Lung Cell Mol Physiol 289: L777-787; Brownstein, DG 1987, J Virol 61: 1670-1671; Brownstein et al. 1981, Am J Pathol 105: 156-163; and Brownstein et al. 1986, Lab Anim Sci 36: 126-129]. For example, 129/SvJ and DBA/2 mice are highly susceptible to lung infection and its resulting pathogenesis while BALB/c and C57BL/6 mice are highly resistant. How host genetics affects Sendai virus replication in the URT and trachea has not been previously reported. Therefore, the *in vivo* dynamics of Sendai virus infection was measured in 129/SvJ, DBA/2, C57BL/6, and BALB/c strains of mice intranasally inoculated with 7,000 PFU of SeVc-luc(M-F*). As expected from previous studies, the extent of infection in the lungs and weight loss correlated with each other and followed the trend C57BL/6 < BALB/c << DBA/2 < 129/SvJ (Figures 2 and 4). In contrast, the URT and trachea were highly permissive to Sendai virus infection, having similarly high levels of bioluminescence for all four strains of mice. Thus, the URT and trachea of BALB/c and C57BL/6 mice were highly permissive to Sendai virus infection despite genetic resistance in the lungs. While it is not necessary to understand the mechanism of action it is believed these results show that genetic susceptibility to Sendai virus infection is tissue specific and that reduced infection in the lungs is not due to lower infection in the URT or trachea. In subsequent experiments on transmission, light-coated BALB/c and 129/SvJ strains of mice were used. Therefore, Sendai virus titers in groups of sacrificed BALB/c mice were measured and found that the *ex vivo* titers correlate with bioluminescence in intact mice (Figure 13a) just as they had for 129/SvJ mice. Compared to 129/SvJ mice, infection in the lungs of BALB/c mice was decreased at least 10-fold as measured by both bioluminescence (Figure 4e) and viral titers (Figure 13b-c). Consequently, the BALB/c mice had only very mild clinical symptoms, including very little weight loss (Figure 4f). In contrast, nasopharyngeal infection in BALB/c mice reached a level similar to that in 129/SvJ mice by 3 dpi, as measured by both bioluminescence (Figure 4e) and viral titer (Figure 13b-c). Overall, it is believed that the bioluminescence imaging studies revealed three conditions in which robust infection in the URT

and trachea was observed despite reduced infection in the lungs and little apparent weight loss: an attenuated virus, a low virus dose, and a resistant strain of mouse.

EXAMPLE XV

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Dynamics of Infection During Contact Transmission

Infection control requires an understanding of how pathogens are transmitted. Sendai virus, the hPIVs, and hRSV are known to transmit primarily via contact with respiratory secretions as opposed to long-range transmission of small-particle aerosols [Iida, T. 1972, J Gen Virol 14: 69-75; van der Veen et al. 1970, Arch Gesamte Virusforsch 31: 237-246; Henrickson, KJ 2003, Clin Microbiol Rev 16: 242-264; Hall et al. 1981, J Pediatr 99: 100-103; and McLean et al. 1967, Can Med Assoc J 96: 1449-1453]. It is also known that growth of Sendai virus [Iida, T. 1972, J Gen Virol 14: 69-75] and influenza virus [Lowen et al. 2007, PLoS Pathog 3: 1470-1476] in the URT promotes transmission. Two longstanding, fundamental questions about PIV transmission that remained unknown were (i) how growth of virus in the lungs of donors influences transmission and (ii) what factors determine the timing of transmission and the tissue-specific spread of infection after transmission. To address these fundamental questions about PIV transmission, BALB/c or 129/SvJ donor mice were inoculated with 70 or 7,000 PFU of SeVc-luc(M-F*) and then placed 3 naïve contact mice in a cage with 1 donor mouse at 1 dpi. Bioluminescence was measured daily in inoculated and contact mice until primary infection cleared, collected sera on day 60, challenged the mice with 7,000 PFU of SeVc-luc(M-F*) on day 63, and then imaged the mice daily for reinfection (Figure 5). It is believed that transmission to every naïve contact mouse was observed by nasopharyngeal bioluminescence and seroconversion, even for resistant BALB/c mice exposed to donor animals inoculated at the lower dose. The timing of transmission was not influenced by the extent of lung infection in donors as lung titers were ~10-fold lower in BALB/c versus 129/SvJ donor mice after 7,000-PFU inoculation (Figure 13c) yet the transmission times (difference in time until detection in inoculated versus transmitted animals) were a similar 3.3 and 3.4 days, respectively (Figure 6f). LRT infection occurred in both strains of mice and may contribute to transmission. Regardless, the primary determinant of transmission appeared to be virus shedding in the URT and trachea. For example, high-titer ($> 10^5$ PFU) shedding in the nasal cavities and trachea of 129/SvJ donor

mice (Fig 4a,b) and contact transmission (Figure 6e,f) both occurred ~ 1 day earlier after 7,000-PFU inoculation compared to 70-PFU. Overall, while it is not necessary to understand the mechanism of action, it is believed these results demonstrate how animals that suffer little apparent weight loss are able to promote efficient transmission of Sendai virus Enders strain.

5 In order to investigate the magnitude of Sendai virus infection after transmission, previous studies measured *ex vivo* titers in groups of contact mice sacrificed different times after exposure to infected cagemates [Iida, T. 1972, J Gen Virol 14: 69-75 and van der Veen et al. 1970, Arch Gesamte Virusforsch 31: 237-246]. These classical studies yielded highly ambiguous results in which titers varied 100-fold from day to day and the progression of infection in the
10 respiratory tract after transmission was not clear. Therefore, non-invasive bioluminescence imaging was used to measure for the first time the temporal and spatial spread of PIV infection throughout the respiratory tract in individual, living mice after transmission. The inoculated dose was varied in donors and the mouse strain so that viral and host determinants of transmission could be investigated. Under all four conditions tested (129/SvJ or BALB/c mice infected at 70
15 or 7,000 PFU), the tropism and magnitude of infection in contact animals after transmission was similar to that observed after direct inoculation with a 70-PFU dose of SeVc-luc(M-F*) delivered intranasally. After transmission, bioluminescence was first observed in the nasopharynx and then spread to the trachea and lungs an average of 0.8 and 1.0 days later, respectively (Figure 14a-d). Robust infection was observed in the nasopharynx and trachea (Fig 6a-d, Fig 14e-h), and low
20 levels of lung infection were consistent with little weight loss after transmission (Figure 6g-h). For all four groups of mice, Sendai virus-specific antibody titers on day 60 were similarly high ($\sim 10^6$) and the animals were universally protected during challenge on day 63 (Figure 5). After challenge a low level of bioluminescence ($< 10^6$ photons/s), but no weight loss, was detected in only 1 contact mouse out of 30, the animal with the lowest level of bioluminescence after
25 primary infection on days 5-12 (Figure 5b, solid black circles). As this individual animal also had the lowest level of Sendai virus-specific antibodies at day 60 before challenge, a threshold level of infection may be required for protective immunity. Overall, while it is not necessary to understand the mechanism of action, it is believed that Sendai virus infection after transmission was observed to be robust enough in the URT and trachea, yet limited enough in the lungs, to
30 induce protective immunity without causing significant weight loss in the matched murine host that is susceptible to Sendai virus infection.

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All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and devices of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred
5 embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention, which are obvious to those skilled in the subject area of vaccine development, infectious disease, molecular biology, diagnostics, biotechnology and-or related fields are
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CLAIMS

1. A recombinant Sendai viral vector comprising a modified Sendai viral vector in which a portion of Z strain genome is added to the Enders Sendai virus strain genome to create an Enders/Z chimera comprising a modified L gene.
2. The recombinant Sendai viral vector of Claim 1, wherein said modified L gene comprises nucleic acid encoding amino acid changes selected from the group consisting of S to G at position 155, R to K at position 258, G to E at position 466, G to E at position 482, S to R at position 581, Q to R at position 717, T to I at position 800, R to K at position 852, and combinations thereof.
3. A recombinant Sendai viral vector of claim 1 with immunogenic properties.
4. The recombinant Sendai viral vector of claim 1 or 2 having a foreign gene(s) inserted in any position(s) including but not limited to one or more than one intergenic junction selected from the group consisting of a N-P, a P-M, a M-F, a F-HN, a HN-L, and combinations thereof.
5. The recombinant Sendai viral vector of claim 3 wherein said foreign gene facilitates virus tracking *in vitro*, *in vivo*, or combinations thereof.
6. The recombinant Sendai viral vector of claim 4 wherein said foreign gene for tracking is selected from the group of a luciferase, a green fluorescent protein, and combinations thereof.
7. The recombinant Sendai viral vector of claims 1,2,3,4,5 or 6 wherein at least a gene start/stop site is manipulated to alter gene transcription.
8. The recombinant Sendai viral vector of claims 1,2,3,4,5,6 or 7 wherein said foreign gene is a respiratory syncytial virus (RSV) F protein.

9. The recombinant Sendai viral vector of claims 1,2,3,4,5,6 or 7 wherein said foreign gene is a respiratory syncytial virus (RSV) G protein.
10. The recombinant Sendai viral vector of claims 1,2,3,4,5,6 or 7 wherein said foreign gene is a parainfluenza virus type 1 (PIV-1) protein.
11. The recombinant Sendai viral vector of claims 1,2,3,4,5,6 or 7 wherein said foreign gene is a parainfluenza virus type 2 (PIV-2) protein.
12. The recombinant Sendai viral vector of claims 1,2,3,4,5,6 or 7 wherein said foreign gene is a parainfluenza virus type 3 (PIV-3) protein.
13. The recombinant Sendai viral vector of claims 1,2,3,4,5,6 or 7 wherein said foreign gene is a parainfluenza virus type 4 (PIV-4) protein.
14. The recombinant Sendai virus vector of claim 4 wherein said foreign gene is a reporter gene.
15. A method of immunizing an animal or a model tissue culture system against infection comprising use of an effective amount of a Sendai viral vector or recombinant Sendai viral vector of Claims 1,2,3,4,5,6 or 7.
16. A composition comprising the Sendai viral vector of Claims 1,2,3,4,5,6 or 7 and a pharmaceutically acceptable carrier or diluent or any carrier, adjuvant or diluent.
17. A method, comprising:
- a) providing:
 - i. a subject in which vaccination is desired wherein said subject includes *in vitro*, *in vivo*, and combinations thereof;
 - ii. the vector of Claim 1,2,3,4,5,6,7 or the composition of Claim 16; and

- b) administering said vector or composition to said subject in an amount effective to elicit an immune response.

5 18. The recombinant Sendai viral vector of Claims 1,2,3,4,5,6,7 or the composition of Claim 16, wherein said foreign gene is inserted between a Sendai virus P gene and a Sendai virus M gene.

10 19. The recombinant Sendai viral vector of Claim 1,2,3,4,5,6,7 or the composition of Claim 16, wherein said foreign gene is inserted between a Sendai virus M gene and a Sendai virus F gene.

15 20. The recombinant Sendai viral vector of Claim 1,2,3,4,5,6,7 or the composition of Claim 16, wherein said foreign gene is inserted between a Sendai virus F gene and a Sendai virus HN gene.

21. A recombinant Sendai viral vector comprising a modified Enders strain Sendai genome with a foreign gene or portion thereof inserted at an intergenic junction(s) selected from the group consisting of a N-P, a P-M, a M-F, a F-HN, a HN-L, and combinations thereof.

20 22. The recombinant Sendai viral vector of Claim 21, wherein said modified Enders strain Sendai genome comprises a modified L gene.

25 23. The recombinant Sendai viral vector of Claim 22, wherein a portion of the L gene of the Enders strain is replaced with the corresponding portion from the Z-strain of Sendai virus.

30 24. The recombinant Sendai viral vector of Claim 21, wherein said foreign gene is a reporter gene.

25. A kit comprising:

a) providing:

- i) the vector of claim 1,2,3,4,5,6 or 7; and
- ii) instructions for use to vaccinate against a targeted pathogen.

5

26. A kit comprising:

a) providing:

- i) the composition of claim 16; and
- ii) instructions for use to vaccinate against a targeted pathogen.

10

27. A kit comprising:

a) providing:

- i) the vector of claim 4; and
- ii) instructions for use to visualize the vector.

15

28. The recombinant Sendai viral vector of claims 1,2,3,4,5,6 or 7 wherein said vector is mixed with at least one other antigen or immunogen.

20

29. A method, comprising:

a) providing:

- i) a vector comprising an Enders Sendai virus strain genome; and
- ii) a Z Sendai virus strain genome; and

25

b) replacing at least a portion of a gene of the Enders strain genome with the corresponding portion from the Z-strain of Sendai virus genome so as to generate a modified Sendai viral vector.

30. The method of claim 29, wherein a portion of the L gene of the Enders strain is replaced with the corresponding portion from the Z-strain of Sendai virus so as to generate a modified Sendai viral vector comprising a modified L gene.

30

31. The method of claim 30, wherein said modified L gene comprises nucleic acid encoding amino acid changes selected from the group consisting of S to G at position 155, R to K at position 258, G to E at position 466, G to E at position 482, S to R at position 581, Q to R at position 717, T to I at position 800, R to K at position 852, and combinations thereof.

32. The method of claim 29, 30, or 31 further comprising, step c) inserting a foreign gene or a portion thereof into said vector wherein said insertion is at an intergenic junction selected from the group consisting of a N-P, a P-M, a M-F, a F-HN, a HN-L, and combinations thereof.

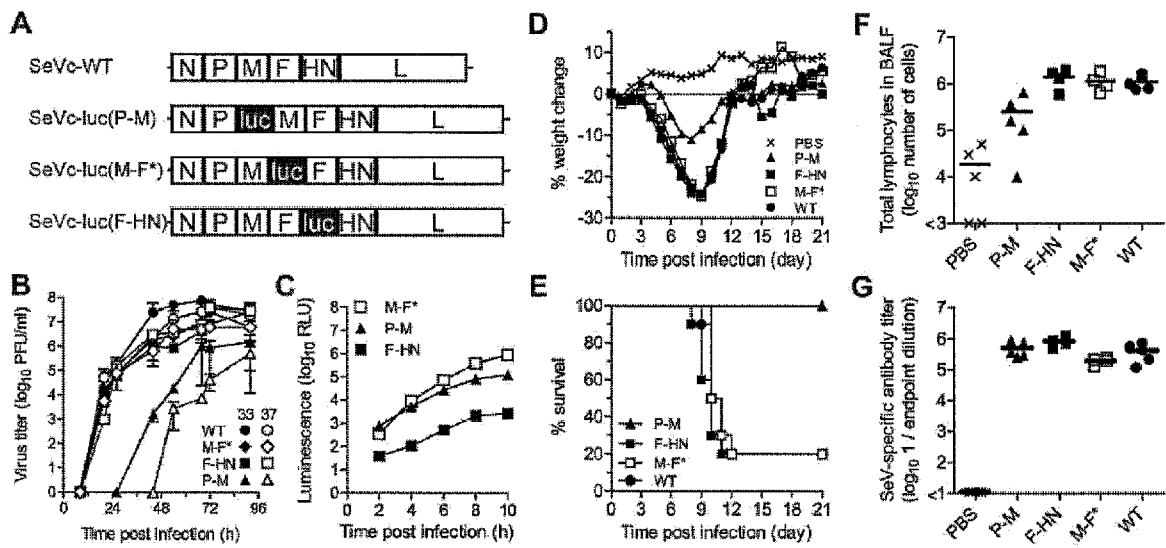


FIG. 1

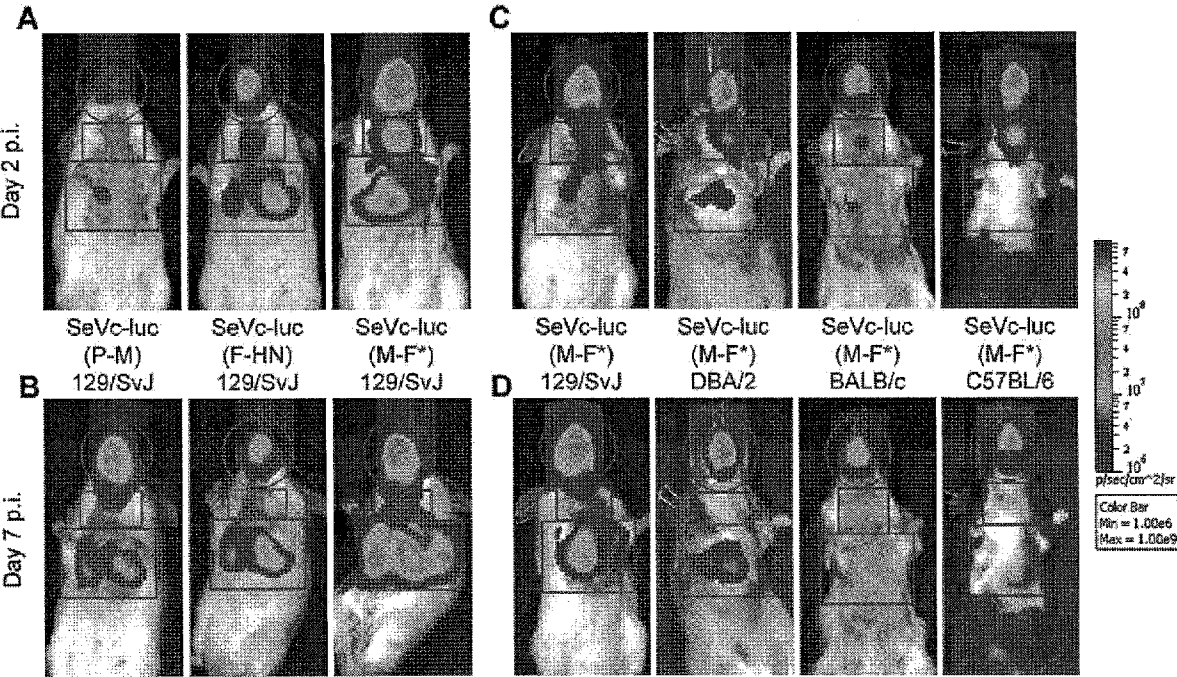


FIG. 2

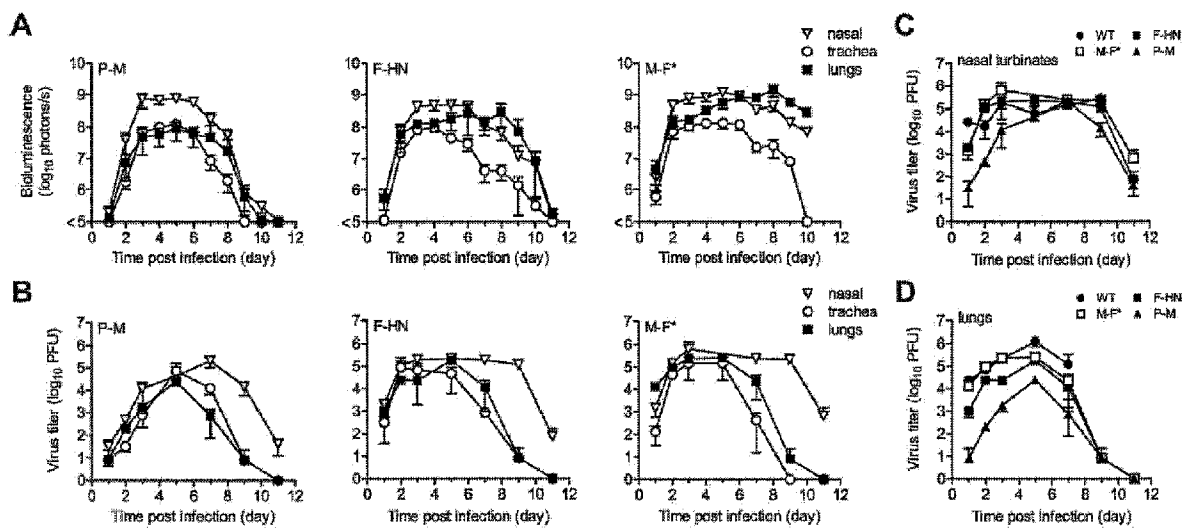


FIG. 3

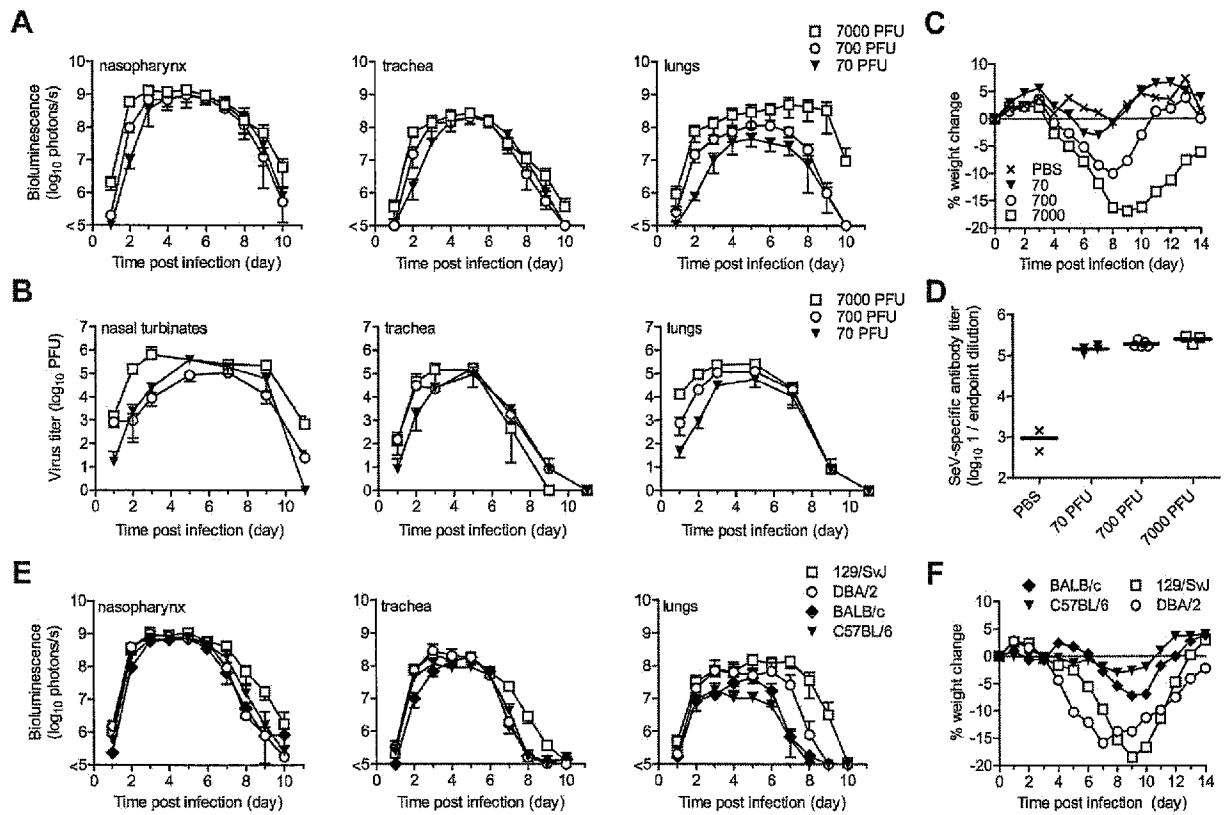


FIG. 4

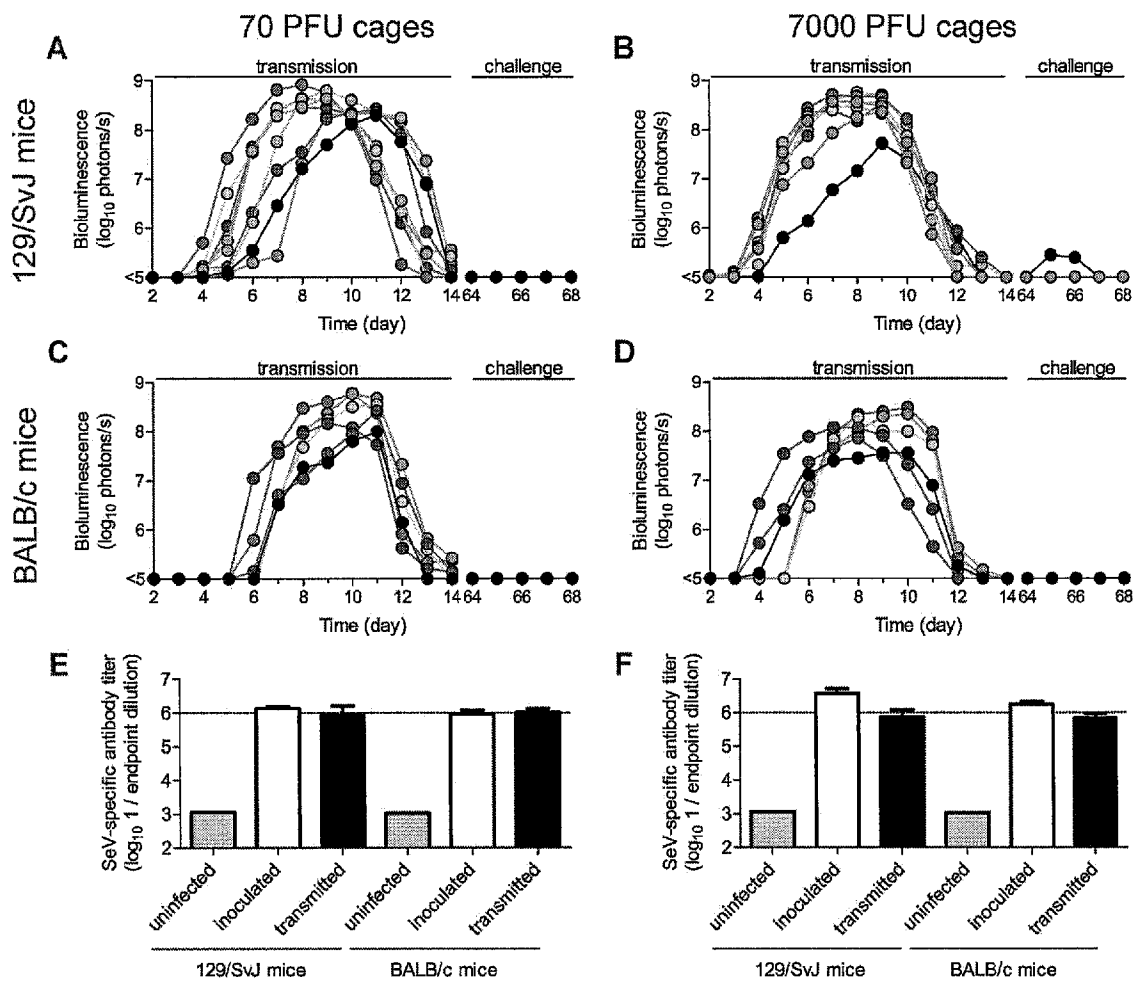


FIG. 5

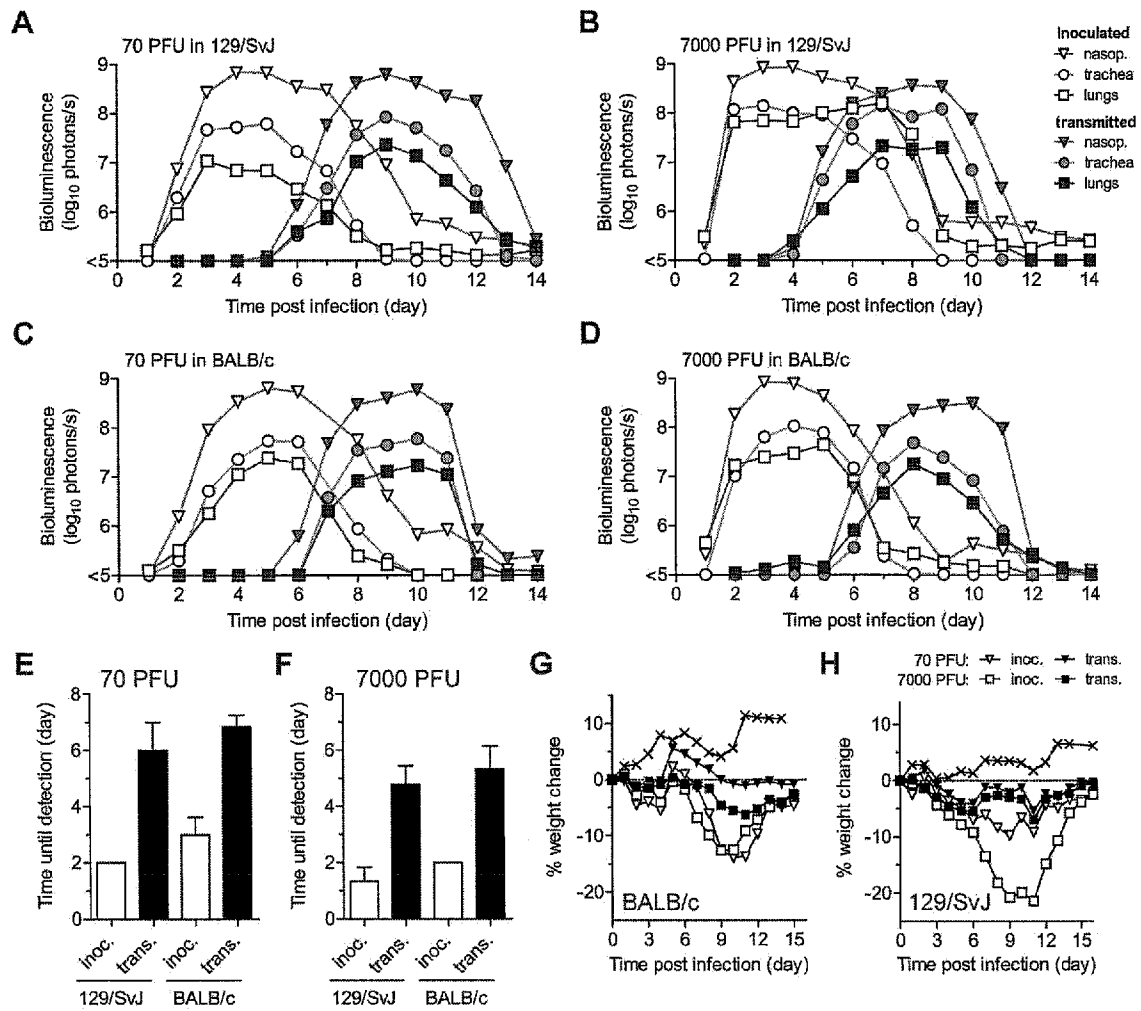


FIG. 6

pSVC plasmid sequence (SEQ. ID NO.:3)

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gcccgggttgttgagcaccttcgatacatttagctctaggaggagcgaaagtattaataagtcgggagg
aggtgctgttatccccggccagaggagcacagtctcagtggtcgtactaggcccaagtgtgactgatgat
gcagacaagttattcattgcaactaccttcctagctcactcattggacacagataagcagcactctcaga
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ttcattgtgaagacgagagatatggaatatgagaggaccacagaatggctgtttggacctatggtcaaca
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aatcaaaagagaacaagactaaggctaccaggtttgaccatctatggagaccctagaagatatgaagta
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FIG. 7A

atccccgggtgaggcatcctaccatcctcagtcataagagagatccaattaattaacagcatcagccagtaa
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FIG. 7A continued

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FIG. 7A continued

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FIG. 7A continued

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FIG. 7A continued

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FIG. 7A continued

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caattatcctgcactatggagttacgccatgggagtcgccgtctgtacagaacaaggcaatgcagcagtag
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cggcaaggaggattctgcaaccatggagatgaaggccgcaataacgggtgtcgatcacgacgaagatgacg
ataccgcagcagtagctgggataggaggaatc

FIG. 7B

P cDNA: (SEQ. ID. NO.:6)

atggatcaagatgccttcattcttaagaagattctgaagttgagagggaggcgccaggaggaagagagt
cgctctcggatggtatcggattcctcgcgctgtcctgtcgcgagtgaaccaactgacatcggaggggacag
aagctggctccacaacacccatcaacactccccaaggaccaggctctgcccatagagccaaaagtgagggc
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aagaactggtacaaactctgtatctcaggatctgggcgatggaggagactccggaatccttgaaaatcct
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caatccacccgggtctagagaccgactcaacaaaaagggtcataggagagaacacatcatctatgaaaga
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cgcaaccgggtgtaccaagagaggggacacagaacccaggggcctcaaacgcacacgcctcctccctcca
aagagaagcccacaatgcactctctcagggtcgtcatagagagcagtcacctaaagcagagctgagaaagc
agcatatgtgaaatcattatccaagtgcaagacagaccaagagggttaaggcagtcattggaactcgtagaa
gaggacatagagtcactgaccaactag

FIG. 7C

C cDNA (SEQ. ID. NO.:8)

ATGCCTTCATTCTTAAAGAAGATTCTGAAGTTGAGAGGGAGGCGCCAGGAGGAAGAGAGTCGCTCTCGGATGTTATCGGAT
TCCTCGATGCTGTCCTGTGCGAGTGAACCAACTGACATCGGAGGGGACAGAAGCTGGCTCCACAACACCATCAACACTCCCC
AAGGACCAGGCTCTGCCATAGAGCCAAAAGTGAGGGCGAAGGAGAAGTCTCAACACCGTCGACCCAAGATAATCGATCAG
GTGAGGAGAGTAGAGTCTCTGGGAGAACAAGCAAGCCAGAGGCAGAAGCACATGCTGGAAACCTTGATAAAACAAAATATAC
ACCGGGCCTTTGGGGGAAGAAGTGGTACAACTCTGTATCTCAGGATCTGGGCGATGGAGGAGACTCCGGAATCCTTGAAA
ATCCTCCAAATGAGAGAGGATATCCGAGATCAGGTATTGAAGATGAAAACAGAGAGATGGCTGCGCACCCCTGATAAGAGGG
GAGAAGACCAAGCTGAAGGACTTCCAGAAGAGGTACGAGGAGGTACATCCCTACCTGATGAAGGAGAAGGTGGAGCAAGTA
ATAATGGAAGAAGCATGGAGCCTGGCAGCTCACATAGTGCAAGAGTAA

FIG. 7D

M cDNA: (SEQ. ID. NO.:10)

atggcagatatctatagattccctaagttctcatatgaggataacgggtactgtggagcccctgcctctga
gaactgggtccagataagaaagccatccccctacatcaggattatcaaggtaggagaccctcctaaacatgg
agtgagatacctagatttattgctcttgggtttctttgagacaccgaaacaaacaaccaatctagggagc
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FIG. 7E

F cDNA (SEQ. ID. NO.:12)

atgacagcatatatccagaggtcacagtgcattctcaacatcactactggttgttctcaccacattggctc
cgtgtcagattcccagggtataggctctctaacataggggtcatagtcgatgaagggaatcactgaagat
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ctatagaactgctgcaaaacgctgtgggggaacaaattcttgctctaaagacactccaggatttctgtgaa
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gtctaactctatgatgtcattttatagacagatcaaaggaacgggtgatagatgtggatctagagaga
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cgtctatttcttacaacatagacggggaggaatgggtatgtgactgtcccagccatatactcagtcgtgc
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aagttgtggacagccttatcccaagtttgcttttgtgaatgggggcgttggttgctaactgcatagcatc
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tataccgaggggacacatatacattagagccgaagatcagacatatgtacacaaacgggtgggtttgatgcg
atggctgagaaaagatga

FIG. 7F

HN cDNA (SEQ. ID. NO.:14)

atggatggtgatagg
ggcaaacgtgactcgtactggtctacctctcctagtggttagcactacaaaattagcatcaggttgggaga
ggtcaagtaaagttgacacatggttgctgattctctcattcaccagtgaggctttgtcaattgccacagt
gatcatctgtatcataatttctgctagacaagggtatagtatgaaagagtactcaatgactgtagaggca
ttgaacatgagcagcaggggaggtgaaagagtcacttaccagtcctaataaggcaagaggttatcgcaaggg
ctgtcaacattcagagctctgtgcaaaccggaatcccagtccttgttgacaaaaacagcagggatgtcat
ccagatgattgataagtcgtgcagcagacaagagctcactcagctctgtgagagtacgatcgagtcacac
catgccgagggaattgcccccttggagccacatagtttctggagatgcccgtgtcggaacccgtatctta
gctcagatcctaaaatctcattgctgcctgggtccgagcttgttatctggttctacaacgatctctggatg
tgttaggctcccttactctcaattggcgaggcaatctatgcctattcatcaaatctcattacacaaggt
tgtgtgacatagggaaatcatatcaggtcctgcagctagggtacatatcactcaattcagatatgttcc
ctgatcttaaccccgtagtgccacacttatgacatcaacgacaatcggaatcatgctctgtggtggc
aaccgggactaggggttatcagcttctccatgccgactgtagacgaaagaaccgactactctagtgat
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ctactgctttcacatcagagatcaatcagaagagcctgaataccttacagccgatgtcttttaagact
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FIG. 7G

L cDNA (SEQ. ID. NO.:16)

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FIG. 7H

tatcatcataaatgggtatagagagaggcatggcggacagtggccccctgtgacttccctgatcacgtg
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FIG. 7H continued

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FIG. 7H continued

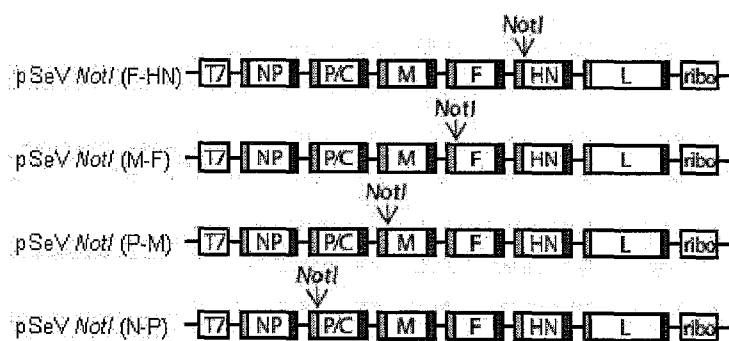


FIG. 8



FIG. 9

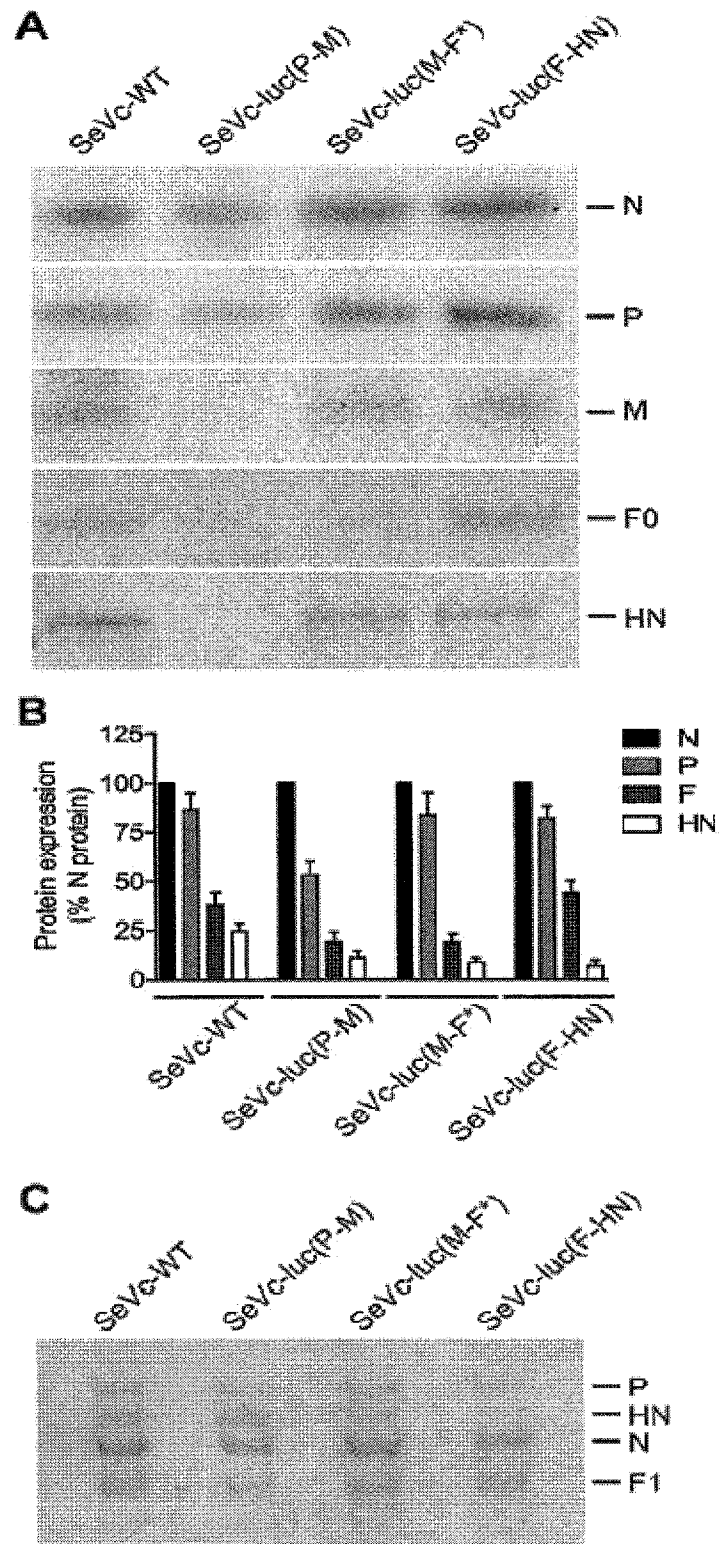


FIG. 10

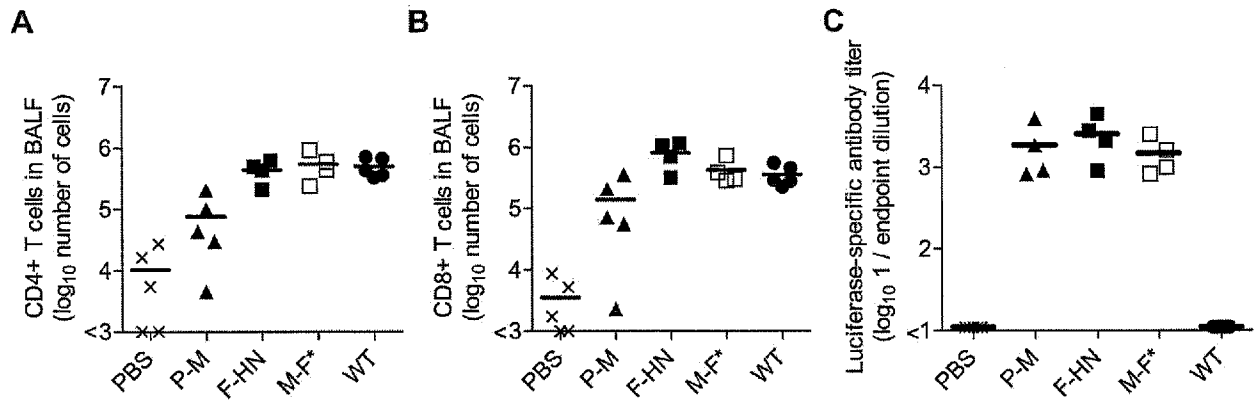


FIG. 11

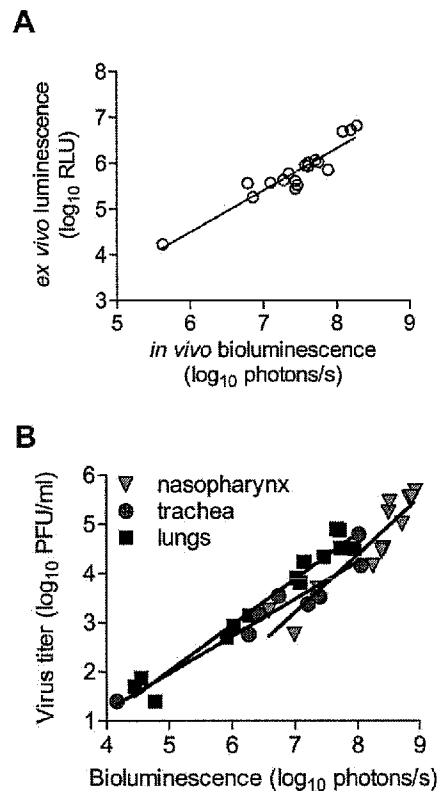


FIG. 12

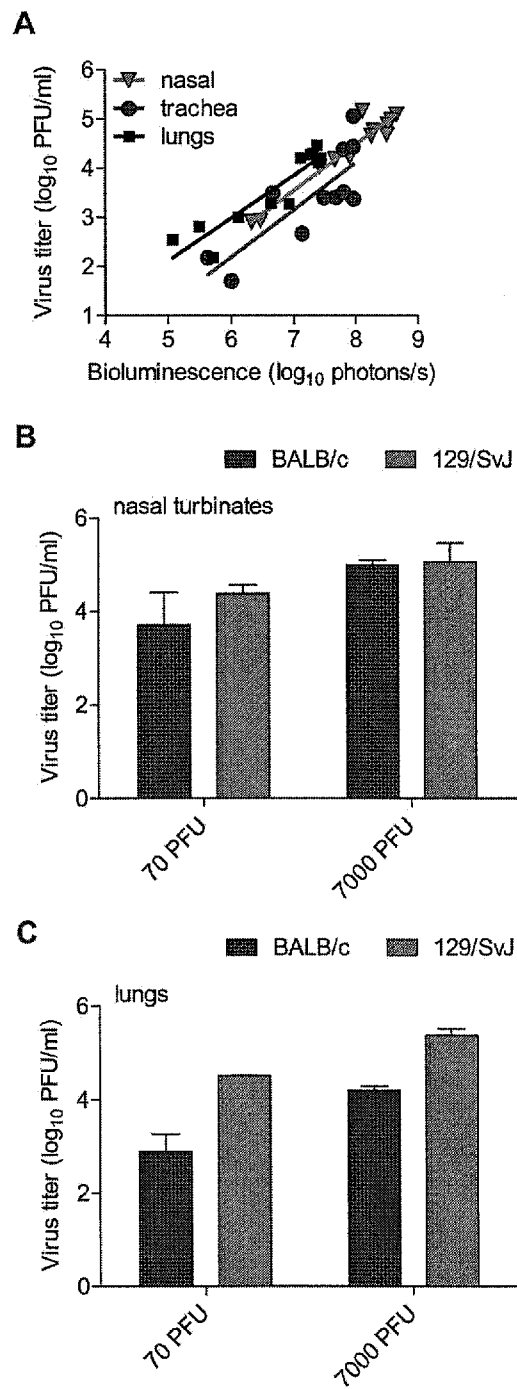


FIG. 13

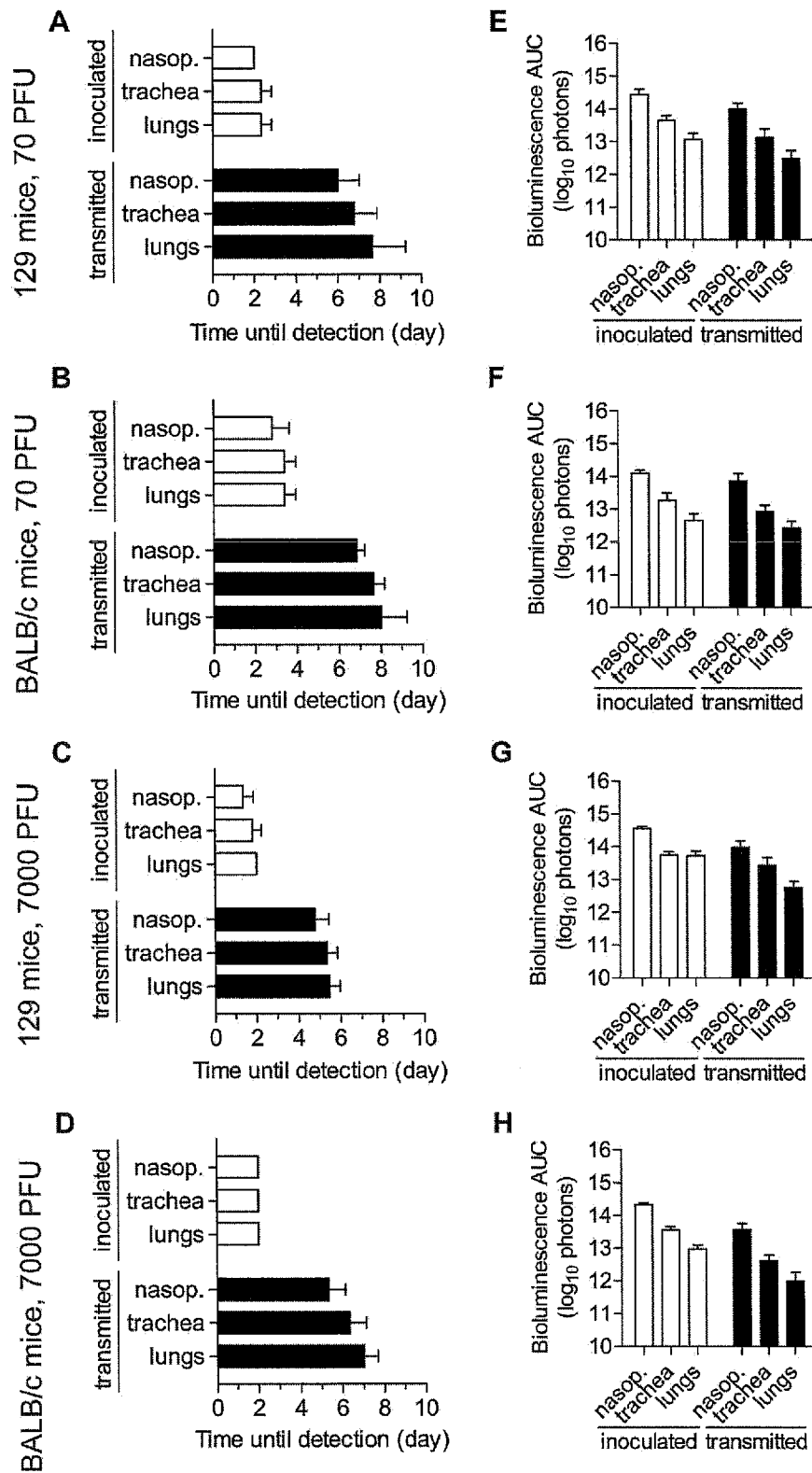


FIG. 14

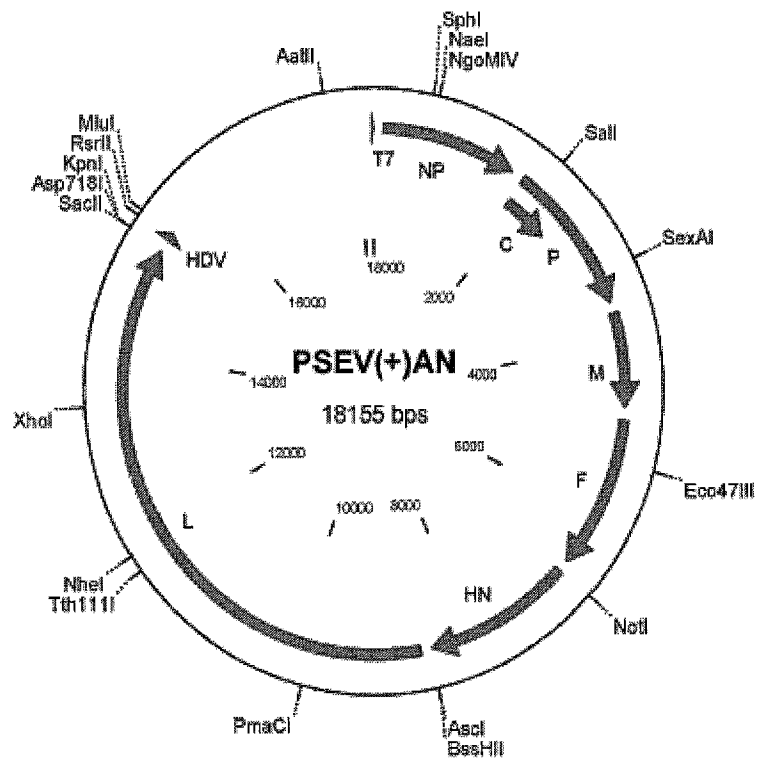
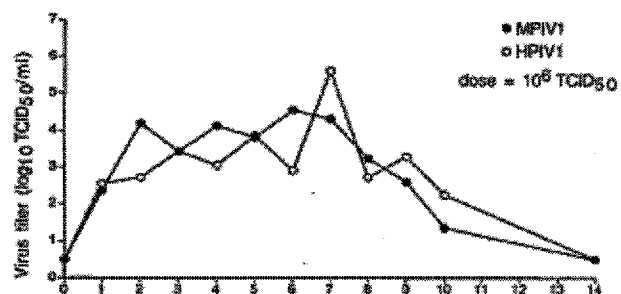


FIG. 15

A
URT



B
LRT

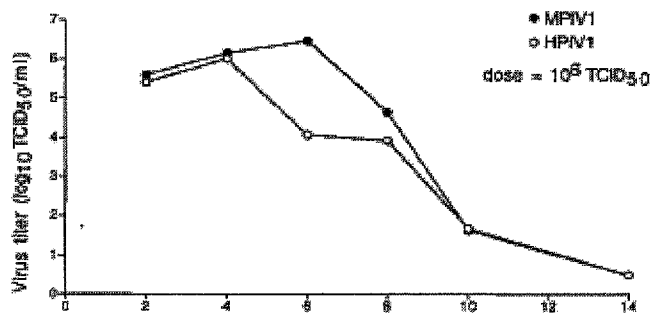


FIG. 16

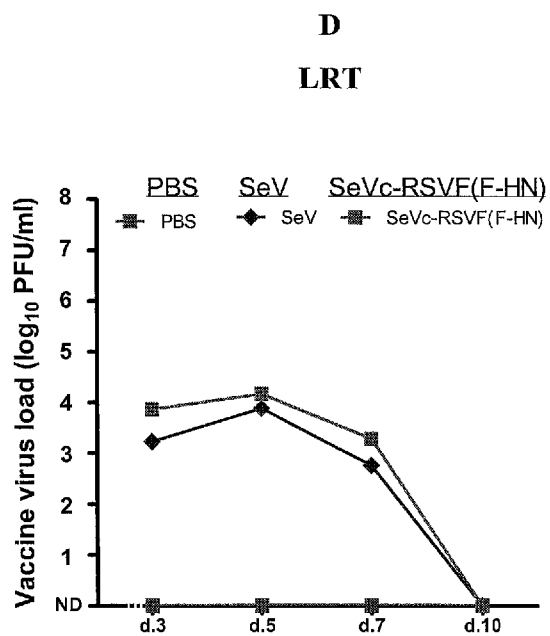
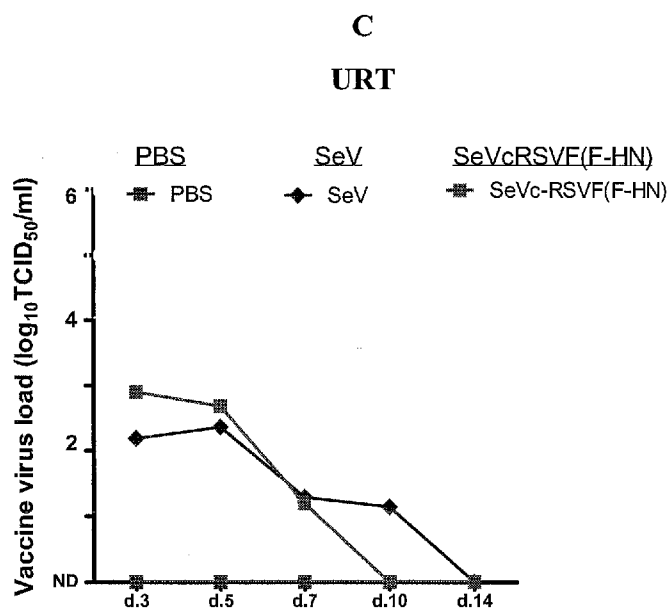


FIG. 16 continued

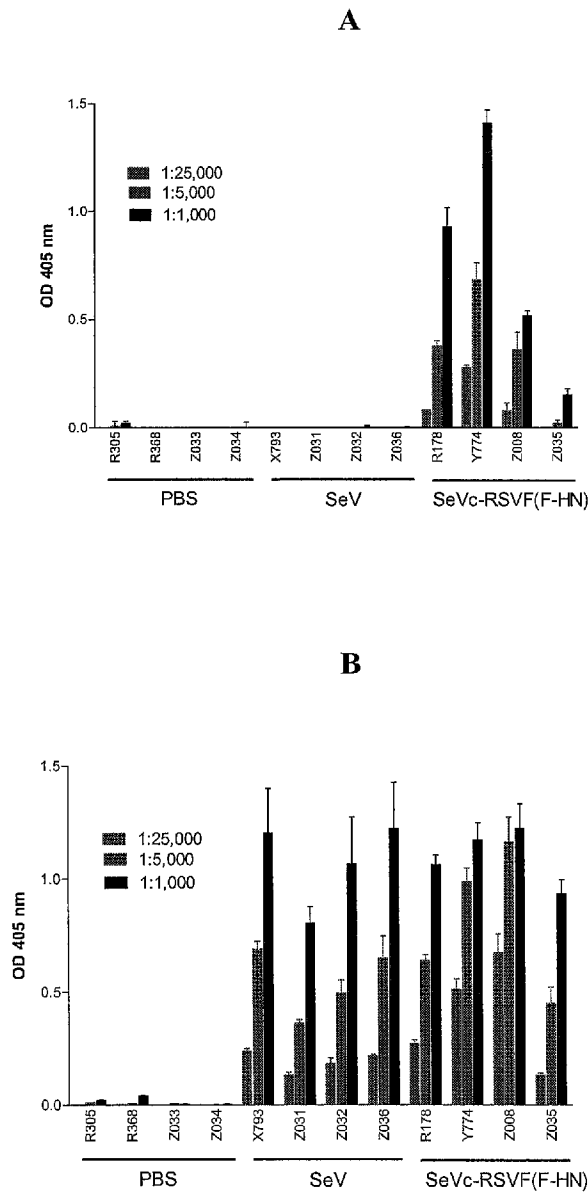


FIG. 17

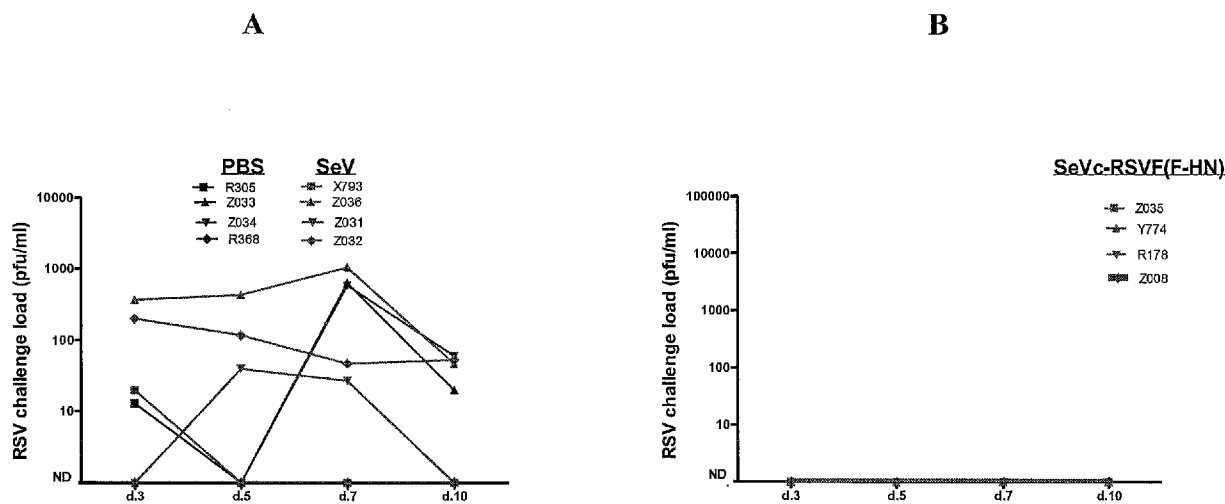


FIG. 18

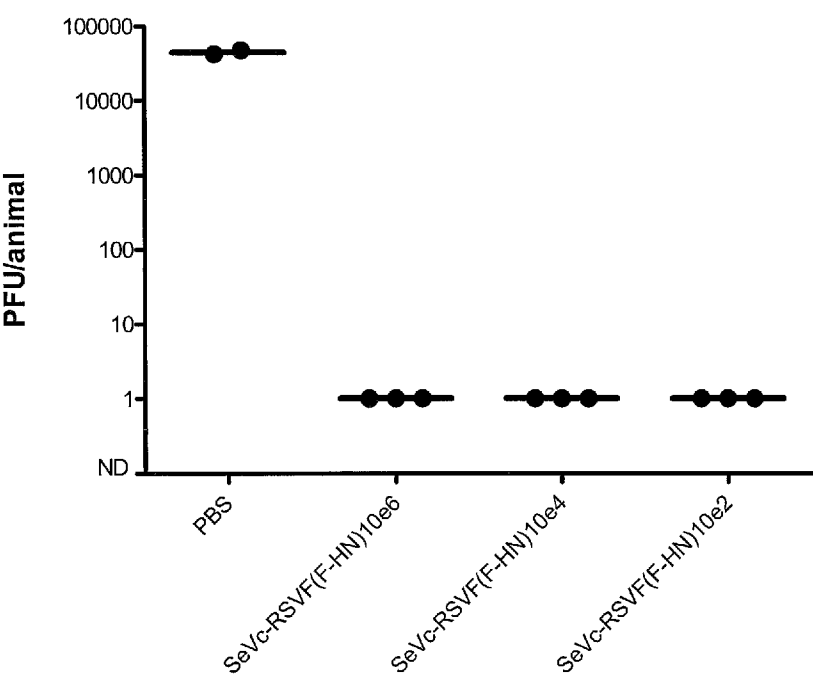


FIG. 19

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 12/33482

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12N 15/86, 7/01; A61K 39/155 (2012.01)

USPC - 435/320.1; 424/211.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8) - C12N 15/86, 7/01; A61K 39/155 (2012.01)

USPC - 435/320.1; 424/211.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

USPC - 424/93.1, 93.2

(Text Search)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PubWEST (PGPB, USPT, USOC, EPAB, JPAB), Google Patents, PubMed and Google Scholar.

Search Terms: modified Sendai Virus, RSV, PIV, PIY, L gene, Z-strain, S155 to G, R258 to K, G466 to E, S581 to R, Q717 to R, T800 to I, R852 to K, N-P, P-M, M-f, F-HN, HN-L, Enders, Sendai, Chimera, mPIV, L protein, L gene, luciferase, GFP, green fluorescent protein

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	CHAMBERS, R. et al. Trafficking of Sendai Virus Nucleocapsids Is Mediated by Intracellular Vesicles. PLoS ONE. 07 June 2010, Vol 5(6), e10994, pages 1-13: fig 1A; abstract; pg 10, col 2, para 1	21 and 24 ----- 1-6, 14, 22-23, 27 and 29 -32
Y	NISHIO, M. et al. Recombinant Sendai viruses with L1618V mutation in their L polymerase protein establish persistent infection, but not temperature sensitivity. Virology. 28 September 2004, Vol 329, pages 289-301: table 1; abstract; pg 290, col 2, para 2	1-6, 14, 22-23, 27 and 29 -32
Y	JONES, B. et al. Human PIV-2 recombinant Sendai virus (rSeV) elicits durable immunity and combines with two additional rSeVs to protect against hPIV-1, hPIV-2, hPIV-3, and RSV. Vaccine. 04 February 2009, Vol 27, pages 1848-1857: abstract; pg 1849, col 1, para 5	3
A	US 2010/0323428 A1 (YOSHIZAKI et al.) 23 December 2010 (23.12.2010) entire document	1-6, 14, 21-24, 27 and 29 -32
A	US 7,704,509 B2 (MURPHY et al.) 27 April 2010 (27.04.2010) entire document	1-6, 14, 21-24, 27 and 29 -32
A	TAKIMOTO, T et al. Recombinant Sendai Virus Expressing the G Glycoprotein of Respiratory Syncytial Virus (RSV) Elicits Immune Protection against RSV. Journal of Virology. June 2004, Vol 78(11), pages 6043-6047, entire document.	1-6, 14, 21-24, 27 and 29 -32

☐ Further documents are listed in the continuation of Box C.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

03 August 2012 (03.08.2012)

Date of mailing of the international search report

25 SEP 2012

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents

P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-3201

Authorized officer:

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PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 12/33482

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 7-13, 15-20, 25-26 and 28
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.