Published in final edited form as:

Vaccine. 2015 August 26; 33(36): 4495–4504. doi:10.1016/j.vaccine.2015.07.023.

Restricted Replication of the Live Attenuated Influenza A Virus Vaccine during Infection of Primary Differentiated Human Nasal Epithelial Cells

William A. Fischer II^{a,e}, Landon S. King^a, Andrew P. Lane^b, and Andrew Pekosz^{c,*}

^aDepartment of Medicine, Division of Pulmonary and Critical Care Medicine, The Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA

^bDepartment of Otolaryngology-Head and Neck Surgery, Center for Sensory Biology, and Department of Molecular Biology and Genetics, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, USA

^cW. Harry Feinstone Department of Molecular Microbiology and Immunology, The Johns Hopkins University, Bloomberg School of Public Health, 615 North Wolfe St Suite 5132, Baltimore MD 21205

Abstract

Live Attenuated Influenza Vaccine (LAIV) strains are associated with cold adapted, temperature sensitive and attenuated phenotypes that have been studied in non-human or immortalized cell cultures as well as in animal models. Using a primary, differentiated human nasal epithelial cell (hNEC) culture system we compared the replication kinetics, levels of cell-associated viral proteins and virus particle release during infection with LAIV or the corresponding wild type (WT) influenza viruses. At both 33°C and 37°C, seasonal influenza virus and an antigenically matched LAIV replicated to similar titers in MDCK cells but seasonal influenza virus replicated to higher titers than LAIV in hNEC cultures, suggesting a greater restriction of LAIV replication in hNEC cultures. Despite the disparity in infectious virus production, the supernatants from H1N1 and LAIV infected hNEC cultures had equivalent amounts of viral proteins and hemagglutination titers, suggesting the formation of non-infectious virus particles by LAIV in hNEC cultures.

Graphical abstract

*Corresponding author contact information: apekosz1@jhu.edu; Tel: 410-502-9306; Fax: 410-955-0105.

^ecurrent address: CEMALB EPA Building Room 543, 104 Mason Farm Road, CB#7310, Chapel Hill, NC 27599-7310
william_fischer@med.unc.edu , Tel: (919) 966-2531 ext 237

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

Nasal epithelial cells; live attenuated influenza vaccine; influenza

1. Introduction

Influenza viruses are global pathogens that infect approximately 10% of the world's population each year causing an excess in morbidity and mortality (1). Annual immunization with inactivated or live attenuated vaccines remains the primary defense against seasonal influenza-related morbidity and mortality. Live Attenuated Influenza Vaccine (LAIV) strains are nasal vaccines that elicit a robust mucosal innate immune response without causing clinical disease. The original vaccines were generated by passaging a seasonal strain of influenza (H2N2, A/Ann Arbor/6/60 or A/Leningrad/134/17/57) at progressively lower temperatures (2, 3). This process selected for virus strains that had acquired multiple mutations in six gene segments conferring three phenotypes: cold adaptation (*ca*; able to replicate at 25°C *in vitro*), temperature sensitivity (*ts*; reduced replication at temperatures greater than 38°C *in vitro*), and attenuation (*att*; reduced ability to cause disease in animal models or humans) (3, 4). Seasonal influenza A vaccines are generated by creating reassortant viruses containing the HA and NA segments from circulating influenza strains and the other 6 segments from the master donor A/Ann Arbor/6/60 or A/Leningrad/134/17/57 vaccine strain which confer the *ca*, *ts*, and *att* phenotypes. These LAIV viruses elicit an immune response by replicating at the cooler temperatures of the nasal epithelium but do not cause clinical symptoms associated with replication in the warmer temperatures of the lower respiratory tract.

Previous studies in non-human or immortalized cell lines have mapped the *ca*, *ts* and *att* phenotypes of A/Ann Arbor/6/60 to multiple mutations within the PB1, PB2, M, PA, and NP segments (5–9) or to the PB1 and PB2 gene segments of A/Leningrad/134/17/57 (4). The attenuation of the master donor vaccine strain is believed to be due to reduced viral RNA synthesis, decreased nuclear-cytoplasmic export of viral RNP and significantly reduced incorporation of M1 into the viral progeny (5, 10). However, because these studies utilized cell culture systems that were either derived from non-human hosts or transformed/immortalized cell lines, the significance of these observations to LAIV replication in human respiratory epithelial cells remains to be determined. Because the core body temperature of ferrets, an animal model often utilized to assess LAIV attenuation, is higher than that of humans (39°C vs. 37°C), the degree of LAIV attenuation seen in this animal model may not accurately reflect that which occurs in the human respiratory tract (11, 12).

In this study, we utilize a primary, differentiated human nasal epithelial cell (hNEC) culture system to characterize the differences in viral replication kinetics, cell-associated and released viral proteins and particles release between wild type (WT) seasonal influenza viruses and antigenically-matched LAIV strains in order to gain a better understanding of LAIV replication in a culture system which represents the primary site for LAIV replication in humans.

2. Materials and Methods

2.1. MDCK cells

Madin-Darby canine kidney (MDCK) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Sigma, St. Louis, MO) containing 10% fetal bovine serum (Atlanta Biologicals, Inc., Atlanta, GA), 100 U of penicillin/ml (Invitrogen, Carlsbad, CA), 100 µg of streptomycin/ml (Invitrogen), and 1 mM sodium pyruvate (Sigma) at 37°C in a humidified environment with air supplemented with 5% CO₂.

2.2. hNEC isolation and culture

Human nasal epithelial cells (hNECs) were obtained from non-diseased hosts during endoscopic sinus surgery for non-infection related conditions and grown in culture at the air-liquid interface (ALI) as previously described (13–15). Once obtained, the mucosal tissue was transferred to phosphate buffered saline (PBS) supplemented by penicillin (100 µg/mL, Gibco, Gaithersburg, MD), streptomycin (100 µg/mL, Gibco), amphotericin B (2.5 µg/mL, Gibco), and gentamicin (50 µg/mL, Gibco). Samples were collected through a cell strainer (BD Falcon, Bedford, MA) and digested in 4° C overnight in Ham's F12 media containing 0.01% protease Sigma Type XIV (Sigma) supplemented with antibiotics as above. The cells were separated by straining into a conical tube to which fetal bovine serum (FBS, Sigma) was added to a final concentration of 10% to inactivate the protease. Cells were centrifuged at 1200 rpm for 10 minutes in 4°C, after which the supernatant was aspirated. The washed hNECs were re-suspended in Bronchial Epithelium Growth Medium (BEGM; Lonza, Walkersville, MD) supplemented with (all sources Sigma, unless otherwise indicated) insulin (5 µg/mL), hydrocortisone (0.072 µg/mL), human epidermal growth factor (25 ng/mL, Upstate Biotechnology), triiodothyronine (6.5 ng/mL), retinoic acid (5×10^{-8} M), transferrin (10 µg/mL, Gibco), epinephrine (0.6 µg/mL), phosphoethanolamine (0.5 5mol/L), ethanolamine (0.5 5mol/L), bovine pituitary extract (10 µg/mL, Upstate Biotechnology), BSA (0.5 mg/mL), calcium (0.11 1mol/L) from CaCl₂·2H₂O, selenium (30 nM) from NaSeO₃, manganese (0.6 nM) from MnCl₂·4H₂O, silicon (0.5 5mol/L) from Na₂SiO₃·9H₂O, molybdenum (1.0 nM) from (NH₄)₆Mo₇O₂₄·4H₂O, vanadium (5.0 nM) from NH₄VO₃, nickel (0.5 nM) from NiSO₄·6H₂O, tin (0.5 nM) from SnCl₂·2H₂O, zinc (3.0 0mol/L) from ZnSO₄·7H₂O, iron (0.15 5mol/L) from FeSO₄·7H₂O, magnesium (60 nM) from MgCl₂·6H₂O, penicillin (100 U/mL), streptomycin (100 µg/mL), and amphotericin B (0.25 µg/mL). The cells were then seeded at a density 1.5×10^4 cells/cm² in collagen coated 100-mm culture dishes. The media was changed initially 24 hours after the cells were grown, and then every 48 hours until cells reached confluence.

Confluent cultures were washed with Hank's Buffered Salt Solution (HBSS) prior to trypsinization, then treated at 37°C for 2 minutes with a solution containing 0.2% Trypsin (Sigma), 1% polyvinylpyrrolidone (Sigma), and 0.02% EGTA (Sigma) in HBSS. The trypsin was then neutralized by the addition of an equal volume of cold soybean trypsin inhibitor at a concentration of 1 mg/mL in Ham's F12 media. Dissociated cells were washed and re-suspended into BEGM media and plated onto human type IV placental collagen (Sigma, Type VI) coated 12-well Falcon filter inserts (0.4-µm pore size; Becton Dickinson, Franklin Lakes, NJ). When confluent, media was removed from the apical chamber and the media in the basolateral chamber was changed to LHC Basal Medium:DMEM-H (Gibco) (50:50) containing the same concentrations of additives as BEGM with the exception that the concentration of epidermal growth factor was reduced to 0.63 ng/mL, and amphotericin B was omitted. Each set of cultures came from an individual patient source and was maintained at the air-liquid interface for at least 3 weeks prior to study. Cultures from different donors were used for each replicate experiment performed to ensure that effects were not donor-specific. Experiments comparing WT and LAIV virus infections were performed on cultures from the same donor to ensure that donor variation was not driving differences observed between the two viruses.

2.3. Viruses

Influenza A viruses were propagated on MDCK cells at 33°C in DMEM containing 4 µg/ml *N*-acetyltrypsin (Sigma), 100 U of penicillin/ml, 100µg of streptomycin/ml, and 0.3% bovine serum albumin (BSA; Calbiochem or Sigma). The viruses used in this study were pairs of seasonal Wild Type (WT) viruses and antigenically matched LAIV strains, including A/California/10/78 WT (H1N1) and A/California/10/78 LAIV, A/Washington/897/90 WT (H3N2) and A/Washington/897/90 LAIV, and A/Alaska/14/6/77 WT (H3N2) and A/Alaska/14/6/77 LAIV that were kindly provided by Brian Murphy and Kanta Subbarao at the NIAID/NIH.

2.4. Virus infections

Low-multiplicity of infection (MOI) growth curves were carried out in triplicate by infecting cell cultures in 12-well plates at an MOI of 0.001 50% tissue culture infective doses (TCID₅₀) per cell. hNEC cultures were incubated with virus diluted in DMEM containing, penicillin, streptomycin, and 0.3% BSA on the apical surface for 1 hour at room temperature. After 1 hour the inoculum was aspirated and the apical and basolateral chambers were washed twice with PBS+ (containing calcium and magnesium). BEGM media was then placed into the basolateral chamber and the cultures were then incubated at either 33°C or 37°C for the indicated times. MDCK cells were infected in a similar fashion except that 4 µg/ml *N*-acetyltrypsin was included in the infectious media containing the virus and, following removal of the virus inoculum and subsequent washing with PBS+, both apical and basolateral medium were replaced with DMEM containing, penicillin, streptomycin, 0.3% BSA and 4 µg/ml *N*-acetyltrypsin. At the times indicated in the figures, 200µl of DMEM containing penicillin, streptomycin, and 0.3% BSA was placed on the apical chamber of hNECs and the cultures were then incubated at either 33°C or 37°C for 10 minutes. The apical media was then removed and stored at -80°C. For MDCK cell infections the media was collected and stored at -80°C and replaced with fresh media. The

amount of infectious virus in each sample was determined by TCID₅₀ assay as previously described using MDCK cells at 33°C (16). High-multiplicity infections were also conducted in 12 well plates at an MOI of 10 using the same infection protocol as described for low-multiplicity growth curves.

2.5. Hemagglutination assay

Hemagglutination titers were calculated from high MOI infections at 24 hours post infection as previously described. Human red blood cells (RBCs) were separated from whole blood using a Ficoll-Paque PLUS assay (GE Healthcare Life Sciences, Pittsburgh, PA). Isolated RBCs, diluted to 0.5% in PBS, were incubated with two-fold serial dilutions of supernatant. Hemagglutination titers were calculated after an overnight incubation at 4°C.

2.6. Immunofluorescence microscopy

Human NEC cultures were stained for immunofluorescence microscopy as described previously (17). At 12 hours post infection (hpi) at an MOI of 10 (high MOI), hNEC cultures were fixed with 4% paraformaldehyde in PBS for 10 minutes at room temperature, permeabilized with 0.2% TX-100, then incubated with goat α -HA0 A/PR/8/34 (1:500 dilution, V-314-511-157; National Institute of Allergy and Infectious Diseases), followed by donkey anti-goat IgG conjugated with Alexa Fluor 555 (1:500; Invitrogen, Carlsbad, CA). Ciliated cells were identified using a mouse anti- β Tubulin IV antibody (1:100; BioGenex, San Ramon, CA) followed by donkey anti-mouse IgG conjugated with Alexa Fluor 488 (1:500; Invitrogen, Carlsbad, CA). All antibodies were diluted in PBS with 3% normal donkey serum and 0.5% BSA. Coverslips were mounted using ProLong Gold antifade reagent (Invitrogen, Carlsbad CA).

Samples were imaged with a Nikon Eclipse 90i microscope. In each experiment, images were taken of 5–10 non-overlapping, adjacent fields of view using a 40x objective. Infected cells and ciliated cells were quantified and the percentage of total cells infected that were ciliated was calculated.

2.7. Western blotting

Samples were analyzed by Western blot analysis as described previously (16, 18). Cell-associated polypeptides were obtained from whole cell lysates of hNEC cultures infected with a high MOI that were lysed in 1% sodium dodecyl sulfate (SDS) in PBS and stored at –20°C. Polypeptides from whole cell lysates and apical supernatants were separated by SDS-PAGE and transferred to polyvinylidene fluoride membrane (Immobilon-FL; Millipore, Billerica, MA). All antibodies were diluted in PBS with 5% skim milk powder and 0.3% Tween 20. The primary antibodies used for western blots were 14c2 (anti-M2 monoclonal antibody; 1:1,000) (19), goat α -HA0 A/PR/8/34 (1:500 dilution, V-314-511-157; National Institute of Allergy and Infectious Diseases), and mouse α -M1 HB-64 (1:100 dilution) (16, 20). Antibodies were detected with species-specific secondary antibodies conjugated to Alexa Fluor 647 (1:500; Invitrogen, Carlsbad, CA), and the blots were imaged using an FLA-5000 phosphorimager (FujiFilm). Bands were quantitated using Multi Gauge software, version 3.0 (FujiFilm). The amount of each protein in whole cell lysates was determined

relative to the amount of β -actin (1:10,000 dilution, Ab 6276; AbCam, Cambridge, MA) in the sample.

2.8. Statistical Analyses

Low-multiplicity growth curves were compared to wild-type control curves using multi-variable analysis of variance (MANOVA) with repeated measures. Significant interactions (* $p < 0.05$) were further analyzed with a Bonferroni post-test. Western blot analyses and infectious virus production from high-multiplicity infections were compared using t tests. All statistical analyses were done using Prism, version 4.0 (GraphPad Software Inc.).

3. Results

3.1. Influenza A virus replication in primary differentiated human nasal epithelial cells

LAIV strains have demonstrated restricted replication in numerous cultures systems at temperatures at or above 39°C (4, 7, 9, 10, 21). In order to assess LAIV replication kinetics in a culture system that more faithfully represents the upper respiratory tract – the primary site of replication of LAIV – we utilized primary, differentiated human nasal epithelial cell (hNEC) cultures. Infections of MDCK and hNECs were carried out at 33°C and 37°C, rather than the typical permissive temperatures (30°C or lower) and non-permissive temperature (39°C or higher) as the former temperatures reflect the true physiologic temperatures of the human nasal epithelium and the lower respiratory tract, respectively.

At 33°C there were no differences in the amount of infectious virus produced between the WT and antigenically-matched LAIV infected MDCK cells (Figure 1A, B, and C). However, in hNECs at 33°C, WT viruses produced between 10 and 1,000-fold greater levels of infectious virus than antigenically-matched LAIV viruses (Figure 1D, E, and F). Although the absolute differences in infectious virus produced by the WT and LAIV viruses differed between the pairs of clinical viruses, the trends were similar and significant. At 37°C, only one WT virus, A/California/10/78, produced significantly greater levels of infectious virus than the antigenically-matched LAIV strain in both MDCK and hNEC cultures (Figure 2A and D). Infectious virus production in MDCK cells infected at 37°C with A/Washington/897/90 (Figure 2B) or A/Alaska/14/6/77 (Figure 2C) did not differ between the WT and antigenically matched LAIV but the WT virus replicated to 10–10,000 fold higher levels compared to the antigenically matched LAIV in hNEC cultures (Figures 2E and F). Together, these data demonstrate that LAIV replication in hNEC cultures, at either 33°C or 37°C, is significantly reduced compared to MDCK cells.

3.2. Cell tropism and numbers of infected cells

Both WT and LAIV virus infection of MDCK cells at an MOI= 0.001 resulted in a complete loss of viable cells by 96 hpi, indicating that both viruses could infect all the cells in the culture (data not shown). After infection of hNEC cultures, viable cells were present during the entire course of infection, indicating that virus infection did not lead to loss of all cells in the cultures. The hNEC cultures are composed of a number of distinct cell types including ciliated, Clara, goblet, and basal cells. To identify the step in the viral life cycle that is

responsible for the reduced titers of LAIV in hNEC cultures, we infected hNECs at an MOI of 10 (high MOI) and compared WT and LAIV replication by several different parameters.

To determine if different numbers of cells were infected by WT and LAIV, viral antigen and cellular markers were identified by indirect immunofluorescence with specific antibodies and analyzed by fluorescence microscopy at 12 hours post high MOI infection. Viral antigen was identified in both ciliated (cells expressing β -tubulin IV) and non-ciliated cells at both 33°C (data not shown) and 37°C (Figure 3A). There was no significant difference in either the total numbers of infected cells (Figure 3B) or in the percentage of infected ciliated cells (Figure 3C) in hNEC cultures infected with LAIV as compared with WT virus at either temperature. These data suggest that a difference in cell tropism or in the numbers of infected cells does not explain the disparity in infectious virus production between WT and antigenically-matched LAIV viruses in hNEC cultures.

3.3. Cell-associated viral protein production

To evaluate whether a differential level of viral protein expression could explain the disparity in infectious virus production between WT and LAIV viruses, western blots of whole cell lysates were performed. At 24 hours after high MOI infection, LAIV infected MDCK cells (data not shown) and hNEC cultures produced an equivalent or greater level of viral proteins HA (Figure 4, as quantified using the HA2 protein band) and M1 (Figure 5 A, B, C and D) at 33°C and 37°C when compared to WT-infected cells. LAIV-infected hNECs produced significantly greater levels of M2 protein at 12 and 24 hours post infection at both 33°C and 37°C (Figure 5 E, F, G, and H). These data indicate that the attenuation in the LAIV production of infectious virus was not due to a lack of viral protein production within infected hNECs since all LAIV protein levels were the same or greater than the levels in IAV infected cells.

3.4. Infectious virus production and viral particle release

Next, we assessed the production of infectious and total virus particles using TCID₅₀ and hemagglutination titers to quantify these two parameters. After high MOI infection, significantly less infectious virus was detected in the apical supernatants of LAIV infected MDCK cells and hNEC cultures when compared to WT virus (Figure 6 A and B) at both 33°C and 37°C. When total virus particles were quantified by hemagglutination of human RBCs, there was no significant difference in virus titers in hNEC supernatants at either temperature (Figure 6C). The large difference in infectious virus titers combined with the lack of a difference in hemagglutination titer indicates that LAIV infection of hNEC cultures leads to a reduction in *infectious* virus production, without a noticeable change in *total* particle production.

3.5. Viral protein release from infected cultures

To confirm that LAIV and WT virus infected hNECs were producing comparable amounts of virus particles, Western blot analysis of apical supernatants was performed to evaluate the relative amount and composition of released viral proteins. After high MOI infection at 33°C and 37°C, there was an equivalent level of HA2 (Figure 7), M1 (Figure 8) and M2 (data not shown) proteins found in the apical supernatant at 12 and 24 hpi. These data are

consistent with the observed lack of difference in hemagglutination titers (Figure 6C) and indicates that the restricted replication of LAIV was the result of impaired *infectious* virus production but not total virus production or release of viral proteins from infected cells.

4. Discussion

In this study we compare the replication kinetics, viral proteins produced, virus particles released, and the composition of released viral particles during infection of primary, differentiated hNEC cultures with WT and antigenically-matched LAIV viruses in order to elucidate the mechanism of attenuated replication during LAIV infection. Traditionally, studies of viral kinetics and virus assembly have been conducted in non-human, immortalized cell lines, including MDCK cells, or in embryonated hen's eggs. Primary, differentiated hNEC cultures showed greater restriction in LAIV infectious virus production at both 33°C and 37°C compared to MDCK cells, highlighting the utility of studying virus infection in primary differentiated cultures as opposed to transformed or immortalized cell lines.

Comparison of viral proteins, infectious virus production and hemagglutination titers in the apical supernatants indicates that the LAIV produces significantly greater levels of non-infectious virus as compared with WT virus infection of hNECs. Because LAIV infected cells produce at least as much, if not more viral proteins than WT virus infected cells, a lack of viral protein production does not explain the attenuation. It is possible that altered ratios of viral proteins or altered subcellular localization of viral proteins in LAIV infected cells may be contributing to LAIV attenuation in hNEC cultures. The greater amounts of M2 protein produced by LAIV infected hNEC cultures correlated with decreased LAIV infectious virus production but overexpression of M2 in MDCK cells does not adversely affect WT influenza virus replication (22, 23). The presence of a presumably truncated form of M2 in LAIV infected cell lysates may contribute to the reduced infectious virus production due to reduced viral RNA packaging as truncations to the C-terminus of M2 can affect viral RNA packaging but not virus particle production (16).

It remains to be determined if the reduced LAIV replication in hNEC cultures is due to host cell factors expressed differentially in hNEC cultures (24), or simply represent an augmented effect of the mutations in LAIV that have been demonstrated to control the *ts*, *ca* and *att* phenotypes in other cell culture systems (8, 9). Several mutations in LAIV have been ruled out with respect to their contribution to the *ts*, *ca* and *att* phenotypes (7, 21). It is intriguing to speculate that perhaps some of those mutations contribute to virus replication in primary respiratory epithelial cell cultures but not to virus replication in other culture systems. Greater attenuation of LAIV replication in primary human epithelial cells derived from adenoid or bronchial tissue compared to MDCK cells has been demonstrated previously (25), but we have extended those observations to show that an altered infectious to non-infectious virus particle ratio is responsible for this difference.

LAIV attenuation in MDCK cells and embryonated eggs is usually studied at temperatures >37°C and alterations in virus replication have been studied extensively (5, 10). In our study, all three LAIV strains replicated to lower titers in hNECs at 33°C but replicated as

efficiently as WT virus in MDCK cells at the same temperature (Figure 1). Only one LAIV strain replicated to lower titers in MDCK cells at 37°C but all three strains were attenuated for replication in hNEC cultures at the same temperature (Figure 2). This suggests a greater attenuation of LAIV replication in hNEC cultures compared to MDCK cells. In addition, we did not detect significant differences in the incorporation of M1 protein into virus particles, suggesting that this defect is not present in LAIV virus particles produced from hNEC cultures at 33°C or 37°C (5, 10). Previous studies compared infection between the original seasonal H2N2 virus (A/Ann Arbor/6/60) and the master vaccine donor strain (5, 7, 10) using permissive temperatures of 25°C and 33°C and non-permissive temperatures of 38.5°C – 40°C. We elected to focus on LAIV infection at the physiologically relevant temperatures of 33°C (upper human respiratory tract) and 37°C (lower human respiratory tract) in order to identify the replication defects that are most likely occurring during infection of humans with LAIV.

The comparison of multiple pairs of seasonal strains and their antigenically matched LAIV viruses reveal provocative differences in infectious virus production between antigenically different viruses. Whereas both H1N1 and H3N2 WT viruses produced significantly more infectious virus than the corresponding antigenically-matched LAIV strains at 33°C and 37°C in hNEC cultures, the absolute differences varied suggesting that the HA and/or the NA proteins may play a role in virus assembly and in modulating LAIV attenuation, as has been suggested previously (26, 27).

Annual influenza vaccination remains the most effective means of decreasing the impact of seasonal influenza, however, the level of protection in individuals older than 60 years of age who account for greater than 90% of influenza related mortality remains limited (28). LAIV represents an important tool in the prophylaxis against seasonal influenza which is not utilized in this population. Engineering LAIV strains to replicate with increased efficiency in hNEC cultures could improve its efficacy in elderly individuals. Among multiple clinical trials in pediatric populations, the LAIV formulations have been shown to have significantly greater efficacy than the trivalent inactivated vaccine especially during times of poor homology between the vaccine and circulating strain of influenza (29–35). Improved understanding of the mechanism underlying the attenuated infectious virus production by LAIV will lead to improved vaccine development. During infection of hNEC cultures, LAIV produces significantly greater levels of non-infectious virus when compared to WT infected cultures. LAIV infected hNEC cultures also produce an altered innate immune response when compared to WT infected hNEC cultures (24). The combination of reduced infectious virus production and an altered epithelial cell specific innate immune responses to LAIV infection, may contribute to the efficacy of LAIV as an influenza virus vaccine.

Acknowledgments

The study was supported by funds from R01AI097417 (AP), HHSN272201400007C (AP), R03AG045088 (WF), KL2TR001109 (WF), and IDSA Young Investigator Award in Geriatrics (WF). We thank Sabra Klein and the members of the Pekosz laboratory for critical discussions of the data and the manuscript. We thank Katherine Fenstermacher for help with our graphical abstract.

References

1. Fischer, WA., II; Hayden, F. Novel Antiviral Agents and Approaches in Pandemic Influenza. In: Vassil St Georgiev, KAW.; McGowan, John J., editors. National Institute of Allergy and Infectious Diseases, NIH, Frontiers in Research. Humana Press; 2008. p. 179-192.
2. Maassab HF. Adaptation and growth characteristics of influenza virus at 25 degrees c. *Nature*. 1967; 213:612–614. [PubMed: 6040602]
3. Murphy BR, Coelingh K. Principles underlying the development and use of live attenuated cold-adapted influenza A and B virus vaccines. *Viral Immunol*. 2002; 15:295–323. [PubMed: 12081014]
4. Isak ova-Sivak I, Chen L-M, Matsuoka Y, Voeten JTM, Kiseleva I, Heldens den Bosch Hv, Klimov A, Rudenko L, Cox NJ, Donis RO. Genetic bases of the temperature-sensitive phenotype of a master donor virus used in live attenuated influenza vaccines: A/Leningrad/134/17/57 (H2N2). *Virology*. 2011; 412:297–305. [PubMed: 21315402]
5. Chan W, Zhou H, Kemble G, Jin H. The cold adapted and temperature sensitive influenza A/Ann Arbor/6/60 virus, the master donor virus for live attenuated influenza vaccines, has multiple defects in replication at the restrictive temperature. *Virology*. 2008; 380:304–311. [PubMed: 18768193]
6. Jin H, Bin L, Zhou H, Kemble G. Genetic studies of Flumist vaccines derived from cold-adapted A/Ann Arbor/6/60. *International Congress Series*. 2004; 1263:153–156.
7. Jin H, Lu B, Zhou H, Ma C, Zhao J, Yang CF, Kemble G, Greenberg H. Multiple amino acid residues confer temperature sensitivity to human influenza virus vaccine strains (FluMist) derived from cold-adapted A/Ann Arbor/6/60. *Virology*. 306:18–24. [PubMed: 12620793]
8. Snyder MH, Betts RF, DeBorde D, Tierney EL, Clements ML, Herrington D, Sears SD, Dolin R, Maassab HF, Murphy BR. Four viral genes independently contribute to attenuation of live influenza A/Ann Arbor/6/60 (H2N2) cold-adapted reassortant virus vaccines. *J Virol*. 1988; 62:488–495. [PubMed: 3336068]
9. Snyder MH, Clements ML, De Borde D, Maassab HF, Murphy BR. Attenuation of wild-type human influenza A virus by acquisition of the PA polymerase and matrix protein genes of influenza A/Ann Arbor/6/60 cold-adapted donor virus. *J Clin Microbiol*. 1985; 22:719–725. [PubMed: 4056002]
10. Odagiri T, Tanaka T, Tobita K. Temperature-sensitive defect of influenza A/Ann Arbor/6/60 cold-adapted variant leads to a blockage of matrix polypeptide incorporation into the plasma membrane of the infected cells. *Virus Res*. 1987; 7:203–218. [PubMed: 3604455]
11. Hussein RH, Sweet C, Collie MH, Smith H. Elevation of nasal viral levels by suppression of fever in ferrets infected with influenza viruses of differing virulence. *J Infect Dis*. 1982; 145:520–524. [PubMed: 7069233]
12. Rappuoli, RaDGG. Influenza Vaccines for the Future. In: Schmidt, A.; Weber, O.; Kaufmann, SHE., editors. Birkhauser Advances in Infectious Diseases. 2. Springer; Italy: 2010.
13. Lane AP, Saatian B, Yu XY, Spannhake EW. mRNA for genes associated with antigen presentation are expressed by human middle meatal epithelial cells in culture. *Laryngoscope*. 2004; 114:1827–1832. [PubMed: 15454780]
14. Ramanathan M Jr, Lane AP. Innate immunity of the sinonasal cavity and its role in chronic rhinosinusitis. *Otolaryngol Head Neck Surg*. 2007; 136:348–356. [PubMed: 17321858]
15. Ramanathan M Jr, Lee WK, Dubin MG, Lin S, Spannhake EW, Lane AP. Sinonasal epithelial cell expression of toll-like receptor 9 is decreased in chronic rhinosinusitis with polyps. *Am J Rhinol*. 2007; 21:110–116. [PubMed: 17283572]
16. McCown MF, Pekosz A. The influenza A virus M2 cytoplasmic tail is required for infectious virus production and efficient genome packaging. *J Virol*. 2005; 79:3595–3605. [PubMed: 15731254]
17. Newby CM, Rowe RK, Pekosz A. Influenza A virus infection of primary differentiated airway epithelial cell cultures derived from Syrian golden hamsters. *Virology*. 2006; 354:80–90. [PubMed: 16876846]
18. Grantham ML, Wu WH, Lalime EN, Lorenzo ME, Klein SL, Pekosz A. Palmitoylation of the influenza A virus M2 protein is not required for virus replication in vitro but contributes to virus virulence. *J Virol*. 2009; 83:8655–8661. [PubMed: 19553312]
19. Zebedee SL, Lamb RA. Influenza A virus M2 protein: monoclonal antibody restriction of virus growth and detection of M2 in virions. *J Virol*. 1988; 62:2762–2772. [PubMed: 2455818]

20. Yewdell JW, Frank E, Gerhard W. Expression of influenza A virus internal antigens on the surface of infected P815 cells. *J Immunol.* 1981; 126:1814–1819. [PubMed: 7217668]
21. Kiseleva IKA, Su Q, Szymkowiak C, Toner TJ, Kwan W-S, Rudenko L, Shaw AR, Youil R. Role of individual genes of A/Leningrad/134/17/57 (H2N2) cold-adapted donor strain in manifestation of the temperature-sensitive phenotype or reassortant influenza A viruses. *International Congress Series.* 2004; 1623:547–550.
22. Stewart SM, Wu W-H, Lalime EN, Pekosz A. The cholesterol recognition/interaction amino acid consensus motif of the influenza A virus M2 protein is not required for virus replication but contributes to virulence. *Virology.* 2010; 405:530–538. [PubMed: 20655564]
23. Grantham ML, Stewart SM, Lalime EN, Pekosz A. Tyrosines in the Influenza A Virus M2 Protein Cytoplasmic Tail Are Critical for Production of Infectious Virus Particles. *J Virol.* 2010; 84:8765–8776. [PubMed: 20573832]
24. Fischer WA II, Chason KD, Brighton M, Jaspers I. Live attenuated influenza vaccine strains elicit a greater innate immune response than antigenically-matched seasonal influenza viruses during infection of human nasal epithelial cell cultures. *Vaccine.* 2014; 32:1761–1767. [PubMed: 24486351]
25. Ilyushina NA, Ikizler MR, Kawaoka Y, Rudenko LG, Treanor JJ, Subbarao K, Wright PF. Comparative Study of Influenza Virus Replication in MDCK Cells and in Primary Cells Derived from Adenoids and Airway Epithelium. *Journal of Virology.* 2012; 86:11725–11734. [PubMed: 22915797]
26. Chen BJ, Leser GP, Morita E, Lamb RA. Influenza virus hemagglutinin and neuraminidase, but not the matrix protein, are required for assembly and budding of plasmid-derived virus-like particles. *J Virol.* 2007; 81:7111–7123. [PubMed: 17475660]
27. Marsh GA, Hatami R, Palese P. Specific residues of the influenza A virus hemagglutinin viral RNA are important for efficient packaging into budding virions. *J Virol.* 2007; 81:9727–9736. [PubMed: 17634232]
28. Molinari NA, Ortega-Sanchez IR, Messonnier ML, Thompson WW, Wortley PM, Weintraub E, Bridges CB. The annual impact of seasonal influenza in the US: measuring disease burden and costs. *Vaccine.* 2007; 25:5086–5096. [PubMed: 17544181]
29. Ashkenazi S, Vertruyen A, Aristegui J, Esposito S, McKeith DD, Klemola T, Biolek J, Kuhr J, Bujnowski T, Desgrandchamps D, Cheng SM, Skinner J, Gruber WC, Forrest BD. Superior relative efficacy of live attenuated influenza vaccine compared with inactivated influenza vaccine in young children with recurrent respiratory tract infections. *Pediatr Infect Dis J.* 2006; 25:870–879. [PubMed: 17006279]
30. Belshe RB, Ambrose CS, Yi T. Safety and efficacy of live attenuated influenza vaccine in children 2–7 years of age. *Vaccine.* 2008; 26(Suppl 4):D10–16. [PubMed: 18611422]
31. Belshe RB, Edwards KM, Vesikari T, Black SV, Walker RE, Hultquist M, Kemble G, Connor EM. Live attenuated versus inactivated influenza vaccine in infants and young children. *N Engl J Med.* 2007; 356:685–696. [PubMed: 17301299]
32. Bridges CB, Thompson WW, Meltzer MI, Reeve GR, Talamonti WJ, Cox NJ, Lilac HA, Hall H, Klimov A, Fukuda K. Effectiveness and cost-benefit of influenza vaccination of healthy working adults: A randomized controlled trial. *Jama.* 2000; 284:1655–1663. [PubMed: 11015795]
33. Fleming DM, Crovari P, Wahn U, Klemola T, Schlesinger Y, Langussis A, Oymar K, Garcia ML, Krygier A, Costa H, Heininger U, Pregaldien JL, Cheng SM, Skinner J, Razmpour A, Saville M, Gruber WC, Forrest B. Comparison of the efficacy and safety of live attenuated cold-adapted influenza vaccine, trivalent, with trivalent inactivated influenza virus vaccine in children and adolescents with asthma. *Pediatr Infect Dis J.* 2006; 25:860–869. [PubMed: 17006278]
34. Halloran ME, Piedra PA, Longini IM Jr, Gaglani MJ, Schmotzer B, Fewlass C, Herschler GB, Glezen WP. Efficacy of trivalent, cold-adapted, influenza virus vaccine against influenza A (Fujian), a drift variant, during 2003–2004. *Vaccine.* 2007; 25:4038–4045. [PubMed: 17395338]
35. Jefferson T, Rivetti A, Harnden A, Di Pietrantonj C, Demicheli V. Vaccines for preventing influenza in healthy children. *Cochrane Database Syst Rev.* 2008:CD004879. [PubMed: 18425905]

Highlights

- LAIV is more attenuated in human nasal epithelial cells compared to MDCK cells
- Equivalent viral proteins are produced in influenza and LAIV infections
- Reduced LAIV infectious virus titers but not total particles compared to influenza

Figure 1.

Attenuation of LAIV infectious virus production at 33°C. MDCK cells (A, B and C) or hNEC cultures (D, E, and F) were infected with WT or an antigenically-matched LAIV at an MOI of 0.001 in triplicate and incubated at 33°C. Virus titers in apical supernatants were measured by calculating the Tissue Culture Infectious Dose 50% (TCID₅₀/ml) in MDCK cells at the indicated hours post infection (HPI). The limit of detection is shown by the dotted line. The mean and standard error of samples collected in triplicate are graphed. The data shown are representative of two independent experiments. Statistically significant differences (*= $p < 0.05$) were determined by MANOVA with a Bonferroni post test.

Figure 2.

Attenuation of LAIV infectious virus production at 37°C. MDCK cells (A, B and C) or hNEC cultures (D, E, and F) were infected with WT or an antigenically-matched LAIV at an MOI of 0.001 in triplicate and incubated at 37°C. Virus titers in apical supernatants were measured by calculating the Tissue Culture Infectious Dose 50% (TCID₅₀/ml) in MDCK cells at the indicated hours post infection (HPI). The limit of detection is shown by the dotted line. The mean and standard error of samples collected in triplicate are graphed. The data shown are representative of two independent experiments. Statistically significant differences (*= $p < 0.05$) were determined by MANOVA with a Bonferroni post test.

Figure 3.

Cell tropism of A/California/10/78 WT and LAIV in hNEC cultures. hNECs were infected with WT and antigenically-matched LAIV at an MOI of 10 at 37°C (A) for 12 hours. Infected cells were identified using an antibody directed against the viral protein Hemagglutinin (HA - red) and ciliated cells were identified using an antibody that recognizes β -tubulin IV (green). Images were obtained with a 40x objective and 5–10 fields were quantified. Cells were counted per 40x field. (B) Total numbers of infected cells per field and (C) the percentage of infected ciliated cells are graphed. The mean and standard error of the mean are graphed. A representative image is shown but quantification is based on three independent experiments, using two samples per timepoint in each experiment.* = $p < .05$ as determined by t-test.

Figure 4.

Cell-Associated Viral HA Protein Synthesis. The level of intracellular viral HA protein produced during hNEC infection with WT and LAIV was analyzed by western blot. hNECs were infected with WT and LAIV at a MOI of 10 at 33°C and 37°C for 12 and 24. (A and C) The expression of viral protein HA and the cellular protein β-actin was determined by Western blotting. (B and D) HA2 protein bands were quantified using fluorescence detection with a phosphoimager and normalized to the cellular protein β-actin. A representative image is shown but quantification is based on three independent experiments, using three samples per timepoint in each experiment. * = $p < .05$ as determined by t-test.

Figure 5.

Cell-Associated Viral M1 and M2 Protein Synthesis. The production of viral M1 and M2 proteins within infected hNECs was analyzed by western blot. HNECs were infected with WT and LAIV at a MOI of 10 at 33°C and 37°C for 12 and 24. (A, C, E and G) The expression of viral proteins M1 and M2 and the cellular protein β-actin was determined by Western blotting. (B, D, F and H) M1 and M2 protein bands were quantified using fluorescence detection with a phosphorimager and normalized to the cellular protein β-actin. A representative image is shown but quantification is based on three independent

experiments, using three samples per timepoint in each experiment. * = $p < .05$ as determined by t-test.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 6.

Virus Titer. MDCK and hNEC cultures were infected with WT and LAIV virus at an estimated MOI of 10 in triplicate and incubated for 24 hpi at 33°C or 37°C. Infectious virus titers of apical supernatants from MDCK (A) and hNEC cultures (B) were determined using the 50% Tissue Culture Infective Dose (TCID₅₀/ml) assay in MDCK cells. Total virus particles in the supernatants of infected hNECs were quantified by determining the hemagglutination (C) human red blood cells. The results are representative of three independent experiments, each performed in duplicate. ** p < 0.01 *** p < 0.001 as determined by t-test.

Figure 7.

Comparison of Viral Protein HA in the Apical Supernatant. HNECs were infected with WT or an antigenically-matched LAIV at a MOI of 10 at 33°C and 37°C and apical supernatants were harvested at 12 and 24 HPI. (A and C) The levels of viral HA protein were determined by western blotting. (B and D) HA protein intensities were quantified using fluorescence detection with a phosphorimager. The mean and standard error are graphed. A representative image is shown but quantification is based on three independent experiments, using three samples per timepoint in each experiment. * = $p < .05$ as determined by t-test.

Figure 8.

Comparison of Viral Protein M1 in the Apical Supernatant. hNECs were infected with WT or an antigenically-matched LAIV at a MOI of 10 at 33°C and 37°C and apical supernatants were harvested at 12 and 24 HPI (A and B). The level of M1 protein in the apical supernatant was evaluated by western blot and the M1 protein intensities were quantified using fluorescence detection with a phosphoimager. The mean and standard error are graphed. A representative image is shown but quantification is based on three independent experiments, using three samples per timepoint in each experiment. * = $p < .05$ as determined by t-test.