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A viral-vectored RSV vaccine induces long-lived humoral immunity in cotton rats

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Abstract

Human respiratory syncytial virus (RSV) is the leading cause of lower airway disease in infants worldwide and repeated infects immunocompetent individuals throughout life. Severe lower airway RSV infection during infancy can be life-threatening, but is also associated with important sequelae including development of asthma and recurrent wheezing in later childhood. The basis for the inadequate, short-lived adaptive immune response to RSV infection is poorly understood, but it is widely recognized that RSV actively antagonizes Type I interferon (IFN) production. In addition to the induction of the anti-viral state, IFN production during viral infection is critical for downstream development of robust, long-lived immunity. Based on the hypothesis that a vaccine that induced robust IFN production would be protective, we previously constructed a Newcastle disease virus-vectored vaccine that expresses the F glycoprotein of RSV (NDV-F) and

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demonstrated that vaccinated mice had reduced lung viral loads and an enhanced IFN- γ response after RSV challenge. Here we show that vaccination also protected cotton rats from RSV challenge and induced long-lived neutralizing antibody production, even in RSV immune animals. Finally, pulmonary eosinophilia induced by RSV infection of unvaccinated cotton rats was prevented by vaccination. Overall, these data demonstrate enhanced protective immunity to RSV F when this protein is presented in the context of an abortive NDV infection.

1. Introduction

Human respiratory syncytial virus (RSV), a negative sense RNA virus in the Paramyxoviridae family, is the major cause of bronchiolitis and pneumonia in infants [1, 2]. RSV outbreaks occur on an annual basis and essentially all persons are infected within the first two years of life. While RSV infection is limited to the upper respiratory tract in most healthy adults and children, severe, even fatal, RSV pneumonia occurs in young infants 2 to 4 months of age, transplant recipients and the elderly [1]. Secondary RSV infections, which are generally limited to the upper respiratory tract, present with mild, cold-like symptoms in healthy adults, but are commonly associated with otitis media in young children [3–5]. In addition, RSV has been associated with the development of asthma, and exacerbation of wheezing in asthmatic patients [6–9].

Immunity to RSV is remarkably ineffective, allowing for repeated infection of immunocompetent children and adults [10, 11] [12]. Unlike other viral pathogens, serum antibody levels are very slow to rise following RSV infection, with a gradual accumulation of protective antibodies only after multiple re-infections [13, 14]. The inability of RSV to induce robust immunity following repeated natural infections likely underlies the difficulties encountered in attempts to design effective, attenuated vaccine strains [15]. We hypothesized that the relative inability of RSV to generate a potent IFN response in mouse [16] or man [17–19] contributes to the relatively ineffective B cell response to this virus. We tested this idea by constructing a vaccine vector based on the Hitchner B1 vaccine strain of Newcastle disease virus (NDV). NDV is an avian paramyxovirus that is nonpathogenic in humans or mice but is known to induce very high type I IFN levels in mice and mammalian cells [20, 21]. NDV “infects” mammalian cells in the sense that viral transcripts and proteins are synthesized, but replication is abortive, with no production of viral particles [22]. We reasoned that construction of a recombinant NDV virus expressing the RSV F protein (NDV-F) would trigger expression of RSV F in the context of robust induction of a type I IFN response. Type I IFNs, produced early in viral infection, induce the anti-viral state and are potent immunomodulators. IFN- α/β s are known to activate natural killer (NK) cells [23, 24], upregulate DC costimulatory molecule expression [21, 25, 26], stimulate clonal expansion and memory formation of CD8⁺ T cells [27–29], as well as antibody production [30–32]. In addition to its capacity for potent IFN induction, NDV’s suitability as a vaccine vector is further enhanced by the absence of virus-specific antibodies in the vast majority of the human population, and its proven safety in human subjects [33].

Previously, we demonstrated that mice immunized by a recombinant NDV construct encoding the F protein of RSV (NDV-F) were protected from RSV challenge and showed an

enhanced Th1 response following secondary infection [34]. By using this vectored vaccine we hoped to couple expression of the RSV F protein gene with the adjuvant effect of type I IFN induction. While the mouse data were encouraging, the relative resistance of the mouse to RSV infection was not evidence that this approach to RSV vaccination was sufficient to immunize a more susceptible species. In this study, we evaluated NDV-F as an RSV vaccine candidate in the cotton rat, a rodent species more susceptible to RSV infection than the mouse [35]. In the mouse, only the alveolar lining epithelium is infected whereas, in cotton rats and human patients, there is widespread infection of nasal mucosa, and limited infection of airway epithelium [36]. While it is not possible to examine cases of non-fatal RSV infection of human subjects, RSV-infected cotton rats develop chronic eosinophilic inflammation of the lower airway following intranasal infection, which persists long after virus is no longer detectable [37]. Given the similarities of RSV tropism and spread in the cotton rat and human subjects, we chose this species to test the NDV-F vaccine in order to test its effects on neutralizing antibody production, but also to test the hypothesis that exposure to RSV antigens in the context of IFN induction would reshape, as well as extend, the immune response to this virus.

We found that two intranasal doses of NDV-F induced robust, long-lived, antigen-specific humoral immunity, prevented RSV infection of the lower airway and largely eliminated RSV replication in the upper airway. This protection occurred without associated vaccine-mediated disease or enhanced pathology following RSV infection of NDV-F vaccinated cotton rats. On the contrary, the prolonged eosinophilic inflammatory response observed previously in the lung after RSV infection of the cotton rat [37] was abrogated by NDV-F vaccination. This observation supported our premise that activation of innate immune responses by the NDV vector acts to bias anti-viral responses away from a Th2-like pattern of inflammation, as well as promoting a prolonged neutralizing antibody response. In addition, and perhaps most important, we were able to demonstrate vaccine efficacy in previously RSV-infected, seropositive animals. The potential to immunize the immune represents a major advance as our ability to safely and effectively immunize the 2 to 4 month old infants who are most susceptible to severe lower airway disease from RSV presents a nearly insurmountable problem.

2. Materials and Methods

2.1 Animals

6–10 week old female cotton rats (*Sigmodon hispidus*) were used in this study (Sigmovir Biosystems, Inc., Bethesda, MD, USA). Animals were housed in groups of 3 in a BSL 2 facility and were offered a commercial pelleted rat chow and water ad libitum. Animals were acclimated for 7 to 10 days prior to vaccination or viral challenge. All procedures used here were conducted humanely. Data were collected from 3–5 cotton rats per cohort per time point, unless otherwise specified. All cotton rat studies were approved by the Institutional Animal Care and Use Committees of New York University School of Medicine (protocol 100504-02) and Rutgers-New Jersey Medical School.

2.2 Vaccination

NDV-F was constructed as previously described [34] then amplified in 10-day-old embryonated chicken eggs. While under isoflurane anesthesia, cotton rats were intranasally (i.n.) vaccinated with 1×10^6 pfu NDV-F, 1×10^6 pfu NDV vector alone, or allantoic fluid as a mock control in a 100 μ l total volume divided equally between nares. Twenty-eight days after the priming vaccination, cotton rats were boosted in the same manner.

2.3 RSV challenge

Human RSV strain A2, originally obtained from ATCC (VR-1540), was passaged on murine STAT1 $^{-/-}$ fibroblast monolayers as previously described [38]. The challenge dose of 1×10^6 pfu RSV was instilled into the nasal cavities of cotton rats under isoflurane anesthesia in a 50 μ l total volume, divided equally between nares. The inoculum was delivered slowly, over approximately 15 seconds, to restrict initial virus infection to the upper airway.

2.4 Plaque assay

Cotton rats were euthanized by CO₂ asphyxiation, and nasal wash fluids and lungs were collected immediately after euthanasia and stored at -80°C until use in viral plaque assay. Viral plaque assay was performed on murine STAT1 $^{-/-}$ fibroblast monolayers as previously described [39].

2.5 Measurement of IFN induction

Allantoic fluid, 10^6 pfu RSV or 10^6 pfu NDV-F were delivered intranasally to 6–8 week old female cotton rats in a volume of 50 μ l. Three animals were used in each treatment group. RNA was purified from lungs harvested 24 hours later using the Qiagen RNeasy Mini Kit. cDNA from each sample was prepared from 1 μ g of RNA using the Biorad iScript cDNA synthesis kit. qPCR was performed using the IQ SYBR Green supermix on a Biorad CFX96 Real time system. IFN induction was measured indirectly by quantifying upregulation of the IFN-stimulated gene, Mx1. Beta actin was used as the housekeeping gene. Primer sequences were adopted from Pletneva et al. [40]. After a 95° incubation for 3 minutes, 39 cycles were performed as follows: 95° for 10 seconds, 65° for 30 seconds.

Mx1 forward primer AAAGTTCTGCTGAGATAGACAGCC

Mx1 reverse primer ATGCTCTTTCCACACTCCTGGTAG

Beta actin forward primer GGCCAACCGTGAAAAGATCACTC

Beta actin reverse primer GTCCGCCTAGAAGCATTTGCG

2.6 Histology and immunohistochemistry

Nasal cavities and lung were collected immediately following CO₂ asphyxiation. Nasal cavities were fixed in neutral buffered formalin then decalcified with 0.35M EDTA in 0.1M Tris (pH 6.95). The lung and decalcified nasal cavities were processed routinely, paraffin-embedded and sectioned at 5 μ m. Tissues sections were stained with hematoxylin and eosin (H&E) or were left unstained for immunohistochemistry (IHC). For IHC, tissue sections were incubated with goat polyclonal RSV antiserum (Biodesign, Saco, Maine, USA) diluted

1:500 followed by incubation with biotinylated rabbit anti-goat IgG antibody then HRP (ScyTek, Logan, UT, USA). Virus detection was accomplished with the streptavidin link and AEC chromagen (Scytek, Logan, UT, USA). IHC-labeled tissue sections were counterstained with hematoxylin.

2.6 RSV F-specific ELISA

Purified RSV F glycoprotein, whose transmembrane anchor was replaced with a 6His tag, was produced by transfection of HEK293F cells and purified as described [41]. For RSV F-specific ELISA, Nunc maxisorb immunoplates (ThermoScientific, Waltham, MA, USA) were coated overnight at 4°C with 0.3 µg purified F protein diluted in 0.1M sodium carbonate buffer. Plates were washed with PBS containing 0.05% Tween 20 (PBS-T) then blocked with PBS containing 10% fetal calf serum (PBS-F). Plates were washed with PBS-T then samples, serially diluted in PBS-F, were added followed by incubation at 25°C for 60 minutes. Wells were washed with PBS-T and secondary antibody diluted in PBS-F was added (chicken anti-cotton rat IgG, Immunology Consultants Laboratory, Inc., Portland, OR, USA). Plates were incubated at 25°C for 60 minutes. After washing with PBS-T, tertiary antibody diluted in PBS-F was added (goat anti-chicken IgG conjugated to HRP, Southern Biotech, Birmingham, AL, USA). Following a second incubation at 25°C for 60 minutes, plates were again washed with PBS-T followed by the addition of tetramethylbenzidine peroxidase substrate (ScyTek, Logan, UT, USA). After incubation at 25°C for 30 minutes, the reaction was stopped by addition of 2N H₂SO₄. Absorbance at 450 nm (A₄₅₀) was determined using an Epoch Microplate Spectrophotometer (BioTek, Winooki, VT, USA). Titers were defined as the highest dilution of the sample resulting in A₄₅₀ four-fold greater than background (PBS-F alone).

Serum neutralization assay—Two different methods were used for detection of RSV neutralizing antibody. Data shown in Figure 4D were collected using a standard plaque reduction assay. Serum dilutions pre-incubated with 200 pfu RSV were used as the inoculum for plaque assay on murine STAT1^{-/-} fibroblast monolayers as previously described [39].

For a more quantitative approach to determining neutralizing antibody titer, a flow cytometric assay was devised. For data shown in Figure 7A, serum samples at a 1/800 dilution were incubated with rg-RSV [42] at a concentration of 3×10^5 pfu/ml. in DMEM + 5% fetal calf serum for 1 hour at 37°C. The serum-virus mixture was then used to inoculate, in duplicate, monolayers of Vero cells (10^5 cells plated in 2 cm² wells). After a 20 hour incubation, cells were resuspended and analyzed by flow cytometry. Only infected cells will express gfp. Percent neutralization was calculated as $100(1 - f_s/f_0)$ where f_s = fraction of gfp+ cells in the sample, and f_0 = the fraction of gfp+ cells in the no-serum control.

2.7 Enumeration of RSV F-specific B Cells

For T cell depletion, mouse anti-cotton rat CD8 and mouse anti-cotton rat CD4 monoclonal antibodies (r&d systems) were labeled with DSB-X according to the manufacturer's protocol (Dynabeads FlowComp Flexi, Invitrogen Life Technologies, Grand Island, NY, USA). Single cell suspensions of cervical lymph nodes, pooled by cohort, were incubated with 0.5 µg each of CD8 and CD4 DSB-X labeled antibody per sample, then T cells were

depleted according to the manufacturer's protocol (Dynabeads FlowComp Flexi, Invitrogen Life Technologies, Grand Island, NY, USA). Cervical lymph node single cell suspensions, depleted of T cells and pooled by cohort, were used in ELISpot assays.

For F-specific ELISpot, multiscreen HTS HA plates (Millipore, Jaffrey, NH, USA) were coated overnight at 4°C with 0.3 µg purified F protein diluted in 0.1M sodium carbonate buffer. Plates were washed with RPMI supplemented with 10% fetal calf serum (cRPMI) and then were blocked with cRPMI for 1 hr at 37°C in a 5% CO₂ incubator. Plates were washed with RPMI then cervical lymph node single cell suspensions were added in duplicate. Plates were incubated 4 – 6 hrs at 37°C in 5% CO₂. Plates were then washed with PBS containing 0.05% Tween-20 (PBS-T). Secondary antibody diluted in PBS-T (chicken anti-cotton rat IgG, Immunology Consultants Laboratory, Inc., Portland, OR, USA) was added and plates were incubated overnight at 4°C. Plates were washed with PBS-T. Tertiary antibody diluted in PBS-T was added (goat anti-chicken AP, Southern Biotech, Birmingham, AL, USA) and plates were incubated at 25°C for 60 minutes. Plates were again washed with PBS-T. NBT/BCIP substrate (Millipore, Jaffrey, NH, USA) was added and plates were incubated in the dark for 30 minutes at 25°C. Plates were washed with tap water. Spots were counted visually.

2.8 BAL fluid analysis

BAL fluids were collected from cotton rats immediately after CO₂ asphyxiation by washing the lung with 2 ml of sterile saline. BAL fluid cell counts were determined by hemocytometer and BAL differentials were determined on Wright-Giemsa stained CytoSpin preparations (ThermoScientific, Waltham, MA, USA). For the 1 month time point, BAL fluid data was available for only 2 NDV vaccinated animals as BAL fluid collected from one NDV vaccinated animals was of poor quality and thus excluded. For the 2 month time point, BAL fluid data was available for only 2 mock vaccinated animals because one of the mock vaccinated animals died prior to sampling. For the 4 days after secondary RSV infection time point, BAL fluid data was available for only 2 mock vaccinated animals as necropsy of a third mock-vaccinated animal revealed pyometra, an abdominal mass and fibrinous pericarditis. BAL fluid analysis was not performed on that animal.

2.9 Statistical analysis

SigmaPlot 12.0 (Systat Software, Inc., San Jose, CA, USA) and GraphPad (GraphPad Software, Inc., La Jolla, CA USA) were used to perform ANOVA and t-test where appropriate. A p-value < 0.05 was considered statistically significant.

3. Results

3.1 NDV-F vaccination induces IFN and a mild inflammatory response

The immunization regimen involved intranasal delivery of 10⁶ pfu of NDV-F into the nasal cavity of cotton rats. It was anticipated that, despite the inability of this live vaccine to replicate in the cotton rat host (data not shown), a limited inflammatory response reflecting the ability of this construct to activate innate immunity would result. To determine the effect of inoculation, the nasal cavity and lung tissues were examined following each of the two

i.n. vaccine doses. Histologic sections from the upper airway taken 24 hours, 48 hours, 72 hours and 7 days following intranasal instillation of 10^6 pfu of NDV-F showed minimal signs of inflammation, indistinguishable from similar sections taken from untreated cotton rats (Data not shown).

To demonstrate IFN induction by NDV-F, lung RNA was harvested 24 hours after i.n. instillation of allantoic fluid, 10^6 pfu RSV or 10^6 pfu NDV-F. While immunological reagents for this species are somewhat limited, the Blanco laboratory [40] has cloned and sequenced the cotton rat *Mx1* gene and shown it to be induced specifically by type I IFN. Fold induction of *Mx1* mRNA following each treatment was determined by quantitative RT-PCR (Fig. 1). As seen in mice, *Mx1* upregulation is minimal in RSV infected animals, but is upregulated 300 fold by NDV-F.

The nasal cavities of inoculated animals were also examined 28 days following priming, and 28 days following boost, for any evidence of disease secondary to immunization alone. No histologic changes were observed at this time point following delivery of allantoic fluid (Fig. 2 A&B) or the NDV vector alone (Fig. 2 E&F), but nasal cavity tissues collected from NDV-F vaccinated animals showed submucosal lymphoplasmacytic infiltrates (Fig 2 C&D), indicative of an acquired immune response. A more pronounced infiltrate was observed following NDV-F boosting (Fig. 2 G&H).

No histologic changes were observed in the lung following delivery of allantoic fluid or a priming dose of NDV vector alone (Fig. 2 I&L, J). However, in response to NDV-F priming, submucosal lymphoplasmacytic infiltrates similar to those observed in the nasal cavity mucosa, were observed surrounding bronchioles (Fig. 2 K), with an increased number of lymphocytes and plasma cells surrounding bronchioles following NDV-F boosting (Fig. 2 N). Similar peribronchiolar infiltrates were observed in response to a boosting dose of NDV vector alone (Fig. 2M), but these were less cellular than those that accompanied NDV-F vaccination. Additionally, following a second dose with NDV vector alone, or either dose of NDV-F, rare eosinophils were observed intermixed with the lymphocytic and plasma cell infiltrates surrounding bronchioles (Fig. 2 K, M&N). Based on these studies it appears that intranasal immunization with NDV-F causes no acute injury to the upper or lower airway, but does induce a mucosal lymphocytic response. The basis for the enhanced, though still mild, inflammatory response to NDV-F when compared with the NDV vector alone is not clear, but is consistent with our ability to boost immunity with a second, boosting vaccine dose.

3.2 NDV-F vaccination protects against RSV infection and reinfection

Tissues were harvested 4 days after RSV challenge of animals that had been immunized with two i.n. doses of allantoic fluid, NDV vector alone, or NDV-F. In the nasal cavity and lung tissue of animals receiving allantoic fluid or NDV vector alone prior to RSV challenge, primary RSV infection was associated with mucosal and submucosal inflammatory infiltrates composed primarily of lymphocytes (Fig. 3 A&B). The lungs from these mock-immunized cotton rats had sparse peribronchiolar lymphohistiocytic infiltrates, with mucus and inflammatory cells in small airways (Fig. 3 D&E). In contrast, both lung and nasal cavity tissues collected from RSV-challenged, NDV-F vaccinated cotton rats exhibited

changes similar to those observed in response to vaccination alone (Fig. 3 C&F), with a more pronounced lymphocytic infiltrate, but lacking the mucus production and mixed inflammatory infiltrate.

Viral load following challenge was assayed by immunohistochemistry (IHC) and by plaque assay. IHC staining with a polyclonal antiviral serum revealed diffuse RSV antigen staining in the nasal cavities and small foci of infected cells in the lower airways of mock and NDV vaccinated animals. In contrast, only rare RSV antigen positive cells were detected in the nasal cavities of the NDV-F vaccinated cotton rats, and none in the lower airways of the immunized animals (Fig. 4 A–F). In support of IHC findings, viable virions were not detected by plaque assay of nasal mucosa and lung collected from animals that had received NDV-F vaccination prior to RSV challenge (Fig. 4G).

Following a second RSV challenge delivered 6 months after the first RSV challenge of mock-vaccinated or NDV-F vaccinated animals, lung and nasal tissues were collected. IHC of nasal cavity and lung collected 4 days after secondary RSV challenge revealed rare, scattered antigen-positive cells in the nasal mucosa of cohorts primed with allantoic fluid or NDV vector only. No RSV antigen was detected in the lungs or nasal cavities of NDV-F vaccinated animals. Plaque assay of nasal cavity mucosa and lung tissues collected after secondary RSV challenge was negative in all animals (not shown).

3.3 NDV-F vaccination induces a long-lived mucosal antibody response

Serum, bronchoalveolar lavage (BAL) fluid and nasal wash (NW) fluid were collected at multiple time points after vaccination and RSV challenge for determination of RSV F-specific IgG levels by ELISA (Fig. 5). NDV-F vaccination induced robust, F-specific IgG responses that were boosted following RSV challenge and then maintained at high levels. While serum antibody levels were similar amongst cohorts 1–2 months after RSV challenge, mucosal F-specific antibody responses remained significantly elevated in BAL and NW fluid samples collected from NDV-F vaccinated animals compared to controls up to 6 months after RSV infection, the latest time point examined. Serum neutralization, assayed by plaque reduction, demonstrated that antibodies produced in response to NDV-F vaccination were neutralizing (Fig. 5 D).

Cervical lymph nodes were collected 4 days or 5 months after RSV challenge of mock and NDV-F primed and boosted animals, and RSV F-specific IgG antibody secreting cells (ASCs) were enumerated by ELISpot (Fig. 5 E). While a response was not detected, or was minimal, by cells harvested from cervical lymph nodes of mock or NDV vaccinated animals, robust F-specific ASC responses were observed in cervical lymph nodes collected from NDV-F vaccinated animals at both the 4 day and 5 month time points. This is a significant finding given the short-lived mucosal protection that follows natural infection, and the importance of augmenting this protection by any RSV vaccine candidate [12]. Reagents to assay IgA in the cotton rat model are not yet available.

3.4 NDV-F vaccination prevents eosinophilic lung inflammation

The cellularity of BAL fluid collected at multiple time points after vaccination and RSV challenge was determined by cell counts, and evaluated for cell type by examination of

Wright-Giemsa stained cytospin preparations (Fig. 6 A&B). While vaccination alone did not increase total BAL fluid cellularity, a small, but not statistically significant, increase in total BAL fluid cellularity was detected in all cohorts after primary RSV challenge. After secondary RSV infection this increase in cellularity was again detected in BAL fluid collected from animals that had been vaccinated with allantoic fluid or NDV vector alone, but not present in BAL fluids collected from NDV-F vaccinated animals.

As we have previously described [37], eosinophils are present in the lungs of naïve cotton rats, in the absence of pulmonary pathology, and the composition of the BAL fluid was not altered by vaccination alone. Consistent with our prior observations, eosinophils were significantly increased one month after primary RSV challenge of control animals receiving vector alone [37], but this increase did not occur in animals vaccinated with NDV-F (Fig. 6). The number of pulmonary eosinophils decreased nearly to baseline 2 months after primary RSV infection, but rose again in response to secondary RSV challenge of control animals that had received only allantoic fluid or NDV vector. This increase in BAL eosinophils following secondary RSV infection, while striking, did not reach statistical significance ($p = 0.06$). Thus, in the cotton rat model, NDV-F vaccination confers long-lasting protection, but also inhibits eosinophilic inflammation in response to RSV infection and reinfection.

3.6 Immunization of RSV-immune animals

Based on our vaccination strategy wherein the RSV F protein is expressed only in NDV-F infected cells, we hypothesized that it might be possible to immunize animals previously exposed to RSV. To test this possibility, 20 cotton rats were infected with RSV, and half of these were immunized with 10^6 pfu of NDV-F administered i.n. at 2 months, and again at 3 months, after primary RSV infection (Fig 7). The 2 month time point for vaccination was chosen to ensure that RSV F protein specific antibodies would be present in serum and at the mucosal surface of the respiratory tree (See Fig 5). Serum antibody titers were determined at 4 months, and again at 9 months after primary RSV infection as outlined in Fig. 7. All remaining animals were re-challenged with RSV at the 9 month time point, and no virus was detected in the lungs or noses of animals in either cohort at 2 or 4 days post virus instillation.

RSV F-specific IgG levels in all animals were measured by an F-protein specific ELISA assay which demonstrated an approximately 3-fold increase in animals that had received NDV-F (1/2500 versus 1/7500), a level maintained for 6 months after the boosting dose of vaccine was administered (Fig. 8B). Neutralizing antibody was assessed by incubating serum samples with rg-RSV, a recombinant virus expressing eGFP [42], and determining % of Vero cell infection by flow cytometry. The result of this assay is shown in Fig. 8A, demonstrating an enhancement of neutralizing antibody in animals immunized with NDV-F in the presence of RSV-neutralizing antibody, an enhancement which remained stable at the 9 month time point. This study suggests that, in addition to the ability of this vaccine to give long-lasting protection, NDV-F is also efficacious in those with pre-existing antibody.

4. Discussion

RSV is a major cause of lower respiratory tract disease in infants and the elderly worldwide. While an effective RSV vaccine has been eagerly sought, no licensed vaccine yet exists.

RSV vaccine development has focused primarily on live-attenuation, a strategy with demonstrated safety in infants, the target population for most RSV vaccines. Unfortunately, live-attenuated RSV vaccines evaluated clinically to date have been poorly immunogenic, an outcome that could be predicted from the poor immune response to natural RSV infection [43–45]. Disappointing immunogenicity of live-attenuated RSV vaccines has led investigators to diversify RSV vaccine strategies (recently reviewed in [46]). Here, we demonstrate that a Newcastle disease virus vectored RSV vaccine, designed to stimulate robust IFN production simultaneous with RSV F protein presentation to the immune system, protects against RSV infection and induces a long-lived humoral immune response.

While prevention of lower airway infection is the major goal of all RSV vaccine candidates, the importance of eliminating upper airway infection cannot be understated. Virus shedding during upper airway infection of otherwise healthy children and adults is the major source of infection for those most at risk for severe lower airway disease, thus it is essential that any RSV vaccine candidate prevent infection of both the upper and lower airways. In addition, the association of upper airway RSV infection with otitis media [47, 48] in young children remains a major clinical concern. NDV-F vaccination of the cotton rat completely protected both the upper and lower airway against RSV challenge as measured by plaque assay of lung and nasal wash. Rare RSV antigen positive cells were detected by immunohistochemistry in the nasal cavities of vaccinated animals following challenge, although the significance of this low level of virus replication is not clear.

A major goal of pre-clinical vaccine evaluation is to establish the safety profile of a vaccine candidate. This is particularly true for RSV vaccine candidates given the history of the 1960's vaccine trial in which enhanced pathology following natural RSV infection of infants that had previously received a formalin-inactivated RSV vaccine (FI-RSV) [49], this is particularly true for RSV vaccine candidates. It has been suggested that this vaccine-enhanced disease may have been related to the presence of non-neutralizing antibody, allowing virus replication and simultaneous immune complex deposition in the lung [50]. FI-RSV has been shown to generate primarily non-neutralizing antibody in mice, and RSV challenge of mice receiving the FI-RSV vaccine resulted in immune complex mediated pathology [50]. It is therefore important to demonstrate the induction of long-lasting, virus-specific adaptive immunity by any vaccine candidate, but also to verify the absence of disease-promoting immune responses.

Delivery of a protective dose of NDV-F was not associated with adverse histopathology. Lymphoplasmacytic infiltrates observed in immunized animals in response to the boosting NDV-F dose and subsequent challenge are consistent with an appropriate adaptive immune response (Fig 1). Additionally, the increase in lung eosinophils detected in cotton rats that been mock vaccinated prior to primary and secondary RSV challenge was not detected in the lungs of NDV-F vaccinated cotton rats. This finding is of particular interest when considered in light of mounting evidence that severe RSV lung disease during infancy is associated with asthma and recurrent wheezing later in childhood [6–9]. While the cotton rat model has become the gold standard for pre-clinical testing of RSV vaccines and treatments, reagents for the study of immune mechanisms in this species are still under development. Nonetheless, our previous characterization of NDV-F in the mouse model demonstrated that

exposure to NDV-F led to an enhanced T_H1 response following RSV challenge [34]. The relatively few immunological reagents and methods available for the cotton rat model make it more difficult to analyze T cell responses in this species, but it is interesting to note the eosinophilic response to RSV infection that occurs in cotton rats [37] and guinea pigs [51], both new world rodents. As shown here, this eosinophilic response to RSV in the cotton rat was abrogated by NDV-F vaccination. In the guinea pig model, eosinophilic inflammation following RSV infection was inhibited by pretreatment with IFN-inducing TLR ligand CpG (cytosine phosphate guanine oligodeoxynucleotides) [51]. Thus it is possible that the adjuvant effect of type I IFN induction, in addition to the prolongation of antibody responses, could also decrease T_H2 responses to inhaled allergens encountered during RSV infections in young children.

RSV-specific secretory and serum antibodies have been correlated with protection of the upper and lower airways, respectively, against re-infection with RSV [52, 53]. A recent study suggests that the ability of this virus to re-infect immunocompetent adults is correlated with a defect in B cell memory. Neutralizing serum and nasal antibody levels in experimentally infected healthy adult volunteers increased post-infection, but returned to baseline levels 6 months later [12]. This differs markedly from the lifelong persistence of influenza specific IgG that follows this infection [54]. Thus, it is essential that any RSV vaccine candidate induce both systemic and mucosal RSV-specific antibodies, but do so in a manner that induces a B cell memory response superior to that induced by RSV infection. It is this requirement that recommends consideration of a vectored RSV vaccine.

This strategy has recently been employed by Pierantoni et al. [55] who have generated vectored RSV vaccines using the chimpanzee Adenovirus and the Modified Vaccinia Ankara virus encoding the RSV F, N and M2-1 proteins (PanAd3-RSV and MVA-RSV). Intranasal vaccination of cotton rats with PanAd3-RSV provided complete protection of the nose and lungs at the highest doses following RSV challenge; intramuscular (i.m.) administration of MVA-RSV gave complete protection of the lungs following RSV instillation, and partial protection of the upper airway. Neutralizing antibody titers using either vaccine were superior to those induced by RSV infection alone, and remained elevated 3 months after vaccination. A combined vaccination protocol, with a priming dose of PanAd3-RSV given i.n. followed by an i.m. MVA-RSV boost protected both the upper and lower respiratory tracts from challenge and induced high levels of neutralizing serum antibody (on the order of 1/1000) 3 weeks after administration of the boosting dose [56]. No enhanced disease was present in vaccinated animals following challenge.

While PanAd3-RSV and MVA-RSV were both efficacious in cotton rats and in young calves seronegative for the closely related bovine RSV, the question of long term B cell memory was not addressed in these studies. In human subjects the neutralizing antibody response to RSV was boosted by either vaccine given i.m., but had waned by 6 months after priming [57]. In our study, NDV-F vaccination induced serum and mucosal F-specific antibodies that were maintained for at least 7 months. Importantly, the mucosal F-specific antibody response, which correlates most closely with protection against reinfection [12], was more robust and longer lived in NDV-F immunized cotton rats than that induced by RSV infection alone. These data lead us to propose that an NDV-vectored RSV vaccine may be the best

available approach to RSV vaccination - a proposition that must now be tested in the clinic. We do not yet know whether an NDV-vectored vaccine will be equally effective in human subjects, but successful trials of NDV-vectored influenza and ebola vaccines in non-human primates support the validity of this approach [58, 59]. The ability of NDV-F to boost neutralizing antibody titers in RSV seropositive animals suggests that vaccination of older children and adults may provide an avenue for protecting a population while avoiding the difficulties associated with vaccination of young infants.

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References

1. Hall CB, Long CE, Schnabel KC. Respiratory syncytial virus infections in previously healthy working adults. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*. 2001; 33(6):792–6. Epub 2001/08/21. DOI: 10.1086/322657 [PubMed: 11512084]
2. Paramore LC, Ciuryla V, Ciesla G, Liu L. Economic impact of respiratory syncytial virus-related illness in the US: an analysis of national databases. *PharmacoEconomics*. 2004; 22(5):275–84. Epub 2004/04/06. [PubMed: 15061677]
3. Alper CM, Winther B, Mandel EM, Hendley JO, Doyle WJ. Rate of concurrent otitis media in upper respiratory tract infections with specific viruses. *Archives of otolaryngology-head & neck surgery*. 2009; 135(1):17–21. Epub 2009/01/21. DOI: 10.1001/archotol.135.1.17 [PubMed: 19153302]
4. Hall CB, Walsh EE, Long CE, Schnabel KC. Immunity to and frequency of reinfection with respiratory syncytial virus. *The Journal of infectious diseases*. 1991; 163(4):693–8. Epub 1991/04/01. [PubMed: 2010624]
5. Simoes EA, Carbonell-Estrany X. Impact of severe disease caused by respiratory syncytial virus in children living in developed countries. *The Pediatric infectious disease journal*. 2003; 22(2 Suppl):S13–8. discussion S8-20. Epub 2003/04/03. DOI: 10.1097/01.inf.0000053881.47279.d9 [PubMed: 12671448]
6. Korppi M, Piippo-Savolainen E, Korhonen K, Remes S. Respiratory morbidity 20 years after RSV infection in infancy. *Pediatric pulmonology*. 2004; 38(2):155–60. Epub 2004/06/24. DOI: 10.1002/ppul.20058 [PubMed: 15211700]
7. Pelaia G, Vatrella A, Gallelli L, Renda T, Cazzola M, Maselli R, et al. Respiratory infections and asthma. *Respiratory medicine*. 2006; 100(5):775–84. Epub 2005/11/18. DOI: 10.1016/j.rmed.2005.08.025 [PubMed: 16289785]
8. Sigurs N, Bjarnason R, Sigurbergsson F, Kjellman B. Respiratory syncytial virus bronchiolitis in infancy is an important risk factor for asthma and allergy at age 7. *American journal of respiratory and critical care medicine*. 2000; 161(5):1501–7. Epub 2000/05/12. [PubMed: 10806145]
9. Sigurs N, Gustafsson PM, Bjarnason R, Lundberg F, Schmidt S, Sigurbergsson F, et al. Severe respiratory syncytial virus bronchiolitis in infancy and asthma and allergy at age 13. *American journal of respiratory and critical care medicine*. 2005; 171(2):137–41. Epub 2004/11/02. DOI: 10.1164/rccm.200406-730OC [PubMed: 15516534]
10. Buchman CA, Doyle WJ, Pilcher O, Gentile DA, Skoner DP. Nasal and otologic effects of experimental respiratory syncytial virus infection in adults. *American journal of otolaryngology*. 2002; 23(2):70–5. Epub 2002/03/15. [PubMed: 11893972]
11. Mills JT, Van Kirk JE, Wright PF, Chanock RM. Experimental respiratory syncytial virus infection of adults. Possible mechanisms of resistance to infection and illness. *Journal of immunology*. 1971; 107(1):123–30. Epub 1971/07/01.

12. Habibi MS, Jozwik A, Makris S, Dunning J, Paras A, DeVincenzo JP, et al. Impaired Antibody-mediated Protection and Defective IgA B-Cell Memory in Experimental Infection of Adults with Respiratory Syncytial Virus. *American journal of respiratory and critical care medicine*. 2015; 191(9):1040–9. DOI: 10.1164/rccm.201412-2256OC [PubMed: 25730467]
13. Wagner DK, Muelenaer P, Henderson FW, Snyder MH, Reimer CB, Walsh EE, et al. Serum immunoglobulin G antibody subclass response to respiratory syncytial virus F and G glycoproteins after first, second, and third infections. *Journal of clinical microbiology*. 1989; 27(3):589–92. Epub 1989/03/01. [PubMed: 2715331]
14. Welliver RC, Kaul TN, Putnam TI, Sun M, Riddlesberger K, Ogra PL. The antibody response to primary and secondary infection with respiratory syncytial virus: kinetics of class-specific responses. *The Journal of pediatrics*. 1980; 96(5):808–13. Epub 1980/05/01. [PubMed: 7365579]
15. Crowe JE Jr. Respiratory syncytial virus vaccine development. *Vaccine*. 2001; 20(Suppl 1):S32–7. Epub 2001/10/06. [PubMed: 11587807]
16. Jewell NA, Vaghefi N, Mertz SE, Akter P, Peebles RS Jr, Bakaletz LO, et al. Differential type I interferon induction by respiratory syncytial virus and influenza A virus in vivo. *Journal of virology*. 2007; 81(18):9790–800. Epub 2007/07/13. DOI: 10.1128/JVI.00530-07 [PubMed: 17626092]
17. Hall CB, Douglas RG Jr, Simons RL. Interferon production in adults with respiratory syncytial viral infection. *Annals of internal medicine*. 1981; 94(1):53–5. Epub 1981/01/01. [PubMed: 6160798]
18. Hall CB, Douglas RG Jr, Simons RL, Geiman JM. Interferon production in children with respiratory syncytial, influenza, and parainfluenza virus infections. *The Journal of pediatrics*. 1978; 93(1):28–32. Epub 1978/07/01. [PubMed: 206677]
19. McIntosh K. Interferon in nasal secretions from infants with viral respiratory tract infections. *The Journal of pediatrics*. 1978; 93(1):33–6. Epub 1978/07/01. [PubMed: 650342]
20. de Maeyer-Guignard J, de Maeyer E. Effect of antilymphocytic serum on circulating interferon in mice as a function of the inducer. *Nature: New biology*. 1971; 229(7):212–4. Epub 1971/02/17. [PubMed: 4325471]
21. Honda K, Sakaguchi S, Nakajima C, Watanabe A, Yanai H, Matsumoto M, et al. Selective contribution of IFN- α /beta signaling to the maturation of dendritic cells induced by double-stranded RNA or viral infection. *Proceedings of the National Academy of Sciences of the United States of America*. 2003; 100(19):10872–7. Epub 2003/09/10. DOI: 10.1073/pnas.1934678100 [PubMed: 12960379]
22. Bukreyev A, Collins PL. Newcastle disease virus as a vaccine vector for humans. *Current opinion in molecular therapeutics*. 2008; 10(1):46–55. Epub 2008/01/30. [PubMed: 18228181]
23. Biron CA, Nguyen KB, Pien GC, Cousens LP, Salazar-Mather TP. Natural killer cells in antiviral defense: function and regulation by innate cytokines. *Annual review of immunology*. 1999; 17:189–220. Epub 1999/06/08. DOI: 10.1146/annurev.immunol.17.1.189
24. Nguyen KB, Salazar-Mather TP, Dalod MY, Van Deusen JB, Wei XQ, Liew FY, et al. Coordinated and distinct roles for IFN- α beta, IL-12, and IL-15 regulation of NK cell responses to viral infection. *Journal of immunology*. 2002; 169(8):4279–87. Epub 2002/10/09.
25. Asselin-Paturel C, Brizard G, Chemin K, Boonstra A, O'Garra A, Vicari A, et al. Type I interferon dependence of plasmacytoid dendritic cell activation and migration. *The Journal of experimental medicine*. 2005; 201(7):1157–67. Epub 2005/03/30. DOI: 10.1084/jem.20041930 [PubMed: 15795237]
26. Fitzgerald-Bocarsly P, Dai J, Singh S. Plasmacytoid dendritic cells and type I IFN: 50 years of convergent history. *Cytokine & growth factor reviews*. 2008; 19(1):3–19. Epub 2008/02/06. DOI: 10.1016/j.cytogfr.2007.10.006 [PubMed: 18248767]
27. Curtsinger JM, Valenzuela JO, Agarwal P, Lins D, Mescher MF. Type I IFNs provide a third signal to CD8 T cells to stimulate clonal expansion and differentiation. *Journal of immunology*. 2005; 174(8):4465–9. Epub 2005/04/09.
28. Gil MP, Salomon R, Louten J, Biron CA. Modulation of STAT1 protein levels: a mechanism shaping CD8 T-cell responses in vivo. *Blood*. 2006; 107(3):987–93. Epub 2005/10/08. DOI: 10.1182/blood-2005-07-2834 [PubMed: 16210337]

29. Kolumam GA, Thomas S, Thompson LJ, Sprent J, Murali-Krishna K. Type I interferons act directly on CD8 T cells to allow clonal expansion and memory formation in response to viral infection. *The Journal of experimental medicine*. 2005; 202(5):637–50. Epub 2005/09/01. DOI: 10.1084/jem.20050821 [PubMed: 16129706]
30. Le Bon A, Schiavoni G, D'Agostino G, Gresser I, Belardelli F, Tough DF. Type I interferons potentially enhance humoral immunity and can promote isotype switching by stimulating dendritic cells in vivo. *Immunity*. 2001; 14(4):461–70. [PubMed: 11336691]
31. Le Bon A, Thompson C, Kamphuis E, Durand V, Rossmann C, Kalinke U, et al. Cutting edge: enhancement of antibody responses through direct stimulation of B and T cells by type I IFN. *Journal of immunology*. 2006; 176(4):2074–8.
32. Coro ES, Chang WL, Baumgarth N. Type I IFN receptor signals directly stimulate local B cells early following influenza virus infection. *Journal of immunology*. 2006; 176(7):4343–51. Epub 2006/03/21.
33. Lorence RM, Pecora AL, Major PP, Hotte SJ, Laurie SA, Roberts MS, et al. Overview of phase I studies of intravenous administration of PV701, an oncolytic virus. *Current opinion in molecular therapeutics*. 2003; 5(6):618–24. Epub 2004/02/06. [PubMed: 14755888]
34. Martinez-Sobrido L, Gitiban N, Fernandez-Sesma A, Cros J, Mertz SE, Jewell NA, et al. Protection against respiratory syncytial virus by a recombinant Newcastle disease virus vector. *Journal of virology*. 2006; 80(3):1130–9. Epub 2006/01/18. DOI: 10.1128/JVI.80.3.1130-1139.2006 [PubMed: 16414990]
35. Prince GA, Jenson AB, Horswood RL, Camargo E, Chanock RM. The pathogenesis of respiratory syncytial virus infection in cotton rats. *The American journal of pathology*. 1978; 93(3):771–91. Epub 1978/12/01. [PubMed: 362946]
36. Sacco RE, Durbin RK, Durbin JE. Animal models of respiratory syncytial virus infection and disease. *Curr Opin Virol*. 2015; 13:117–22. Epub 2015/07/16. DOI: 10.1016/j.coviro.2015.06.003 [PubMed: 26176495]
37. Grieves JL, Yin Z, Durbin RK, Durbin JE. Acute and Chronic Airway Disease After Human Respiratory Syncytial Virus Infection in Cotton Rats (*Sigmodon hispidus*). *Comp Med*. 2015; 65(4):315–26. [PubMed: 26310461]
38. Durbin JE, Hackenmiller R, Simon MC, Levy DE. Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. *Cell*. 1996; 84(3):443–50. Epub 1996/02/09. [PubMed: 8608598]
39. Gitiban N, Jurcisek JA, Harris RH, Mertz SE, Durbin RK, Bakaletz LO, et al. Chinchilla and murine models of upper respiratory tract infections with respiratory syncytial virus. *Journal of virology*. 2005; 79(10):6035–42. Epub 2005/04/29. DOI: 10.1128/JVI.79.10.6035-6042.2005 [PubMed: 15857989]
40. Pletneva LM, Haller O, Porter DD, Prince GA, Blanco JC. Interferon-inducible Mx gene expression in cotton rats: cloning, characterization, and expression during influenza viral infection. *J Interferon Cytokine Res*. 2006; 26(12):914–21. Epub 2007/01/24. DOI: 10.1089/jir.2006.26.914 [PubMed: 17238834]
41. Chaiwatpongsakorn S, Epand RF, Collins PL, Epand RM, Peebles ME. Soluble respiratory syncytial virus fusion protein in the fully cleaved, pretriggered state is triggered by exposure to low-molarity buffer. *Journal of virology*. 2011; 85(8):3968–77. DOI: 10.1128/JVI.01813-10 [PubMed: 21307202]
42. Hallak LK, Collins PL, Knudson W, Peebles ME. Iduronic acid-containing glycosaminoglycans on target cells are required for efficient respiratory syncytial virus infection. *Virology*. 2000; 271(2):264–75. DOI: 10.1006/viro.2000.0293 [PubMed: 10860881]
43. Gomez M, Mufson MA, Dubovsky F, Knightly C, Zeng W, Losonsky G. Phase-I study MEDI-534, of a live, attenuated intranasal vaccine against respiratory syncytial virus and parainfluenza-3 virus in seropositive children. *The Pediatric infectious disease journal*. 2009; 28(7):655–8. Epub 2009/06/02. DOI: 10.1097/INF.0b013e318199c3b1 [PubMed: 19483659]
44. Wright PF, Belshe RB, Kim HW, Van Voris LP, Chanock RM. Administration of a highly attenuated, live respiratory syncytial virus vaccine to adults and children. *Infection and immunity*. 1982; 37(1):397–400. Epub 1982/07/01. [PubMed: 7107009]

45. Wright PF, Shinozaki T, Fleet W, Sell SH, Thompson J, Karzon DT. Evaluation of a live, attenuated respiratory syncytial virus vaccine in infants. *The Journal of pediatrics*. 1976; 88(6): 931–6. Epub 1976/06/01. [PubMed: 178852]
46. Mazur NI, Martinon-Torres F, Baraldi E, Fauroux B, Greenough A, Heikkinen T, et al. Lower respiratory tract infection caused by respiratory syncytial virus: current management and new therapeutics. *Lancet Respir Med*. 2015; 3(11):888–900. DOI: 10.1016/S2213-2600(15)002556 [PubMed: 26411809]
47. Chonmaitree T, Revai K, Grady JJ, Clos A, Patel JA, Nair S, et al. Viral upper respiratory tract infection and otitis media complication in young children. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*. 2008; 46(6):815–23. DOI: 10.1086/528685 [PubMed: 18279042]
48. Heikkinen T, Ojala E, Waris M. Clinical and Socioeconomic Burden of Respiratory Syncytial Virus Infection in Children. *The Journal of infectious diseases*. 2017; 215(1):17–23. DOI: 10.1093/infdis/jiw475 [PubMed: 27738052]
49. Kim HW, Canchola JG, Brandt CD, Pyles G, Chanock RM, Jensen K, et al. Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. *American journal of epidemiology*. 1969; 89(4):422–34. Epub 1969/04/01. [PubMed: 4305198]
50. Polack FP, Teng MN, Collins PL, Prince GA, Exner M, Regele H, et al. A role for immune complexes in enhanced respiratory syncytial virus disease. *The Journal of experimental medicine*. 2002; 196(6):859–65. [PubMed: 12235218]
51. Sutton TC, Tayyari F, Khan MA, Manson HE, Hegele RG. T helper 1 background protects against airway hyperresponsiveness and inflammation in guinea pigs with persistent respiratory syncytial virus infection. *Pediatr Res*. 2007; 61(5 Pt 1):525–9. Epub 2007/04/07. DOI: 10.1203/pdr.0b013e3180459f5b [PubMed: 17413871]
52. Shinoff JJ, O'Brien KL, Thumar B, Shaw JB, Reid R, Hua W, et al. Young infants can develop protective levels of neutralizing antibody after infection with respiratory syncytial virus. *The Journal of infectious diseases*. 2008; 198(7):1007–15. Epub 2008/08/16. DOI: 10.1086/591460 [PubMed: 18702606]
53. Walsh EE, Falsey AR. Humoral and mucosal immunity in protection from natural respiratory syncytial virus infection in adults. *The Journal of infectious diseases*. 2004; 190(2):373–8. Epub 2004/06/25. DOI: 10.1086/421524 [PubMed: 15216475]
54. Fujimoto C, Takeda N, Matsunaga A, Sawada A, Tanaka T, Kimoto T, et al. Induction and maintenance of anti-influenza antigen-specific nasal secretory IgA levels and serum IgG levels after influenza infection in adults. *Influenza Other Respir Viruses*. 2012; 6(6):396–403. DOI: 10.1111/j.1750-2659.2011.00330.x [PubMed: 22226319]
55. Pierantoni A, Esposito ML, Ammendola V, Napolitano F, Grazioli F, Abbate A, et al. Mucosal delivery of a vectored RSV vaccine is safe and elicits protective immunity in rodents and nonhuman primates. *Mol Ther Methods Clin Dev*. 2015; 2:15018.doi: 10.1038/mtm.2015.18 [PubMed: 26015988]
56. Taylor G, Thom M, Capone S, Pierantoni A, Guzman E, Herbert R, et al. Efficacy of a virus-vectored vaccine against human and bovine respiratory syncytial virus infections. *Sci Transl Med*. 2015; 7(300):300ra127.doi: 10.1126/scitranslmed.aac5757
57. Green CA, Scarselli E, Sande CJ, Thompson AJ, de Lara CM, Taylor KS, et al. Chimpanzee adenovirus- and MVA-vectored respiratory syncytial virus vaccine is safe and immunogenic in adults. *Sci Transl Med*. 2015; 7(300):300ra126.doi: 10.1126/scitranslmed.aac5745
58. DiNapoli JM, Nayak B, Yang L, Finneyfrock BW, Cook A, Andersen H, et al. Newcastle disease virus-vectored vaccines expressing the hemagglutinin or neuraminidase protein of H5N1 highly pathogenic avian influenza virus protect against virus challenge in monkeys. *Journal of virology*. 2010; 84(3):1489–503. Epub 2009/11/20. DOI: 10.1128/JVI.01946-09 [PubMed: 19923177]
59. DiNapoli JM, Yang L, Samal SK, Murphy BR, Collins PL, Bukreyev A. Respiratory tract immunization of non-human primates with a Newcastle disease virus-vectored vaccine candidate against Ebola virus elicits a neutralizing antibody response. *Vaccine*. 2010; 29(1):17–25. Epub 2010/11/03. DOI: 10.1016/j.vaccine.2010.10.024 [PubMed: 21034822]

Highlights

- A Newcastle Disease Virus vectored vaccine expressing the RSV F protein protects the nose and lungs of cotton rats from RSV challenge without inducing vaccine-enhanced pathology.
- This vaccine induces long-lived neutralizing antibody.
- This vaccine candidate can be used to immunize RSV immune animals.

Figure 1. IFN induction by NDV-F

Cohorts (N = 3) of cotton rats were sacrificed 24 hours after receiving allantoic fluid, 10^6 pfu RSV or 10^6 pfu NDV-F. IFN induction was inferred by measuring upregulation of the IFN stimulated Mx1 gene transcription by quantitative RT-PCR.

Figure 2. Inflammation following NDV-F vaccination and boost

Cohorts of cotton rats were sacrificed 28 days after the priming intranasal immunization, or 1 month after a second, boosting dose given on day 28. Mock-immunized animals were given allantoic fluid alone, or the NDV vaccine vector. Representative sections from the nasal cavity and lung after a single priming dose (A-D, and I-K), or after both a priming and a boosting dose (E-H, L-N), are pictured. A lymphohistiocytic submucosal infiltrate is present in the nasal cavity (C, D), and surrounding bronchioles (K), after priming with NDV-F, with increased cellularity 1 month following the boosting dose (G, H, N). A minimal peribronchiolar infiltrate is detected only after priming and boosting with the NDV vector. Rare eosinophils are indicated (*). Images were captured at 100× or 400× (insets) magnification.

Figure 3. Histology of the nasal cavity and lung following primary RSV challenge

Nasal cavity (A-C) and lung (D-F) were collected 4 days after primary RSV challenge from animals that had been previously mock-vaccinated, or given a priming and boosting dose of NDV-F. Respiratory mucosa in the noses of infected animals was disrupted by lymphoplasmacytic infiltrates (A and B), whereas, the nasal cavities of NDV-F vaccinated animals (C) were similar to those observed following vaccination alone (Fig. 2). RSV infection of the lung induced mild peribronchiolar infiltrates of lymphocytes, plasma cells and rare eosinophils and mucus production, with plugging of small airways (D and E). RSV challenge of NDV-F vaccinated animals resulted in lymphoplasmacytic peribronchial infiltrates (F), but without obstruction of small airways by mucus and necrotic cells. Images were taken at 100× (D-E), 200× (A-C) or 400× (insets) magnification.

Figure 4. Viral burden following primary RSV challenge

Nasal cavity and lung were collected 4 days after primary RSV challenge from controls and animals that had been previously vaccinated. RSV immunohistochemistry revealed marked to complete reduction in antigen-positive cells in nasal cavity (A-C) and lung (D-F) collected from NDV-F immunized animals compared to controls. Virus was detected by plaque assay (G) only in samples collected from animals mock-immunized with allantoic fluid or vector alone. Images were taken at 200× (A-C), 100× (D-F) or 400× (insets) magnification.

Figure 5. Humoral immune responses to vaccination and RSV challenge

Serum, BAL fluid and NW fluid were collected at multiple time points after vaccination and RSV challenge and F-specific IgG levels were determined by ELISA (A-C). Virus neutralization assay (D) revealed that F-specific antibodies induced by NDV-F vaccination were neutralizing. Cervical lymph nodes (CLNs) were collected 4 days and 5 months after RSV challenge of vaccinated animals and F-specific IgG secreting cells (ASCs) were enumerated by ELISpot (E).

Figure 6. BAL fluid cellularity following vaccination and RSV challenge

BAL fluid was collected at multiple time points after vaccination and RSV challenge. Cellularity (A) of BAL fluid collected from animals in all cohorts increased slightly, but not significantly, in response to primary RSV challenge then decreased gradually over time. Cellularity increased in BAL fluid collected from mock and NDV, but not NDV-F, vaccinated animals in response to secondary RSV challenge. The percentage of eosinophils (B) in BAL fluid collected from mock and NDV, but not NDV-F vaccinated animals,

increased in response to primary and secondary RSV challenge. BAL fluid cellularity and eosinophil percentage were not affected by vaccination alone. * $p < 0.05$

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Figure 7. Immunization of RSV-immune animals

Cotton rats were infected with RSV on day 0. Two months post infection, 20/40 animals were primed with NDV-F, and boosted with a second dose 28 days later. At 4 months and 9 months post infection serum samples were taken from cohorts of immunized and control animals (N = 5). At the 9 month time point, all animals were given a second challenge of RSV, and sacrificed day 2 and day 4 post re-infection.

Figure 8. Serum antibody titers following post-infection immunization

Serum was harvested from cotton rats 4 or 9 months following primary RSV infection. (A) Levels of neutralizing antibody were determined by incubation of a 1/800 dilution of each serum with rgRSV, which was then used to inoculate A549 cells. Percentage of GFP-expressing cells was used to calculate % neutralization. * $p=0.0027$; ** $p<0.0001$. (B) Levels of RSV F specific antibody in each sample were determined by ELISA. * $p=0.0114$; ** $p=0.0004$. Levels of neutralizing and RSV F specific antibody were significantly higher in the NDV-F vaccinated animals at 4 and 9 months post primary infection as determined by the unpaired t test.