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Engineering Temperature Sensitive Live Attenuated Influenza Vaccines from Emerging Viruses

Bin Zhou^{1,2,3,†}, Yan Li^{1,†}, Scott D. Speer¹, Anju Subba^{1,3}, Xudong Lin^{1,3}, and David E. Wentworth^{1,2,3,*}

¹Wadsworth Center, New York State Department of Health, State University of New York, Albany, New York 12201

²Department of Biomedical Sciences, School of Public Health, State University of New York, Albany, New York 12201

³J. Craig Venter Institute, 9704 Medical Center Drive, Rockville, Maryland 20850

Abstract

The licensed live attenuated influenza A vaccine (LAIV) in the United States is created by making a reassortant containing six internal genes from a cold-adapted master donor strain (*ca* A/AA/6/60) and two surface glycoprotein genes from a circulating/emerging strain (*e.g.*, A/CA/7/09 for the 2009/2010 H1N1 pandemic). Technologies to rapidly create recombinant viruses directly from patient specimens were used to engineer alternative LAIV candidates that have genomes composed entirely of vRNAs from pandemic or seasonal strains. Multiple mutations involved in the temperature-sensitive (*ts*) phenotype of the *ca* A/AA/6/60 master donor strain were introduced into a 2009 H1N1 pandemic strain rA/New York/1682/2009 (rNY1682-WT) to create rNY1682-TS1, and additional mutations identified in other *ts* viruses were added to rNY1682-TS1 to create rNY1682-TS2. Both rNY1682-TS1 and rNY1682-TS2 replicated efficiently at 30°C and 33°C. However, rNY1682-TS1 was partially restricted, and rNY1682-TS2 was completely restricted at 39°C. Additionally, engineering the TS1 or TS2 mutations into a distantly related human seasonal H1N1 influenza A virus also resulted pronounced restriction of replication *in vitro*. Clinical symptoms and virus replication in the lungs of mice showed that although rNY1682-TS2 and the licensed FluMist®-H1N1pdm LAIV that was used to combat the 2009/2010 pandemic were similarly attenuated, the rNY1682-TS2 was more protective upon challenge with a virulent mutant of pandemic H1N1 virus or a heterologous H1N1 (A/PR/8/1934) virus. This study demonstrates that engineering key temperature sensitive mutations (PB1-K391E, D581G, A661T; PB2-P112S, N265S, N556D, Y658H) into the genomes of influenza A viruses attenuates divergent human virus lineages and provides an alternative strategy for the generation of LAIVs.

Keywords

Influenza A virus; live attenuated vaccine; temperature sensitive; RNA polymerase; heterologous protection

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*Corresponding Author: J. Craig Venter Institute 9704 Medical Center Dr. Rockville, MD 20850 Phone: 301-795-7625 Fax: 301-795-7052 dwentworth@JCVI.org.

†Contributed equally to this study

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1. INTRODUCTION

Annual influenza A epidemics infect 250-500 million people, resulting in 3-5 million cases of severe illness and 250,000-500,000 deaths worldwide [1]. Much higher infection rates and case fatality rates have been seen in past pandemics. The notorious “Spanish flu” of 1918-1919 infected one third of the world’s population and caused an estimated 20-100 million deaths [2].

Vaccination is the most important strategy to prevent influenza infections and alleviate the severity in epidemics and pandemics. The majority of influenza vaccines currently administered in the United States are inactivated vaccines, which have proven to be safe and fairly effective over decades of use in humans. Live attenuated influenza vaccines (LAIVs) can elicit IgA mucosal immunity and cellular immunity and are therefore believed by some to be a superior approach [3-8]. There is still controversy regarding LAIVs versus inactivated vaccines, but comparative efficacy studies in humans support the idea that LAIVs have the potential to elicit better protection than the split inactivated vaccines in children [9-15].

Research on LAIVs has a very strong foundation that was formed when Maassab created the cold-adapted (*ca*) temperature-sensitive (*ts*) H2N2 influenza A virus A/Ann Arbor/6/60 (*ca* A/AA/6/60) by *in vitro* passage at successively lower temperatures in the 1960’s [16]. The tremendous efforts of many scientists and clinicians culminated in the licensure of *ca* A/AA/6/60-based vaccines in humans in 2003. These *ca* A/AA/6/60-based LAIVs are marketed as FluMist®, and they are produced by creating reassortant viruses that are typically encoded by six “internal” viral RNA segments (vRNAs) (PB2, PB1, PA, NP, M, and NS) from the master donor strain *ca* A/AA/6/60, plus the two glycoprotein encoding vRNAs (HA and NA) from a virus that antigenically matches the strains predicted to circulate in the upcoming influenza season [17;18]. The six internal vRNAs of *ca* A/AA/6/60 in FluMist® vaccines confer the temperature sensitive (*ts*) phenotype, which restricts virus replication to the cooler regions of the respiratory tract and attenuates the virus. The *ts* phenotype of *ca* A/AA/6/60-based LAIVs is stable in animal models and in clinical studies of virus shed from children [18]. Using classical experimental reassortment techniques and sequence analysis Snyder *et. al.*, [17], Cox *et. al.*, [18], Herlocher *et. al.*, [19], identified the vRNA segments and a few of the specific mutations responsible for the *ts* phenotype of the *ca* A/AA/6/60 master donor virus. Subsequently, Jin *et al.* introduced the mutations into a parental-like strain of A/AA/6/60 (E10SE2) by reverse genetics and showed that five amino acid mutations in three vRNAs (PB1, PB2, and NP) play critical and additive roles in the *ts* phenotype of *ca* A/AA/6/60 [20]. Incorporating these five mutations into the more distantly related A/PR/8/34 virus also resulted in a *ts* phenotype [21].

Since the development of the *ca* A/AA/6/60 based vaccine, Subbarao *et. al.*, [19;20], Parkin *et. al.*, [21], Jin *et. al.*, [22], and others have made many attempts to impart temperature sensitivity to influenza A viruses other than A/AA/6/60 in order to generate alternative live attenuated vaccines. Recently, Perez and colleagues generated experimental LAIVs by insertion of an HA-tag into the PB1 gene of partially attenuated *ts* viruses and successfully protected mice, chickens, and pigs from infection [23-26]. However, all of the aforementioned approaches are intended to create a universal backbone for *ts* LAIVs, instead of aiming to engineer critical attenuating mutations into the genome of contemporary or emerging viruses in order to generate a LAIV directly from pandemic or epidemic strains. Recombinant LAIVs encoded by the entire genomes of contemporary or emerging viruses, which have been engineered to contain attenuating mutations, have important implications for combating seasonal influenza epidemics and intermittent pandemics. For example, this strategy is likely to provide better protection than *ca* A/AA/6/60-based reassortants when

antigenic drift results in a mismatch between the vaccine strain and the re-emerging strain or when a new pandemic reassortant virus emerges. This is because many of the immune epitopes on the eight-to-nine proteins encoded by the six internal vRNAs differ between the contemporary or emerging pandemic viruses and the master donor strain that is currently used to create LAIVs [27-35].

The 2009/10 influenza pandemic was caused by the emergence of a reassortant H1N1 virus (H1N1pdm) that contained vRNAs derived from swine (HA, NP, NA, M, NS), human (PB1), and avian (PB2, PA) lineage viruses, and it is a good illustration of the fact that novel internal vRNAs often accompany changes in the HA or NA. This pandemic virus provided a unique opportunity to test the hypothesis that mutations responsible for temperature sensitivity and attenuation can be rationally engineered into the genome of novel emerging viruses in order to create LAIVs. We used multi-segment reverse transcription polymerase chain reaction (MRT-PCR) and accelerated reverse-genetics technologies [36] to engineer temperature-sensitive attenuating mutations into the swine, human, and avian gene constellation of the H1N1pdm virus. To ensure substantial attenuation and genetic stability of the experimental LAIVs, multiple *ts* mutations identified in several unrelated *ts* viruses (A/AA/6/60, A/Ud/307/72, A/GL/0389/65) [19;37;38] were introduced into the genome of an H1N1pdm virus (A/New York/1682/2009), and the genome of a seasonal H1N1 virus (A/New York/1692/2009). Two experimental H1N1pdm temperature sensitive LAIV (TS-LAIV) candidates (rNY1682-TS1 and rNY1682-TS2) and two seasonal H1N1 candidates (rNY1692-TS1 and rNY1692-TS2) were created. The vaccine potential of the experimental LAIVs were analyzed *in vitro* and *in vivo* and the rNY1682-TS1 and rNY1682-TS2 candidates were compared to the licensed monovalent FluMist®-H1N1pdm LAIV in the mouse model.

2. MATERIALS AND METHODS

2.1. Biosafety and animal care

All experiments with infectious virus were performed using procedures and facilities that met or exceeded the requirements set forth by the U.S. Department of Health and Human Services for propagation of influenza A viruses. *In vitro* experiments with infectious H1N1pdm viruses were conducted using enhanced biosafety level 2 laboratory practices and procedures as described by the Centers for Disease Control and Prevention interim biosafety guidelines. Experiments involving animals were performed in a biosafety level 3 containment laboratory approved for such use by the Centers for Disease Control and Prevention and the U.S. Department of Agriculture.

The animal studies were conducted under approved animal care and use protocols at the Wadsworth Center, NYSDOH. All animal experiments were conducted in compliance with the requirements of federal and state regulatory agencies and used husbandry and procedures to limit discomfort, distress, pain or injury.

2.2. Cell culture

Human embryonic kidney (293T) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Madin-Darby canine kidney (MDCK) cells were maintained in Eagle's minimum essential medium (EMEM), supplemented with 5% FBS.

2.3. Generation and propagation of recombinant viruses

Recombinant wild type H1N1pdm influenza A virus A/New York/1682/2009 (rNY1682) was created by reverse genetics directly from a human swab specimen collected in New

York state in April 2009 [36]. Mutations were introduced into the NY1682 PB1 and PB2 bidirectional reverse-genetics plasmids to generate three mutant plasmids: PB1-Mut3 (pYL6A6), PB2-Mut1 (pYL8A1), PB2-Mut4 (pYL14A11) (Fig. 1A). PB1-Mut3 contains three mutations in PB1 (K391E, D581G, A661T). PB2-Mut1 contains an N265S mutation in PB2. PB2-Mut4 contains four mutations (P112S, N265S, N556D, Y658H). The rNY1682-TS1 candidate was rescued by co-transfection of reverse-genetics plasmids containing the PB1-Mut3 and PB2-Mut1, along with reverse-genetics plasmids containing the wild type PA, HA, NP, NA, M, and NS gene segments of NY1682 into 293T/MDCK co-cultured monolayers as previously described [39]. The rNY1682-TS2 candidate was rescued by co-transfection of reverse-genetics plasmids containing PB1-Mut3, PB2-Mut4, and the six other wild type NY1682 gene segments. Three days post-transfection, supernatants were collected, and viruses were propagated in MDCK cells at 33°C. A similar reverse genetics approach was used to rescue a co-circulating seasonal H1N1 virus rA/NY/1692/2009 (rNY1692) directly from a swab specimen [36]. Bi-directional reverse genetics clones containing dsDNA copies of vRNA segments representing PB2 (pSS2B4), PB1 (pSS5A1), PA (pSS2B8), NP (pSS4A2), NA (pSS1C3), M (pSS1B8), and NS (pSS6A1) were created by M-RT-PCR and In-Fusion cloning as previously described [36]. Clones containing the HA segment of this virus were not recovered using this method, likely because of toxicity. Therefore, the HA gene segment was cloned (pSS10D1) by insertion into a recently developed low copy reverse-genetics plasmid [40]. The TS1 and TS2 mutation constellations described for the H1N1pdm candidates were subsequently engineered into the PB1 and PB2 clones from the seasonal virus to create PB1-Mut3 (pSS7A1), PB2-Mut1 (pSS9A1), and PB2-Mut4 (pSS11A1). The corresponding rNY1692-TS1 and rNY1692-TS2 viruses were rescued in the same way as the rNY1682 vaccine candidates. All of the rescued viruses, were passaged once in MDCK cells to generate seed stocks (P1), which were propagated in confluent MDCK cells in T-150 flasks (10^3 to 10^4 TCID₅₀ inoculated into each flask). When more than 20% of the cells showed CPE, supernatants were collected from the flasks, centrifuged at 4°C for 4,000 rpm X 5 min, and the cleared supernatants were aliquoted, frozen in dry-ice ethanol bath, and stored in -80°C freezer for *in vitro* and *in vivo* studies (working stocks, P2). To be consistent with the reverse-genetics rescued viruses for *in vitro* and *in vivo* comparison, the licensed 2009 live attenuated monovalent H1N1pdm vaccine (FluMist®-H1N1pdm, Medimmune, CA) was also propagated and working stocks (P2) prepared using the same procedure. All virus propagation was done at 33°C. The working stocks of rNY1682 LAIVs and FluMist were sequenced and the presence of engineered mutations and the absence of unwanted mutations were confirmed. The titers of the viruses were determined by 50% tissue culture infectious dose (TCID₅₀) assays in MDCK cells. Viral titers of all working stocks (P2) were between $10^{7.5}$ and 10^8 TCID₅₀/ML and the viruses were diluted in EMEM to equal titers for *in vitro* and *in vivo* studies.

2.4. Mini-genome replication assay

To construct the pPolI-NS-EGFP plasmid (pYL22A5), the EGFP gene, flanked by the NY1682 NS noncoding region, was cloned into the recombination-based influenza reverse-genetics plasmid pG26A12 (modified from pHH21[41]) [36] between the RNA polymerase I promoter and terminator for expression of vRNA-like negative-sense EGFP RNA, which can be replicated and transcribed by the viral RNA polymerase complex. HEK-293T cells in 35 mm tissue culture dishes were co-transfected with 1 µg of each pYL22A5 and plasmids to express the NY1682 PB1 (WT or PB1-Mut3), PB2 (WT, PB2-Mut1, or PB2-Mut4), PA, NP proteins. Forty-eight hours post transfection, EGFP expression was examined using fluorescence microscopy. All results shown are the representative pictures of at least three independent experiments.

The luciferase-mediated mini-genome replication assay was performed similarly. Briefly, HEK-293T cells in 24-well plates were cotransfected with 0.2 µg each of pPolI-NS-Luc plasmid (pBZ81A36) [42] and various combinations of the polymerases and NP plasmids. To control for transfection efficiency, 0.02 µg of the Renilla luciferase plasmid pRL-TK (Promega) was also cotransfected. Forty-eight hours post-transfection, luciferase production was assayed using the dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions. Firefly luciferase expression was normalized to Renilla luciferase expression (relative activity). At each temperature, the relative activity of WT polymerase was set at 100%, and the activities of the mutants were determined relative to that of the WT. All results shown are the averages from triplicate experiments, and the standard deviation is shown.

2.5. Replication kinetics in cell cultures

Confluent monolayers of MDCK cells were infected at an MOI of 0.01 TCID₅₀/cell with the H1N1 pdm (rNY1682, rNY1682-TS1, or rNY1682-TS2) or the seasonal H1N1 (rNY1692, rNY1692-TS1 and rNY1692-TS2) LAIV candidates or the at 30, 33, 37 and 39°C. The viral titers in the inocula were determined to confirm that equal MOI was used for each virus in the inoculation. One hour post-inoculation (hpi), inocula were removed, and cells were washed twice and dishes were refilled with EMEM containing 0.15% BSA fraction V, 3 µg/ml TPCK-trypsin, and 1% antibiotic-antimycotic. Supernatants were collected at 2, 24, 48, 72, and 96 hpi. To compare the rNY1682-TS LAIV to the commercial live attenuated vaccine (FluMist®, H1N1pdm), MDCK cells were infected at an MOI of 10 TCID₅₀/cell. At 1 hpi, supernatants were collected and cells were washed four time and dishes were refilled as above. Supernatants were further collected at 3, 6, 9, 12, 24 hpi. All virus titers were determined by TCID₅₀ assay using MDCK cells.

2.6. Mouse studies

Virus attenuation was studied in 6-week-old female BALB/cJ mice (Jackson Laboratory, Bar Harbor, ME) that had been separated into microisolator cages at 5 weeks of age and allowed to acclimate to the ABSL3 laboratory for 6-8 days prior to the studies. Groups of 6-week-old female BALB/cJ mice were anesthetized with isoflurane and inoculated intranasally with 10⁴ TCID₅₀ of the rNY1682, rNY1682-TS1, rNY1682-TS2 or FluMist®-H1N1pdm LAIV in 50 µl of EMEM diluent, or were mock inoculated with EMEM to serve as controls. Body weights and clinical symptoms of the mice (n=11/group) were monitored daily for 14 days. Three mice in each group were euthanized at 2 and 4 days post inoculation (dpi). Nasal washes were collected by insertion of a 26-gauge needle into the trachea and washing of the nasal turbinates with 1 ml of virus collection medium (VCM: EMEM supplemented with 0.15% BSA fraction V and 1% antibiotic-antimycotic), which was collected from the nostrils. Lungs were homogenized using a 5 mm stainless steel BB in a Tissue Lyser II (Qiagen) in 1 ml of VCM for virus titration.

For vaccination, specific antibody titration, and virus challenge studies, groups of ABSL3 acclimatized 6-week-old female BALB/cJ mice were anesthetized with isoflurane and intranasally inoculated with 10⁴ TCID₅₀ of one of the rNY1682-WT, rNY1682-TS1, rNY1682-TS2, or FluMist®-H1N1pdm viruses in 50 µl of EMEM diluent, or were mock inoculated with EMEM, to serve as controls (n = 11/group). Serum was isolated from blood samples obtained from mouse tail veins at 21 dpi. The levels of HA-specific antibodies present in sera were assessed by Luminex assay in which the beads were conjugated to H1N1pdm HA glycoprotein. Luminex assay is high-throughput and more sensitive than the classical hemagglutinin inhibition (HAI) assay and requires less serum, which is an important factor since only 10 to 50 µl of blood can be collected from mouse tail veins.

At 30 dpi, 11 mice per group were challenged intranasally with 5×10^4 TCID₅₀ (~100 LD₅₀ in 6-week-old mice) of a mouse-adapted variant of NY1682 (A/NY/1682/2009-MAP7) created by serial passage through BALB/cJ mouse lungs [42]. Disease symptoms and weight changes in the vaccinated mice were monitored for 14 days and three mice from each virus group were euthanized at 2 and 4 days post challenge. Lungs were homogenized in 1 ml of VCM and titers were determined by TCID₅₀ assay. In the heterologous protection study, mice were vaccinated similarly but were challenged with 5×10^4 TCID₅₀ of a recombinant reassortant virus rNY1682:PR8-HA/NA, which comprises the 6 internal gene segments from NY1682-MAP7 and the HA, NA gene segments from A/PR/8/1934. Disease symptoms and weight changes in the vaccinated mice were monitored for 14 days and three mice from each virus group were euthanized at 3 and 6 days post challenge. Lungs were homogenized in 1 ml of VCM and titers were determined by TCID₅₀ assay. Both A/NY/1682/2009-MAP7 and rNY1682:PR8-HA/NA cause substantial disease in naïve mice and animals that became moribund or lost greater than 25% of their starting body weight were euthanized for humane reasons.

3. RESULTS

3.1. Generation of experimental H1N1pdm live attenuated influenza vaccines (LAIVs)

We created the recombinant wild type H1N1pdm virus (A/New York/1682/2009 (rNY1682)) directly from an original clinical specimen using M-RT-PCR genomic amplification and recombination-based cloning techniques [36]. LAIV candidate rNY1682-TS1 was designed to incorporate five mutations in the PB1, PB2, and NP genes that were identified as the major determinants responsible for the temperature sensitive phenotype of *ca* A/AA/6/60 [37;43]. These amino acid mutations include three mutations in PB1 (PB1-Mut3: K391E, D581G, A661T) and one mutation in PB2 (PB2-Mut1: N265S) (Fig.1A). No mutation was introduced into the NP vRNA because the NY1682 wild-type virus already contained NP-D34G, which was shown to play a role in the *ts* phenotype of *ca* A/AA/6/60 [37].

Since the temperature sensitive (*ts*) phenotype of the *ca* A/AA/6/60 master donor strain is a polygenic trait and is related to the gene constellation, the four mutations introduced to create rNY1682-TS1 might not be sufficient to achieve an ideal *ts* phenotype in the novel H1N1pdm virus gene constellation. Thus, to enhance attenuation and safety of the TS-LAIV prototype (NY1682-TS1) we also introduced three additional PB2 mutations (P112S, N556D, Y658H), which were previously identified and confirmed as *ts* markers in *ts*A/Ud/307/72 and *ts*A/GL/0389/65 [19;38]. The rNY1682-TS2 LAIV candidate was designed to incorporate these additional PB2 mutations (PB2-Mut4: P112S, N265S, N556D, Y658H) into the rNY1682 genome (Fig. 1A). This panel of engineered viruses consists of rNY1682-WT, rNY1682-TS1 and rNY1682-TS2, and they are all encoded entirely by vRNAs derived from NY1682. The presence of desired mutations in the TS-LAIVs rescued by reverse genetics was confirmed by sequencing (data not shown).

3.2. Influence of the various mutations on the viral RNA polymerase activity at various temperatures

A mini-genome replication assay system was used to determine if the mutations introduced into the PB1 and PB2 genes of NY1682 H1N1pdm confer temperature sensitivity to the viral RNA dependent RNA polymerase (RDRP). A pPolI-NS-EGFP plasmid expressing vRNA-like EGFP RNA under the control of a human PolI promoter was co-transfected with four plasmids expressing PB1 (PB1-WT or PB1-Mut3), PB2 (PB2-WT, PB2-Mut1, or PB2-Mut4), PA and NP proteins into HEK-293T cells and they were incubated at various temperatures. At 48 h post-transfection, EGFP expression in the cells was examined using

fluorescence microscopy. All combinations of the RNA polymerase complex resulted in similar EGFP expression levels at 30°C and 33°C (Fig. 1B). Reduction of EGFP expression was not observed at any temperature when PB2-Mut1 was used in the polymerase complex (Fig. 1B). In contrast, at 37°C, EGFP expression in PB2-Mut4 complex was weaker than that expressed by the PB2-WT or PB2-Mut1 (Fig. 1B). Additionally, the PB1-Mut3/PB2-Mut4 combination showed very little EGFP expression at 37°C (Fig. 1B). At 39°C, PB1-Mut3 alone and the PB1-Mut3/PB2-Mut1 combination were weaker than the PB2-WT, but still visible; whereas, PB2-Mut4 and the PB1-Mut3/PB2-Mut4 combination had no visible EGFP expression (Fig. 1B). To better quantitate the influence of the specific *ts* mutations and the synergistic effects observed in the EGFP expression experiments we analyzed the same combination of wild type and mutant polymerase subunits using a luciferase-mediated mini-genome replication assay (Fig. 1C). This quantitative data is consistent with EGFP expression data and shows that PB2-Mut4 alone causes a 1000-fold reduction in activity at 37°C and 10,000-fold reduction at 39°C (Fig. 1C). Furthermore, the PB1-Mut3/PB2-Mut4 combination acts synergistically resulting in 10,000-fold reduction at 37°C (Fig. 1C). Collectively the mini-genome data demonstrate that the PB1-Mut3 and PB2-Mut4 each demonstrated *ts* phenotypes in the context of the other WT RDRP subunits (PB2-Mut4 > PB1-Mut3) and combining the PB1-Mut3 and PB2-Mut4 resulted in a synergistic effect at 37°C, and 39°C (Fig. 1B, and 1C).

3.3. The replication of H1N1pdm rNY1682 TS-LAIVs was restricted *in vitro*

To test if the temperature sensitive polymerases can result in impaired growth of the viruses at restrictive temperatures, we rescued recombinant viruses to analyze the *ts* effects of these mutations on a complete viral infection. The replication kinetics of rNY1682-TS1 and rNY1682-TS2 were compared to the kinetics of rNY1682-WT using Madin-Darby canine kidney (MDCK) cell cultures at various temperatures (Fig. 2). At lower temperatures (30°C and 33°C), all of the viruses replicated with similar kinetics and reached uniformly high titers ($\sim 10^8$ TCID₅₀/ml), which is important for the development of vaccine candidates (Fig. 2A, B). At 37°C, rNY1682-WT and rNY1682-TS1 replicated with similar kinetics while rNY1682-TS2 displayed slower replication kinetics. Nevertheless, all of the viruses eventually reached fairly high titers ($\sim 10^{7.5}$ TCID₅₀/ml) (Fig. 2C). Although rNY1682-WT replicated efficiently at 39°C (10^8 TCID₅₀/ml), rNY1682-TS1 was restricted and required 4 days to reach a peak titer of 10^5 TCID₅₀/ml (Fig. 2D). Furthermore, rNY1682-TS2 was not detectable at any of the time points collected at 39°C (Fig. 2D), indicating that the additional mutations (P112S, N556D, Y658H) engineered into the PB2 gene of rNY1682-TS2 conferred an enhanced *ts* phenotype and restricted the virus completely.

To compare the replication kinetics of the rNY1682 LAIVs to the licensed live attenuated monovalent H1N1pdm vaccine (FluMist®-H1N1pdm, Medimmune, CA), we infected MDCK cells with high MOI of each virus and the viral titers were determined at various time points. The replication of both the rNY1682-TS2 and FluMist®-H1N1pdm were partially restricted at 37°C (Fig. 2E) and completely restricted at 39°C (Fig. 2F). No statistically significant difference between these two viruses was observed at any time points compared.

3.4. The TS1 and TS2 constellation of mutations also restricted a human seasonal H1N1 virus

To demonstrate that combining critical substitutions responsible for the *ts* phenotypes of *ca* A/AA/6/60, A/Ud/307/72, A/GL/0389/65 would also restrict other human virus lineages we used the same strategy to generate LAIVs from a seasonal H1N1 influenza A virus that co-circulated with the H1N1pdm virus. An infectious clone set was created directly from clinical specimen using M-RT-PCR and recombination-based cloning technologies, and the

TS1 mutations (PB1-K391E, D581G, A661T, and PB2-N265S) or TS2 mutations (PB1-K391E, D581G, A661T, and PB2-P112S, N265S, N556D, Y658H) were introduced into this seasonal H1N1 influenza A strain (A/New York/1692/2009 [NY1692]), creating LAIV candidates rNY1692-TS1 and rNY1692-TS2. Both rNY1692-TS1 and rNY1692-TS2 showed replication kinetics similar to the wild-type rNY1692 virus at lower temperatures (30°C and 33°C) (Fig. 3A and B). However, in contrast to the H1N1pdm TS-LAIVs, the rNY1692-TS2 was severely restricted at 37°C and both rNY1692-TS1 and rNY1692-TS2 were completely restricted at 39°C (Fig. 3C and D). These results illustrate that to some extent, the temperature sensitivity rendered by the mutations depends on the different virus strains, but that the TS2 mutations are sufficient to thoroughly restrict both the pandemic and seasonal strains at higher temperatures (*e.g.*, 39°C).

3.5. H1N1pdm rNY1682-TS2 LAIV candidate was attenuated in mice

Because the seasonal influenza virus is less pathogenic than the H1N1pdm virus in mice [44] and the seasonal H1N1 TS-LAIVs are more restricted than the corresponding H1N1pdm TS-LAIVs *in vitro*, only the H1N1pdm TS-LAIVs were examined *in vivo*. A licensed live attenuated monovalent H1N1pdm vaccine (FluMist®-H1N1pdm, Medimmune, CA) was also included in these experiments for comparison. For consistency, we propagated the original FluMist®-H1N1pdm vaccine stock in MDCK cells using the same conditions used for the generation of the rNY1682-TS1 and rNY1682-TS2 working stocks. Six-week-old female BALB/cJ mice were inoculated intranasally with 10⁴ TCID₅₀ of the rNY1682-WT, rNY1682-TS1, rNY1682-TS2, or FluMist®-H1N1pdm viruses, or were mock-inoculated. Clinical signs of disease, such as ruffled fur, were observed in mice inoculated with rNY1682-WT virus as early as 2 days post-inoculation (dpi), but were not observed in the mice inoculated with rNY1682-TS1, rNY1682-TS2, or FluMist®-H1N1pdm (data not shown). Weight loss became evident in rNY1682-WT inoculated mice at 3 dpi and they did not recover until 10-11 dpi (Fig. 4A). In contrast, mice inoculated with rNY1682-TS2 and FluMist® LAIVs continued to gain weight at the same rate as the mock-inoculated mice (Fig. 4A). Statistical analysis showed that the weight loss caused by rNY1682-WT virus was statistically different from rNY1682-TS2 from 4-10 dpi ($p < 0.001$, ANOVA analysis). Interestingly, mice inoculated with the rNY1682-TS1 virus experienced slight and transient weight loss (2~3%) at 7-8 dpi, which was significantly different from rNY1682-TS2 inoculated mice ($p < 0.001$, ANOVA analysis). However, statistical analysis showed that the weight loss caused by rNY1682-TS1 was significantly less than that caused by rNY1682-WT from 4-9 dpi ($p < 0.001$, ANOVA analysis).

Average viral titers in the lungs of rNY1682-TS2 and FluMist®-H1N1pdm infected mice were approximately 5-6 log₁₀ lower than those of rNY1682-WT infected mice at 2 and 4 dpi (Fig. 4B). Whereas, the titers of rNY1682-TS1 in the lungs of infected mice were similar to that of rNY1682-WT infected mice, indicating that rNY1682-TS1 was only modestly attenuated *in vivo*. Although the viral titers in the upper respiratory tracts of mice showed similar LAIV-dependent trends, the effects of the *ts* mutations were less dramatic. The average titer of rNY1682-TS1 in nasal washes was approximately 1 log₁₀ lower than rNY1682-WT, whereas the rNY1682-TS2 and FluMist®-H1N1pdm showed 2-3 log₁₀ reductions in titer compared to rNY1682-WT (Fig. 4C). Nevertheless, both the rNY1682-TS2 and FluMist®-H1N1pdm replicated more efficiently in the upper respiratory tracts than in the lower respiratory tracts, which was an objective of this approach because replication at the cooler temperatures within the respiratory tract will promote a strong adaptive immune response.

To assess the stability of the *ts* phenotype of the rNY1682-TS2, viruses recovered from mouse lungs and nasal washes at 4 dpi (mice in Fig. 4B and C) were examined and found to maintain a *ts* phenotype *in vitro* (data not shown). Furthermore, sequence analysis of the

PB1, PB2 and PA genes of these viruses showed that the *ts* mutations were present, and that there were no potential compensatory mutations at a second site within these vRNAs.

3.6. The H1N1pdm rNY1682-TS2 LAIV candidate protected mice from lethal homologous challenge

Mice were immunized by intranasal inoculation with 10^4 TCID₅₀ of the rNY1682-WT, rNY1682-TS1, rNY1682-TS2, FluMist®-H1N1pdm, or were mock immunized. Luminex assays demonstrated that the rNY1682-WT virus infection elicited the highest level of antibodies to HA (6400 Units), followed by rNY1682-TS1 (4200 Units), rNY1682-TS2 (2000 Units) and FluMist®-H1N1pdm (800 Units) at 21 dpi (Fig. 5A). The reason that rNY1682-TS2 elicited higher antibody titer than FluMist®-H1N1pdm did may be that the former replicated to higher titers in the cooler part of the respiratory tract than the latter did (Fig. 4C).

The immunized mice were challenged with 100 MLD₅₀ of a mouse-adapted variant of NY1682 (NY1682-MAP7) [42] at 30 dpi. The challenge virus NY1682-MAP7 was derived from rNY1682-WT and the HA used in the FluMist®-H1N1pdm vaccine was derived from A/CA/07/09. To identify amino acid differences between the two viruses and determine if they influence antigenicity we sequenced the virus stocks and performed hemagglutinin inhibition assays. The nucleotide sequence of the virus stocks used in these experiments showed that the proteins were 99% identical (560/566). There are six amino acid differences (S100P, K136E, A203D, D239G, D293N, and V338I, amino acid positions are based on the use of the start codon and include the signal peptide) between the HA's of rNY1682-viruses and the MDCK propagated FluMist®-H1N1pdm LAIV. Hemagglutinin inhibition assays performed with H1N1pdm (rNY1682) antisera showed viruses possessing either rNY1682 HA or FluMist HA had the same hemagglutinin inhibition titer (data not shown). This illustrates that these two viruses show equivalent cross neutralization and is consistent with previous reports indicating that these substitutions don't influence antigenicity of H1N1pdm viruses [45-47].

Immunization with rNY1682-TS1 and rNY1682-TS2 or prior infection by the rNY1682-WT virus protected the mice from the lethal challenge. No disease symptoms were observed in the mice immunized with the rNY1682-TS1 or rNY1682-TS2 LAIV candidates or in control mice infected with the rNY1682-WT. In contrast, disease symptoms, including ruffled fur, hunched posture, and weight loss, were observed in the mock-immunized mice as early as 2 days post challenge (dpc); the symptoms progressed to severe disease, and the animals showed statistically significant weight loss ($p < 0.001$, ANOVA analysis) compared to the mice immunized by the TS-LAIVs or by infection with rNY1682-WT (Fig. 5B). The mock-immunized mice became moribund, and succumbed to infection by 5-6 dpc. Interestingly, the FluMist®-H1N1pdm immunized mice experienced intermediate weight loss after lethal challenge, which was significant compared to the mice immunized with rNY1682-TS1, rNY1682-TS2, or by rNY1682-WT infection from 2-6 dpc ($p < 0.01$, ANOVA analysis). The differences in the protection generated by the various vaccine candidates were more obvious when viral titers in the mouse lungs post challenge were determined. Compared to the high titers of virus found in the lungs of mock-immunized mice at 2 dpc (1×10^8 TCID₅₀/ml) and at 4 dpc (3×10^7 TCID₅₀/ml), average viral titers in the FluMist®-immunized mice were reduced by approximately 2 log₁₀ at 2 dpc (3×10^6 TCID₅₀/ml) and at 4 dpc (1×10^5 TCID₅₀/ml) (Fig. 5C). The rNY1682-TS2-immunized mice showed better protection from lung replication after lethal challenge than those immunized by the FluMist®-H1N1pdm vaccine. At 2 dpc, the viral titer was 2×10^5 TCID₅₀/ml in one mouse and close to the limit of detection (3×10^1 TCID₅₀/ml) in the other mice immunized with rNY1682-TS2. Furthermore, the challenge virus was not detectable in any of the rNY1682-TS2 immunized mice at 4 dpc (Fig. 5C). Finally, the rNY1682-TS1-immunized mice

showed protection that was nearly identical to WT infection (Fig. 5C). These results demonstrate that both the rNY1682-TS1 and rNY1682-TS2 LAIV candidates induced strong immunity to H1N1pdm viruses, and that they provided better protection than the currently licensed live attenuated H1N1pdm vaccine under these experimental conditions.

3.7. The H1N1pdm rNY1682-TS2 LAIV candidate provided stronger heterologous protection than FluMist®-H1N1pdm

We used a heterologous challenge study to further compare the highly attenuated rNY1682-TS2 LAIV with the licensed FluMist®-H1N1pdm vaccine. We used reverse genetics to create a lethal reassortant challenge virus (rNY1682:PR8-HA/NA) that contained 6 gene segments (PB2, PB1, PA, NP, M, NS) from rNY1682-MAP7 and the HA and NA from an antigenically distinct H1N1 virus (A/Puerto Rico/8/1934) isolated in 1934. Briefly, mice were immunized with 10^4 TCID₅₀ of rNY1682-TS2, FluMist®-H1N1pdm, or were mock immunized. At 30 dpi, the immunized mice were challenged with 5×10^4 TCID₅₀ of rNY1682:PR8-HA/NA. Mice were monitored for clinical disease for 14 days and lungs were collected at 3 dpc and 6 dpc. Although the rNY1682-TS2 immunized mice showed weight loss (maximum 16% at 5 dpi), they survived the normally lethal challenge. In contrast the FluMist®-H1N1pdm and mock immunized mice showed more dramatic weight loss and had to be euthanized for humane reasons 5-7 dpc (Fig. 6A). However, unlike the homologous challenge experiments, the viral titers in the lungs of mice immunized with FluMist®-H1N1pdm were similar to that of the rNY1682-TS2 or mock immunized groups at 3 dpc ($P > 0.05$, ANOVA analysis) (Fig. 6B). This indicates that antibodies to the HA and NA induced from immunization didn't significantly inhibit the initial replication of the antigenically unrelated challenge virus and suggests that the difference in disease symptoms between rNY1682-TS2 and FluMist®-H1N1pdm immunized mice (Fig. 6A) results from enhanced T cell mediated immunity.

4. DISCUSSION

This study demonstrates that introducing seven amino acid mutations into the novel genome of the H1N1pdm virus created an effective LAIV (rNY1682-TS2). *In vitro*, rNY1682-TS2 replicated efficiently at lower temperatures but was extremely restricted at higher temperatures. *In vivo*, rNY1682-TS2 and FluMist®-H1N1pdm showed similar levels of attenuation; nevertheless, rNY1682-TS2 elicited better protection from virus replication and disease when mice were challenged with either a lethal homologous variant of NY1682 (H1N1pdm), or a heterologous virus (rNY1682:PR8-HA/NA). In contrast to the FluMist®-H1N1pdm vaccine, of which all 6 internal segments are from *ca* A/AA/6/60 (H2N2), the rNY1682-TS2 vaccine candidate comprises 6 internal genes from a 2009 H1N1 pandemic virus and thus may induce a more effective T cell response to the contemporary challenge viruses, which contain internal protein expressing gene segments from the pandemic virus.

Previously, it was shown that five *ts* mutations from *ca* A/AA/6/60 (PB1-K391E, D581G, A661T; PB2-N265S, and NP-D34G) were sufficient to impart strong temperature sensitivity to A/PR/8/34 [22]. This study shows that engineering the critical *ca* A/AA/6/60 mutations into the seasonal H1N1 genome restricted virus replication *in vitro*, but that these same mutations only partially restricted the H1N1pdm virus (rNY1682-TS1) *in vitro* and poorly attenuated it *in vivo*. These results are consistent with a recent study showing that introduction of the critical *ca* A/AA/6/60 mutations into the genome of a North American triple reassortant swine H3N2 virus only partially attenuated the virus [25] and illustrates that emerging reassortant viruses may require additional mutations to ensure satisfactory attenuation. The results of the TS1 LAIVs also suggest that N265S does not have the same impact on avian lineage PB2's as it does on human lineage PB2's. So we also generated the rNY1682-TS2 LAIV candidate, in which the three mutations (K391E, D581G, A661T) in

the PB1 gene were based upon the *ca* A/AA/6/60 master donor strain and the four mutations in the PB2 gene were from *ca* A/AA/6/60 (N265S), *ts* A/Ud/307/72 (P112S, N556D) and a reassortant *ts* virus containing an A/GL/0389/65 PB2 (Y658H) [38;43]. The three additional *ts* mutations (P112S, N556D, and Y658H) transferred from the other two *ts* mutant viruses played an important role in further attenuating the H1N1pdm virus. Whether or not all eight *ts* mutations (PB1-K391E, D581G, A661T; PB2-P112S, N265S, N556D, Y658H; NP-D34G) are required to achieve the degree of attenuation of rNY1682-TS2 remains to be determined. Nevertheless, based on the strong replication restriction of the H1N1pdm TS2 virus (Fig. 2) and the seasonal H1N1 TS2 virus (Fig. 3) at higher temperatures, we speculate that engineering these eight *ts* mutations into the PB1, PB2, and NP genes is likely to attenuate most influenza A virus strains to a sufficient degree. Furthermore, since both the pandemic and seasonal TS2-LAIVs still replicated to comparable titers as their WT counterparts at lower temperatures (Fig. 2A, B and Fig. 3A, B), over-attenuation is unlikely to happen when TS2-based LAIVs are administered intranasally, where the temperature is much lower than the body core temperature.

A major concern of the TS-LAIV approach is the genetic stability of the attenuation phenotype. Our examination of the rNY1682-TS2 virus recovered from mouse lungs and nasal washes at 4 dpi illustrated that the viruses present in the tissues maintained the *ts* phenotype, and we did not identify any reverting or compensatory mutations in the PB1, PB2, or PA, supporting the idea that introducing a sufficient number of mutations into multiple vRNAs impairs the TSAIV's ability to revert. However, extensive testing of the genetic/phenotypic stability of the rNY1682-TS2 LAIV is required to show that these mutations will be as stable as the mutations in *ca* A/AA/6/60-based FluMist® vaccines. It is possible that prolonged replication of the virus at gradually elevated temperatures, or in immune compromised animals, may generate some revertants that have lost the attenuating phenotype [21;48-51]. Considering that the licensed vaccine FluMist® has many point mutations in multiple vRNAs that both attenuate the virus and enhance its safety by inhibiting the rapid generation of revertants [43;52;53], additional *ts* or *non-ts* mutations could be introduced into the genome of emerging viruses (e.g., NY1682 (H1N1pdm)) to further enhance their safety. For instance, *ts* mutations can also be incorporated into other vRNAs such as the M and NS [54-56]. Furthermore, cold-adaptive mutations and other mutations that enhance LAIV replication at cooler temperatures should be identified and introduced into the rNY1682-TS2 mutant, which are likely to stabilize the *ts* phenotype and will increase yield [47;52;53;57-59]. However, a practical vaccine must have a reasonable balance between attenuation and immunogenicity.

This study demonstrates that we can engineer previously defined mutations into emerging pandemic virus in order to rationally create a temperature-sensitive LAIV that is more effective than a licensed LAIV in mice. The promising results obtained using this approach indicate that future studies to fully understand the correlates of protection induced by these engineered vaccine prototypes (e.g., rNY1682-TS2) and to analyze/improve their safety and efficacy in other animal models are warranted.

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ABBREVIATIONS

ca	cold adapted
ts	temperature sensitive
LAIV	live attenuated influenza vaccines

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Highlights

- Attenuating mutations engineered into multiple vRNAs of pandemic influenza viruses
- Critical mutations from three *ts* viruses required to attenuate 2009 pandemic H1N1
- Recombinant LAIV candidate genome replication restricted at elevated temperatures
- Rationally designed LAIV more effective in mice than 6:2 reassortant currently used

Fig. 1. Engineering TS-LAIV candidates and effects of temperature on the activity of viral RNA polymerase complex with various PB1 and PB2 gene segments

(A) Schematic diagram of PB1 and PB2 proteins depicting the putative attenuating temperature sensitive mutations designed to create TS-LAIV candidates. TS-1 candidates contain a PB1 with three mutations (PB1-Mut3) and a PB2 with one mutation (PB2-Mut1); TS2 candidates contain a PB1 with three mutations (PB1-Mut3) and a PB2 with four mutations (PB2-Mut4). (B) Viral polymerase activity was analyzed at various temperatures. HEK-293T cells were transfected with pPolI-NS-EGFP plasmid that expresses vRNA-like EGFP RNAs, which can be transcribed by the viral RNA polymerase. The HEK-293T cells were also co-transfected with plasmids expressing the NY1682 PB1 (WT or PB1-Mut3), PB2 (WT, PB2-Mut1, or PB2-Mut4), PA and NP, to generate different viral RNA polymerase complexes. Transfected cells were incubated at 30°C, 33°C, 37°C, and 39°C. EGFP expression was examined using fluorescence microscopy at 48 h post-transfection. The name of the mutant subunit (shown on the top of the panel) in the polymerase complex is used to represent the entire polymerase complex. Representative pictures of at least three independent experiments are shown. (C) Similar to (B) but pPolI-NS-EGFP plasmid was replaced by the pPolI-NS-Luc plasmid that expresses negative-sense virus-like RNA encoding a destabilized firefly luciferase enzyme that can be transcribed by the viral RDRP. Cells were also cotransfected with a Renilla luciferase expression plasmid to control for transfection efficiency. Forty-eight hours posttransfection, both firefly and Renilla luciferase production levels were measured, and Renilla expression was used to normalize the data. At each temperature, the firefly/Renilla relative activity of WT polymerase was set as 100%,

and the relative activity of mutant polymerases was expressed of percentage of that. The averages of triplicate experiments are shown, with error bars that represent SD.

Fig. 2. Replication of H1N1pdm rNY1682-WT, rNY1682-TS1, rNY1682-TS2 viruses, and the FluMist®-H1N1pdm vaccine at various temperatures
MDCK cells were inoculated with an MOI of 0.01 TCID₅₀/cell and incubated at 30° 50 C (A), 33°C (B), 37°C (C), and 39°C (D), and culture supernatants were collected at 2, 24, 48, 72, and 96 hpi. To compare the rNY1682-TS2 to the replication of FluMist®-H1N1pdm LAIV, MDCK cells were inoculated with an MOI of 10 TCID₅₀/cell and incubated at 37° 50 C (E), and 39°C (F). Culture supernatants were collected at 1, 3, 6, 9, 12, and 24 hpi. Note, the 1 hpi supernatants were collected prior to removal and washing the inocula from the cell monolayers. Viral titers were determined by TCID₅₀ assay using MDCK cells and the limit of detection (LOD) of these assays is indicated by the dotted line. Error bars represent the standard deviation. In (A), (B), (C), and (D), “*” indicates viral titer of rNY1682-TS2 is lower than that of rNY1682-WT ($P < 0.001$, Bonferroni post-test of ANOVA analysis). In (E) and (F), viral titer of rNY1682-TS2 was not statistically different from that of FluMist®-H1N1pdm vaccine.

Fig. 3. Replication of seasonal H1N1 rNY1692-WT, rNY1692-TS1, and rNY1692-TS2 viruses at various temperatures

MDCK cells were inoculated with an MOI of 0.01 TCID₅₀/cell and incubated at 30°C (A), 33°C (B), 37°C (C), and 39°C (D). Culture supernatants were collected at 2, 24, 48, 72, and 96 hpi and viral titers were determined by TCID₅₀ assay using MDCK cells. *, viral titer of rNY1682-TS2 is lower than that of rNY1682-WT ($P < 0.001$, Bonferroni post-test of ANOVA analysis). The limit of detection (LOD) of these assays is indicated by the dotted line.

Fig. 4. Replication of rNY1682-WT, rNY1682-TS1, rNY1682-TS2 and FluMist®-H1N1pdm in the respiratory tracts of mice

Six-week-old female BALB/cJ mice were inoculated intranasally with 10^4 TCID₅₀ of rNY1682-WT (**WT**), rNY1682-TS1 (**TS1**), rNY1682-TS2 (**TS2**), FluMist®-H1N1pdm (**FluMist®**), or were mock inoculated. **(A)** Body weight of inoculated mice ($n = 11$ /group) was recorded daily and is represented as the percent of each animals weight on the day of inoculation (day 0). *, WT caused significant weight loss compared to mock infected mice ($P < 0.01$, Bonferroni post-test of ANOVA analysis). **(B)** Viral titers in the lung homogenates of inoculated mice ($n = 3$ /group) were determined for each mouse by TCID₅₀ assay at 2 and 4 dpi. **(C)** Viral titers from nasal airway of inoculated mice ($n = 3$ /group). The average of each group is shown with error bars determined by standard deviation (A, B, C). Data were analyzed by ANOVA analysis and selected P values are shown for specific comparisons. The limit of detection for virus titration is indicated by the dotted line (B, C).

Fig. 5. TS-LAIV candidates protect mice from lethal homologous infection

Groups of mice ($n = 11/\text{group}$) were immunized by intranasal inoculation with rNY1682-WT (**WT**), rNY1682-TS1 (**TS1**), rNY1682-TS2 (**TS2**), FluMist®-H1N1pdm (**FluMist®**), or inoculation media alone (**Mock**). (**A**) H1N1pdm viral HA-specific antibody levels in sera collected at 21dpi were determined by Luminex assay. (**B**) Mice were challenged intranasally, at 30 dpi, with 5×10^4 TCID₅₀ (~100 MLD₅₀) of a lethal mouse-adapted variant of NY1682, and their weight was monitored for 14 days post-challenge, error bars represent $\pm$ SD (days 1-4, $n=8$; days 5-14, $n=5$). All mice in the mock-immunized group were euthanized for humane reasons by day 5. *, Challenge in FluMist® immunized mice caused significant weight loss compared to that of the TS1, TS2, and WT immunized mice. ($P < 0.01$, Bonferroni post-test of ANOVA analysis). (**C**) Viral titers in the lungs were determined at 2 and 4 days post challenge. Data were analyzed by ANOVA analysis and selected P value is shown to compare the TS2 and FluMist® immunized mice at 2 dpc. Each dot represents the antibody titer (A) or virus titer (C) of an individual mouse, and horizontal lines indicate the average of the group. Mock indicates animals that were mock immunized and subsequently challenged. The limit of detection of the assays is indicated by the dotted line.

A. Morbidity **B. Lung** **Fig. 6. TS2-LAIV candidate protects mice from heterologous challenge**

Groups of mice were immunized by intranasal inoculation with rNY1682-TS2 (**TS2**), FluMist®-H1N1pdm (**FluMist®**), or diluent alone (**Mock**). (**A**) Mice were challenged intranasally, at 30 dpi, with 5×10^4 TCID₅₀ of a lethal reassortant virus that comprises the internal protein expressing gene segments from NY1682 and the HA and NA glycoprotein gene segments from A/PR/8/1934. The weight of the mice was monitored for 14 days post-challenge and it is displayed as the percentage of the animals starting weight. Error bars represent $\pm$ the standard deviation. *, Challenge of FluMist® immunized mice caused significant weight loss compared to that TS2 immunized mice. ($P < 0.01$, Bonferroni post-test of ANOVA analysis). (**B**) Viral titers in the lungs of the mice were determined at 3 and 6 days post challenge. No significant differences were observed between the TS2 and FluMist® groups (ANOVA analysis). Each dot represents the virus titer of an individual mouse, and horizontal lines indicate the average of the group. Mock indicates animals that were mock immunized and subsequently challenged. The limit of detection is indicated by the dotted horizontal line.