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A nanoemulsion-adjuvanted intranasal H5N1 influenza vaccine protects ferrets against homologous and heterologous H5N1 lethal challenge

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ABSTRACT

Background: Flu vaccines administered intramuscularly (IM) have shown seasonally fluctuating efficacy, 20–60%, throughout the last 15 years. We formulated a recombinant H5 (rH5) in our Nanovax® (NE01) (rH5/NE01) adjuvant for intranasal vaccination in ferrets. We evaluated the regimen, one vs two immunization, and cross clade protection a ferret challenge model.
Methods: Plant derived recombinant H5 (rH5) antigen was formulated with NE01 and administered intranasally to ferrets. Immunogenicity (IgG), hemagglutination inhibition (HI), and protection against lethal challenge, were measured following one or two immunizations. Protection against homologous (strain A/Indo) and heterologous (strain A/Vn) was evaluated in ferrets following two immunizations.
Results: IN immunization with rH5/NE01 induced significant IgG levels after one and two immunizations. One vaccination did not induce any HI while low HI was measured after two immunizations. Homologous challenge with H5N1 A/ Indonesia showed 100% survival, with minimal weight loss in animals vaccinated twice compared to the unvaccinated controls. Analysis of nasal wash from these challenged ferrets vaccinated twice showed decreased viral shedding compared to unvaccinated controls. Interestingly, animals that received one vaccination showed 88% survival with moderate weight loss. Cross clade protection was evaluated using an increased antigen dose (45 μg rH5). Vaccinated animals demonstrated increased IgG and HAI antibody responses. Both homologous (A/Indo) and heterologous challenge (A/Vietnam) following two immunizations showed 100% survival with no loss of body weight. However viral clearance was more rapid against the homologous (day 3) compared to the heterologous (day 5) post challenge.
Conclusion: Intranasal administration of NE01 adjuvant-formulated rH5 vaccine elicited systemic and probably mucosal immunity that conferred protection against lethal challenge with homologous or heterologous viral strains. It also enhanced viral clearance with decreased shedding. These outcomes strongly suggest that intranasal immunization using NE01 against flu infections warrants clinical testing.

1. Introduction

Influenza is a preeminent global health concern. Seasonal influenza A and B strains cause between 3 and 5 million cases of severe illness worldwide each year, including up to 500,000 deaths, with significant economic and social burden [1,2]. Highly pathogenic H5N1 avian influenza A virus has emerged and spread worldwide in migrating wild-bird populations killing millions of birds. The H5N1 avian influenza A virus also has caused infections with high morbidity upon spread from infected birds to humans during outbreaks in at least 16 countries. From 2003 to 2019, 53% of WHO-confirmed human A/H5N1 influenza infection cases resulted in deaths [3]. The high rate of mutation and genetic re-assortment of influenza viruses within animal reservoirs including birds may increase the risk of pathogenicity and enhanced transmission to humans. The majority of humans have minimal pre-existing immunity to the H5 hemagglutinin. This has raised concern regarding the potential for widespread epidemics caused by H5 influenza in humans, and the need for global pandemic preparedness.

Abbreviations: IM, intramuscular; IN, intranasal; rH5, recombinant H5; HI, hemagglutination inhibition; HA, hemagglutinin; MDCK, Madin-Darby canine kidney; NE01, nanoemulsion; TCID50, 50% tissue culture infective dose; GMT, geometric mean titers.

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Vaccine strategies provide the best approach to prevent severe respiratory disease caused by influenza, and parenteral non-adjuvanted and adjuvanted split-virus vaccines against H5N1 influenza have been licensed and stockpiled for intramuscular (IM) administration [4]. These vaccines are designed to elicit neutralizing antibody responses against hemagglutinin (HA), the surface glycoprotein of the influenza virus. Functional hemagglutination inhibiting (HAI) or virus-neutralizing antibodies are considered essential for protection against infection as these antibodies have been shown to prevent viral attachment to target cells [4–8]. None-the-less, reliable correlates of protection specifically against H5 influenza remain to be established [4,9]. The HA1 globular head region of HA containing the receptor-binding domain is highly variable due to antigenic drift, strong selective pressure induced by the host immune response, and continuous evolution of the influenza virus in animal reservoirs and humans [10]. Therefore, HAI antibodies typically confer only strain-specific neutralization with minimal cross-protection against drifted viruses.

A novel nanoemulsion mucosal adjuvant (NE01) in combination with plant-derived recombinant H5 hemagglutinin antigen (rH5) has been developed and tested in our laboratories as a safe, thermo-stable, easy-to-administer, and fully protective intranasal vaccine (designated rH5-NE01) against highly pathogenic H5N1 influenza as demonstrated in animal models [11]. The rH5 antigen, derived from H5N1 A/Indonesia/05/2005 (HAI-05) is manufactured under cGMP compliance in *Nicotiana benthamiana* tobacco plants, purified using a series of downstream processing steps, and has been characterized extensively *in vitro* and *in vivo* in animal models and humans [12–14]. The NE01 mucosal adjuvant ($W_{80}5EC$) is an oil-in-water nanoemulsion produced by high-speed homogenization of refined soybean oil, purified water, nonionic detergent, organic solvent and cationic surfactant. The NE01 nanoemulsion has been shown to adjuvant multiple different antigens including split-inactivated influenza virus [15,16] and recombinant H5 [11]. An intranasal nanoemulsion-based seasonal influenza vaccine formulated with Fluzone® HA antigens was safe, well-tolerated, and immunogenic as compared to parenteral Fluzone® or inactive control alone in a phase I clinical trial [17].

Previous studies in the ferret pre-clinical model have demonstrated full (100%) protection against mortality and respiratory disease while preventing loss of body weight, rise in body temperature, and viral-shedding after lethal challenge using homologous H5N1 (A/Indonesia/5/2005) influenza virus following three intranasal vaccinations with 20 μg rH5-NE01 [11]. This immunization regimen activated 90–100% seroconversion for HAI antibody responses against A/Indonesia. The current studies were conducted to assess the immunogenicity, rH5 antigen dose, and homologous (A/Indo) or cross-clade (AVn) protection following only one or two intranasal vaccinations using the rH5-NE01 mucosal vaccine in the ferret challenge model. These studies demonstrate significant protection after a single vaccination, and full protection against both the homologous A/Indo and the heterologous A/Vn following two (prime-boost) vaccinations, even in the absence of serum HAI antibody responses. This mucosal immunization strategy may provide a rapid path for advancement of the novel mucosal rH5-NE01 pandemic influenza vaccine to evaluation in humans.

2. Materials and methods

2.1. Nanoemulsion adjuvant

NE01 (60%) (formerly identified as $W_{80}5EC$) was used to prepare the intranasal nanoemulsion-based vaccines. The NE01 adjuvant was manufactured under engineering specifications by high-shear homogenization of water, ethanol, cationic and non-ionic surfactants, and highly-refined soybean oil to form an oil-in-water nanoemulsion with mean particle size of approximately 460 nm [18,19].

2.2. Plant-derived recombinant influenza hemagglutinin rH5 antigen

Recombinant H5 (rH5) from the H5N1 A/Indonesia/05/05 strain of influenza virus was engineered, manufactured, and purified by Fraunhofer USA Center for Cell and Molecular Biology (FhCMB) as previously described [13,14]. Briefly, the HA sequence, encompassing amino acids 17–532 was selected for expression in tobacco plants. The native signal peptide was replaced with the signal peptide from the tobacco PR-1a signal sequence at the N-terminus. In addition, an endoplasmic reticulum retention signal and a hexahistidine (6xHis) affinity purification tag were added to the C-terminus. The resulting sequence was inserted into the pGR-D4 vector, which was engineered as a binary expression vector [20]. Agrobacteria carrying this engineered gene encoding the rH5 antigen were introduced into *Nicotiana benthamiana* plants by vacuum infiltration, and subsequently leaf tissue was harvested, and homogenized. Resultant plant-derived protein extracts were purified by a series of immobilized metal affinity column (IMAC) and other chromatography steps. The purified rH5 antigen (1.2 mg/ml) was characterized for purity (≥90%) based on SDS-PAGE (molecular mass) and reverse-phase chromatography, identity by Western blot analysis, and potency by SRID [12,13,20].

2.3. Nanoemulsion vaccine preparation

Different concentrations of the rH5 antigen were prepared by dilution in formulation buffer consisting of 150 mM NaCl, 50 mM Tris (pH 8.0), 8% sucrose and 10 mM histidine. The nanoemulsion vaccines were prepared by simple mixing of one volume 60% NE01 adjuvant with two volumes of rH5 antigen (1:2 vol/vol) in formulation to yield the final rH5 antigen dose in 20% NE01. This formulation was designated as rH5-NE01.

2.4. Intranasal immunization of ferrets using rH5-NE01 mucosal vaccine

Ferret studies were conducted at Southern Research (SR), Birmingham AL. The studies were performed in compliance with the Humane Care and Use of Laboratory Animals Policy under an inter-institutional animal assurance from the NIH, Office of Laboratory Animal Welfare (OLAW) issued to BlueWillow Biologics. All ferret protocols were reviewed and approved by the Southern Research Institutional Animal Care and Use Committee (IACUC). Castrated and de-scented male Fitch ferrets, 5–8 months of age (1–1.4 Kg body weight), were supplied by Triple F Farms (Sayre, PA). The ferrets were confirmed as seronegative for circulating human influenza A, B and H5N1 influenza strains. To obtain groups with comparable body weights, all ferrets were assigned to their respective treatment groups using a computer-generated randomization procedure. The ferrets (n = 8 animals per group) were immunized intranasally (IN) by pipette using either one, or a series of two vaccinations at a four-week interval in a volume of 0.5 mL rH5-NE01 (0.25 mL per nare) per animal. Control animals (n = 6–8 per group) received formulation buffer (0.5 mL) intranasally as a placebo. The ferrets were challenged using a lethal infectious dose of H5N1 influenza virus at 4 weeks following the final vaccine dose as described below.

2.5. H5N1 highly pathogenic avian influenza virus challenge of ferrets

Stock viruses of H5N1 Strain A/Indonesia/5/2005 (A/Indo/5/05) (clade 2.1.3.2) or H5N1 Strain A/Vietnam/1203/04 (clade 1) were received from the Centers for Disease Control and Prevention. The stock virus was amplified at SR in embryonated hen's eggs according to standard protocols. Ferrets were challenged in an A/BSL3 facility using approximately l0 times the 50% lethal dose of A/Indonesia/05/2005 or A/Vietnam/1203/2004 diluted in DPBS. Groups of ferrets (n = 6–8 animals per group) intranasally received an H5N1 virus inoculation volume of 1.0 mL per animal (0.5 mL per nare) using a pipette at 4 weeks following the final vaccination. The animals were evaluated post-challenge (pc) for survival, H5N1-viral shedding and clinical signs of infection that included body temperature, body weight loss, and total lethargy by activity scores for each ferret using a scale from 0 (normal activity)- 3 (very lethargic and non-responsive). Activity scores: 0 = alert and playful; 1 = alert but playful only when stimulated; 2 = alert but not playful when stimulated; and, 3 = neither alert nor playful when stimulated [21]. Animals that met at least one of the morbidity criteria, >20% weight loss, neurological symptoms, recumbency, or respiratory distress, were removed and euthanized. An implantable micro-identification and transponder chip device (IPTT-300 Chip; Biomedic Data Systems Inc.; Seaford, DE) was used to monitor body temperature (°F). H5N1-viral shedding in 1 mL nasal washes was determined by titration on Madin-Darby canine kidney (MDCK) cells and reported as $TCID_{50}$ per ml.

2.6. Determination of serum rH5-specific IgG by ELISA

ELISA plates (96-well) were coated using plant rH5 antigen at 2 μg/ml in DPBS and subsequently blocked using 5% BSA (Sigma Aldrich). The secondary antibody for rH5-specific IgG detection was horseradish peroxidase conjugated Anti-ferret IgG (Abcam). TMB (NEOGEN Enhanced K-Blue® TMB Substrate) was used as the substrate for detection. The endpoint titer was determined by extrapolating from the closest OD values above and below the cut-off value (3 times the average blank OD value) and calculating the average of these two values.

2.7. Hemagglutination inhibition assays

Ferret serum samples were pre-treated with receptor-destroying enzyme (RDEII; Denka Seiken Co. Ltd., Tokyo, Japan) to remove non-specific inhibitors of hemagglutination. A range of two-fold serial serum dilutions starting at 1:10 was evaluated in the hemagglutination inhibition (HAI) assay using 4 hemagglutinating units (HAU) of H5N1 A/Indonesia/05/2005xPR8 or A/Vietnam/1203/2004xPR8 influenza virus strains (obtained from CDC and propagated at Southern Research, Birmingham, Al), and 1% horse erythrocytes [22–24]. The HAI titer was determined as the reciprocal of the lowest serum dilution that showed non-agglutination [25]. Serum samples were run in triplicate and the Geometric mean titers (GMT) for each animal were used to calculate GMT serum antibody titers for each study group. All negative titers are reported as 5 and all titers that were at least 4-fold higher (GMT = 20) were considered significant.

2.8. Statistical analysis

Group means and percent loss/change from baseline were calculated for body weights, body temperatures, activity scores, and nasal wash titers. Geometric mean titers were calculated for HAI and rH5-specific serum IgG antibody titers. Mann-Whitney analysis was utilized to detect statistical differences; P values<0.05 were considered significant. Comparison of survival curves was evaluated using the Log-rank (Mantel-Cox) test. Graph-Pad Prism software version 7.04 (GraphPad Software, San Diego, CA) was utilized for statistical analysis.

3. Results

3.1. Evaluation of the intranasal rH5-NE01 vaccine candidate for immunogenicity and protection against challenge using highly pathogenic H5N1 A/Indonesia influenza virus in ferrets

3.1.1. Immunogenicity and protection after one versus two intranasal vaccinations using rH5-NE01 in ferrets

To evaluate the mucosal rH5-NE01 vaccine for immunogenicity and protection against H5N1 A/Indonesia (A/Indo) influenza virus challenge, ferrets (8 animals per group) were immunized intranasally using one verses two vaccinations at a four-week interval with 20% NE01+20 μg rH5 plant-derived antigen. Control animals (6 ferrets per group) received formulation buffer alone. As shown in Fig. 1, a single vaccination using the rH5-NE mucosal vaccine activated rH5-specific IgG (GMT: 2.1×10^4) with 100% seroconversion (8/8 animals) (Fig. 1A) in the absence of detectable serum HAI antibody responses (Fig. 1B) when evaluated at 24 days (3.4 weeks) post-vaccination. By comparison, following 2 vaccinations, rH5-specific IgG had increased to 2.7×10^5 (GMT) (100% seroconversion) when evaluated at 24 days post-vaccination; however, only 13% of these animals (1/8) produced HAI antibody against H5N1 A/Indonesia. These findings indicate that vaccination using only one or two doses of the mucosal rH5-NE01 vaccine was sufficient to activate full seroconversion for rH5-specific IgG yet with very low, or undetectable HAI antibody responses in serum.

These ferrets were challenged intranasally using a lethal dose of H5N1 A/Indonesia virus at four weeks following the final (x1 or x2) vaccination. As shown in Fig. 2, ferrets immunized using two doses of the intranasal rH5-NE01 vaccine were protected fully from H5N1 challenge as demonstrated by 100% survival (8/8 animals) (Fig. 2A), minimal loss of body weight (<5% mean weight loss) (Fig. 2B) and normal activity scores (0 = alert and playful) (Fig. 2C). In contrast, survival of only 2/6 animals (33%) together with severe weight loss (>15–20%), and greatly increased relative inactivity scores corresponding to lethargy and reduced responsiveness to stimuli using a rating scale as described in the Materials & Methods section, was observed in the control group that intranasally received formulation buffer. All animals showed a peak rise in body temperature at approximately 24 h post-challenge (Fig. 2D). Thus, immunization using this vaccine dose (20 μg rH5 antigen) did not prevent fever when compared to control animals. Finally, ferrets immunized using two vaccine doses showed significantly reduced viral shedding in nasal washes with time post-challenge when compared to control animals that received formulation buffer, or animals immunized using a single vaccine dose (Fig. 3).

Interestingly, ferrets immunized using only a single dose of the intranasal rH5-NE01 vaccine also were protected substantially from H5N1 virus challenge as shown by 88% survival (7/8 animals), moderate levels of weight loss (up to 10% on average), and significant improvement in activity scores relative to control animals (Fig. 2). Notably all surviving animals (7/8) in this single-vaccination group had returned to normal body weight by day 10 post-challenge (pc).

These results indicate that even a single rH5-NE01 vaccine (20 μg/dose) may provide significant protection against lethal challenge using highly pathogenic homologous H5N1 virus (A/Indo). However, maximum protection was achieved using a prime-boost immunization strategy consisting of two intranasal rH5-NE01(20 μg/dose) vaccine doses.

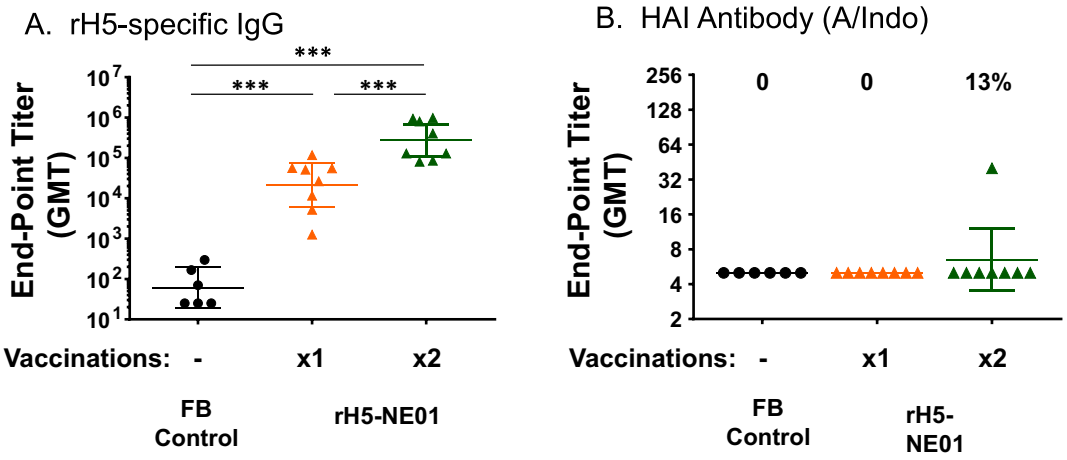

Fig. 1. Comparison of serum rH5-specific antibody responses following one verses two intranasal vaccinations. Ferrets received one (x1) or two (x2) intranasal vaccinations using 20 μg rH5+20% NE01 adjuvant or formulation buffer (FB) as a control. Serum IgG (A) and HAI antibodies (B) were evaluated in sera obtained 3.4 weeks following the last vaccination. Data are shown as geometric mean titer (GMT) with 95% confidence interval (CI); ***P < 0.001 (Mann-Whitney). Percentage of animals with HAI GMT ≥ 40 is shown at top (right).

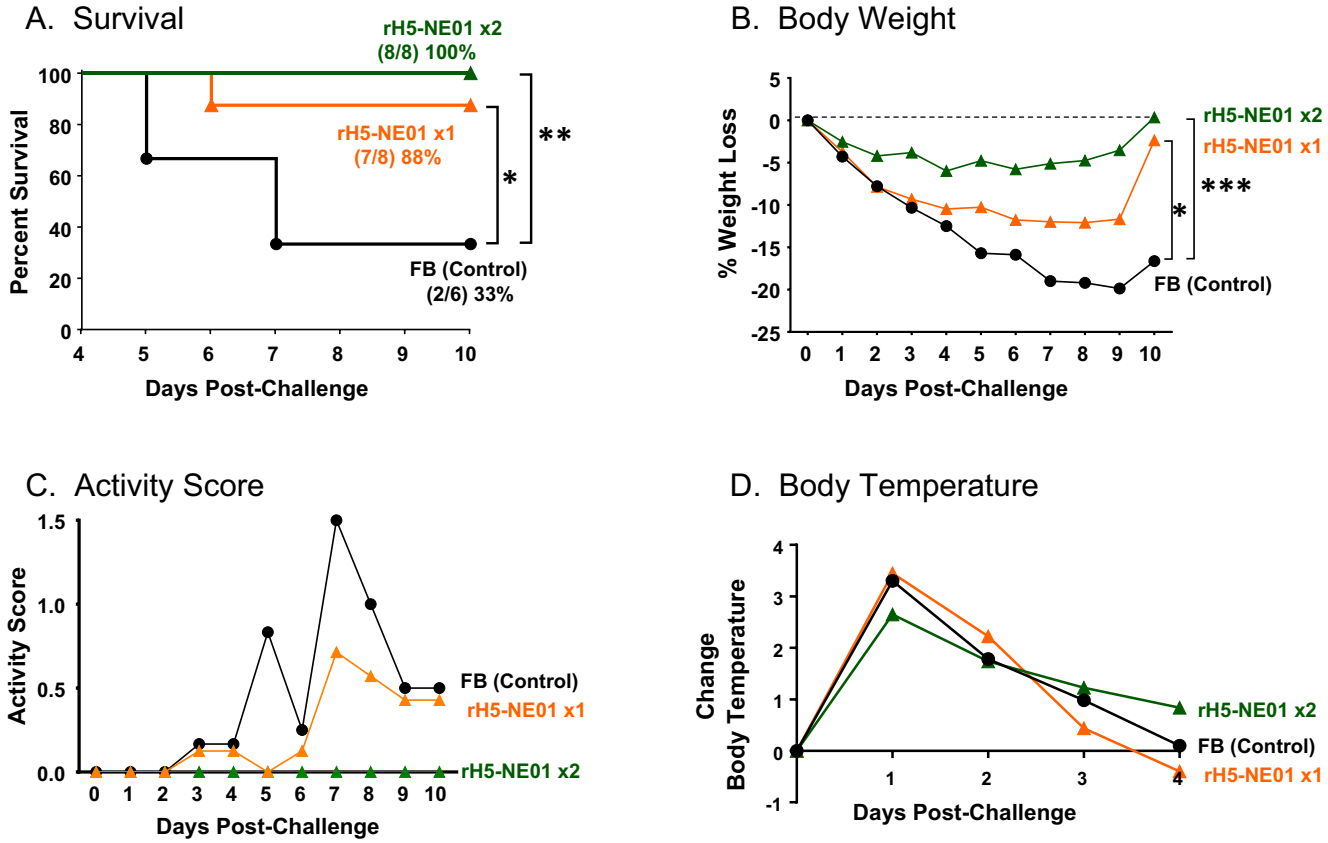

Fig. 2. Comparison of survival and morbidity indicators following H5N1 A/Indonesia influenza virus challenge after one verses two intranasal vaccinations. Ferrets were immunized using 20 μg rH5-NE01 or formulation buffer (FB) as a control as described in the legend of Fig. 1. The ferrets were challenged intranasally using H5N1 A/Indonesia 4 weeks following the final vaccination. **A.** Survival: the number of animals per group, percent survival and statistical significance is shown at right; *P < 0.05; **P < 0.01 (Log-Rank, Mantel-Cox); **B.** Body Weight is shown as mean percent weight loss relative to Day 0 (pre-challenge); *P < 0.05; ***P < 0.001 (Mann-Whitney); **C.** Activity Scores (mean) were determined as described under the Materials and Methods section. **D.** Body Temperature is shown as mean change in body temperature relative to Day 0.

3.1.2. Immunogenicity and protection against homologous (A/Indo) or heterologous (A/Vn) influenza virus challenge after two intranasal vaccinations using an increased dose (45 μg/dose) rH5-NE01 in ferrets

To evaluate rH5-NE01 vaccine for a cross clade protection, we increased the vaccine dose from 20 μg/dose to 45 μg/dose which may increase the cross-reactive antibody proportion in the total rH5 specific antibody population. Sixteen animals per group were immunized with rH5-NE01 or formulation buffer twice, four weeks apart. Serum antibody responses were evaluated at 24 days (3.4 weeks) following the final vaccination as shown in Fig. 4. These animals demonstrated 100% seroconversion for rH5-specific IgG (GMT: 3.3×10^5) (Fig. 4A); 69% seroconversion for

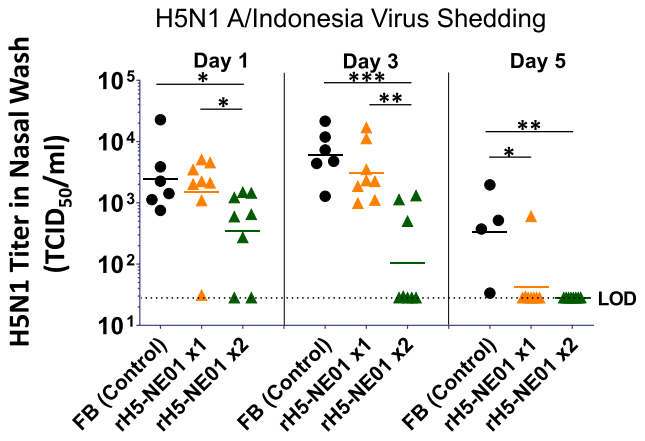

Fig. 3. H5N1 A/Indonesia virus shedding in nasal wash post-challenge following one verses two intranasal vaccinations. Ferrets were immunized intranasally (x1 or x2) using 20 μg rH5-NE01 or formulation buffer (FB) as a control and challenged with H5N1 A/Indonesia four weeks following the final vaccination as described in the legend of Fig. 2. Data are shown as Tissue culture infectious dose per ml ($TCID_{50}$/ml) for each animal and geometric mean (bar) for each group. *P < 0.05; **P < 0.01; ***P < 0.001 (Mann-Whitney); LOD: Limit of detection.

HAI antibody against A/Indo (11/16 animals ≥ 40) (GMT: 82) (Fig. 4B); and, only 25% seroconversion for HAI antibody responses against A/Vn (4/16 animals ≥ 40) (GMT: 13) (Fig. 4C).

These findings indicate that increasing the rH5 antigen dose from 20 μg to 45 μg rH5-NE01 activated equivalent rH5-specific IgG responses (GMT ~3×10^5, 100% seroconversion) while improving the level and seroconversion rate of HAI antibody responses against A/Indo from 13% to 69% (Figs. 1 and 4). None-the-less, 6/16 animals (38%) did not produce detectable serum HAI antibodies against A/Indo, and 12/16 animals (75%) did not produce HAI antibodies against A/Vn strain.

Four weeks post the second immunization, animals from each group were randomly split into two 8-animal groups to assess the protective efficacy of the rH5-NE01 vaccine against a lethal challenge using homologous H5N1 A/Indonesia (clade 2.1) or heterologous A/Vietnam (clade 1) strains of influenza virus. All animals that received the rH5-NE01 vaccine were protected completely against both homologous and heterologous challenge as determined by 100% survival (Fig. 5A) and no loss of body weight (pc) (Fig. 5B). These animals also showed normal body temperature or significantly reduced fever (Fig. 6A), normal activity scores (Fig. 6B); and significantly reduced shedding in nasal washes with return to background by days 3–5 following lethal challenge with either A/Indo or A/Vn strains of H5N1 virus (Fig. 7A & B).

In contrast, animals that received formulation buffer as a control showed poor survival (1/8; 13%), severe loss of body weight (>15–20%), rapid rise in body temperature (>3.5 °F) with fever spiking on day 1 for A/Indo and days 2–3 for A/Vn, and significant changes in inactivity scores (>1–2) indicating lethargy and poor response to stimuli when evaluated with time post-challenge (Figs. 5 and 6). Beginning on day 6–7, control animals showed evidence of dehydration, neurological symptoms (dragging hind limbs, stumbling, circling and ataxia), dyspnea, hypoactivity, and cold-to-the-touch. Animals that met at least on endpoint illness criteria or had an activity score of 3, were euthanized.

These studies demonstrate broad and effective cross-clade protection against highly pathogenic H5N1 virus challenge following 2 intranasal doses using the mucosal rH5-NE01 vaccine in the pivotal pre-clinical ferret challenge model. This complete protection against homologous or heterologous challenge did not correlate directly with serum HAI antibody levels against these strains of H5N1 virus.

4. Discussion

Previous studies in our laboratories have established strong proof-of-concept for a novel mucosal nanoemulsion-adjuvanted rH5-NE01 vaccine against pandemic influenza in the pivotal preclinical ferret protection model [11]. These studies demonstrated high immunogenicity and complete (100%) protection against mortality, morbidity and viral-shedding following lethal challenge using highly pathogenic H5N1 A/Indonesia influenza virus after three intranasal vaccinations using 20 μg rH5 plant-derived antigen in combination with 20% NE01 adjuvant. The current studies were designed to evaluate i. immunogenicity and protection in the ferret challenge model of one or two intranasal vaccinations with rH5-NE01 vaccine, ii. the ability for this new intranasal adjuvant/delivery to elicit efficient protective immunity that can protect against viral infections at their port of entry, and iii. whether cross clade protective immunity is induced using this adjuvant and route of immunization.

Substantial protection in the ferret model was achieved after only 1 intranasal vaccination, while full protection required 2 vaccinations using 20 μg rH5-NE01 to enable 100% survival, reduction in viral shedding, and complete prevention of illness following lethal H5N1 homologous (A/Ind) virus challenge. These levels of protection using the mucosal rH5-NE01 vaccine were accomplished under conditions that induced 100% seroconversion for

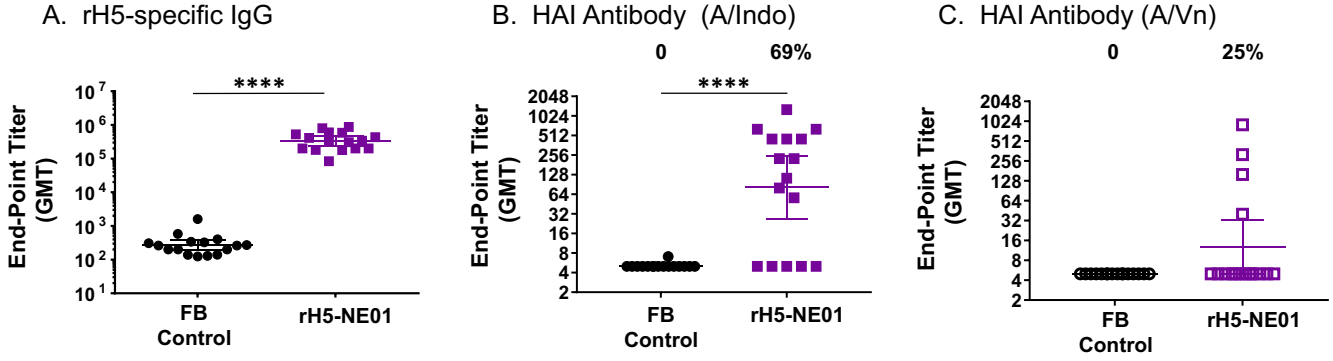

Fig. 4. Serum rH5-specific antibody responses against homologous vaccine strain (A/Indo) or heterologous (A/Vn) influenza virus strain following two intranasal vaccinations. Ferrets (n = 16 animals per group) received two intranasal vaccinations at a 4 week interval using 45 μg rH5+20% NE01 adjuvant or formulation buffer (FB) as a control. Serum was obtained 24 days (3.4 weeks) following the final vaccination. Data are shown as geometric mean titer (GMT) with 95% confidence interval (CI); ****P < 0.0001 (Mann-Whitney). Percentage of animals with HAI GMT ≥ 40 is shown at top.

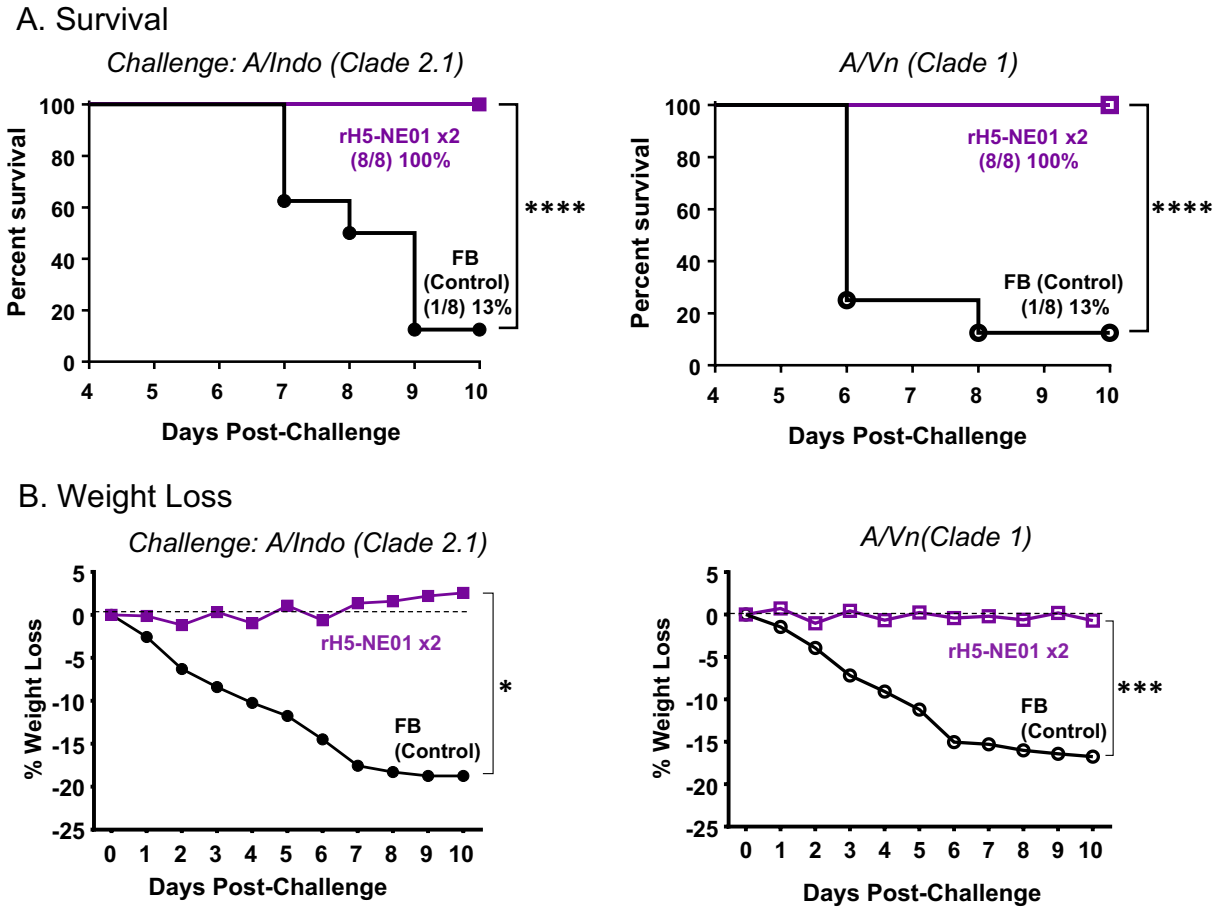

Fig. 5. Survival and weight loss post-challenge using homologous (A/Indo) or heterologous (A/Vn) H5N1 virus challenge following two intranasal vaccinations. Ferrets (n = 16 per group) were immunized using 45 μg rH5-NE01 or formulation buffer (FB) as a control as described in the legend of Fig. 4. Animals (n = 8 per group) were challenged intranasally using H5N1 A/Indonesia or A/Vietnam influenza virus strains 4 weeks following the second vaccination. **A.** Survival: percent survival and statistical significance is shown at right; ****P < 0.0001 (Log-Rank, Mantel-Cox); **B.** Body Weight is shown as mean percent weight loss relative to Day 0 (pre-challenge); *P < 0.05; ***P < 0.001 (Mann-Whitney).

rH5-specific IgG yet with very low or absent serum HAI antibody production, a standard correlate of protection for conventional intramuscular influenza vaccines [6,9,26]. An increase in the rH5 antigen dose from 20 μg to 45 μg rH5-NE01 was shown to augment the level and seroconversion rate of HAI antibody responses. However, 6/16 animals (38%) still did not produce serum HAI antibodies against A/Indonesia (clade 2.1), and 12/16 animals (75%) did not produce HAI antibodies against the A/Vietnam (clade 1) virus strain. Regardless of serum HAI antibody titer, these animals were protected fully against homologous (A/Indo) or heterologous (A/Vn) lethal H5N1 influenza virus challenge as evidenced by 100% survival and absence of morbidity when compared to control animals. These observations suggest that immune effectors other than serum HAI antibodies may be contributing to the protection observed; potentially including mucosal immunity induced by intranasal immunization using the rH5-NE01 vaccine.

Recently non-HAI antibodies directed against the stem/stalk region (HA2) of the HA molecule have been identified [27]. These anti-HA-stem antibodies, which are activated at low frequency during the response to influenza virus infection, recognize epitopes that are distal to the receptor binding site of H5 HA, and do not inhibit influenza virus binding to cells or hemagglutination [28–30]. Instead, these antibodies prevent viral entry by inhibition of fusion between influenza viral and host cell membranes. Our data showing cross-clade protection against heterologous H5N1 A/Vietnam after immunization using the rH5-NE01 mucosal vaccine in the present studies, even with low or absent serum HAI antibodies, may suggest that our intranasal administration route together with the monomeric nature of the HA antigen, had induced non-hemagglutinating cross protective antibodies.

Nanoemulsion adjuvant has been shown to enhance a balanced Th1, Th2, and Th17 type of cell-mediated immune response [15,18,19,31–33] that may be critical both for systemic and mucosal anti-viral immunity [34]. IFNγ-producing T-cells have been detected in the peripheral blood of ferrets prior to H5N1 challenge even after a single (D. Smith, unpublished observations) or multiple [11] intranasal immunizations using the rH5-NE01 mucosal vaccine. In addition to its anti-viral and immunomodulatory activities, IFNγ enhances immunoglobulin heavy-chain class-switching toward isotypes that mediate efficient FcγR binding [35,36], up-regulation of FcγR, and activation both of CTLs and ADCC-effector cells such as macrophage/monocytes, neutrophils, and NK cells. These findings are consistent with the hypothesis that cross-reactive cell-mediated responses in combination with anti-rH5 IgG antibody responses may enhance protection against both homologous and heterologous H5N1 virus challenge vaccination with the NE-rH5 mucosal vaccine.

Induction of mucosal immunity is a hallmark of nanoemulsion-based intranasal vaccines including activation of Th1 and Th17-type cellular immune responses ([19,37,38]) and local production of IgG and IgA antibodies at the sites of viral entry and infection in the nasal and respiratory mucosa [11,18]. In contrast, conven-

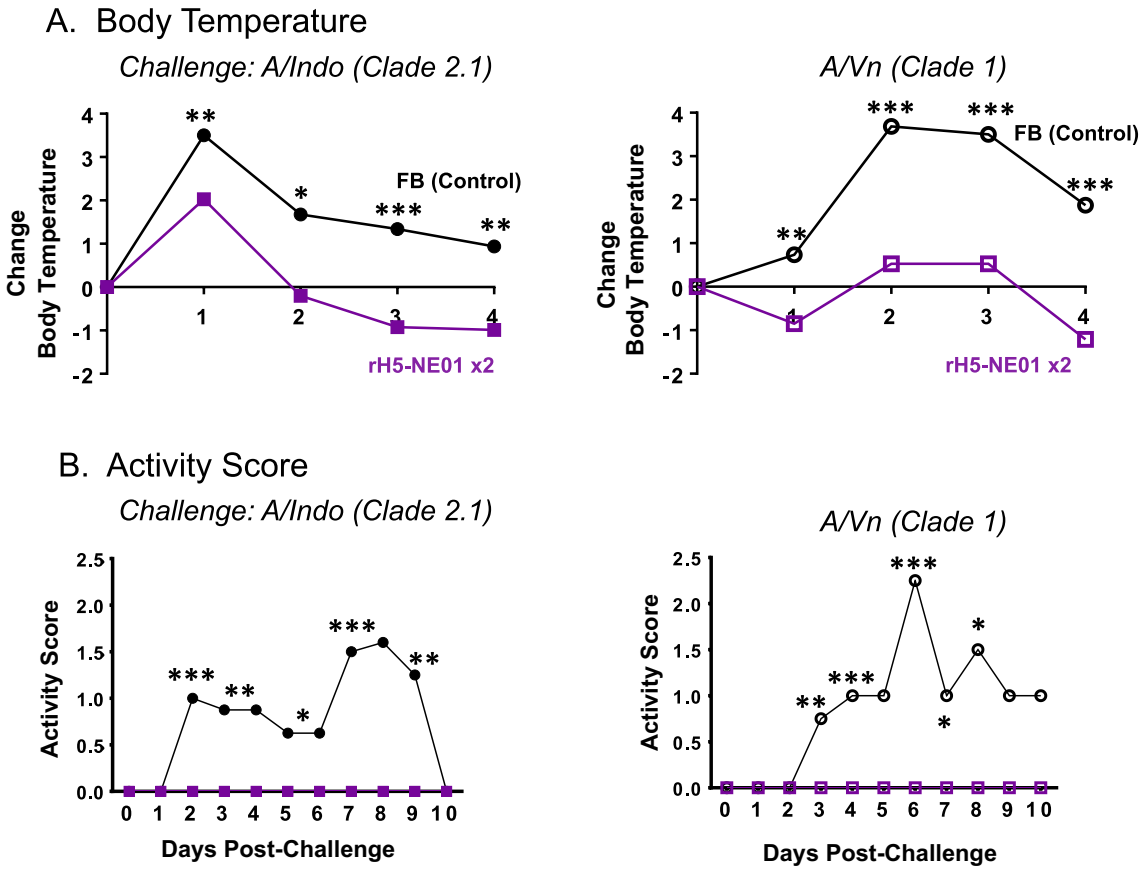

Fig. 6. Body temperature and activity scores post-challenge using homologous (A/Indo) or heterologous (A/Vn) H5N1 virus following two intranasal vaccinations. Ferrets were immunized using 45 μg rH5-NE01 or formulation buffer (FB) as a control and challenged with either H5N1 A/Indo or A/Vn strains of influenza virus (8 animals per group) 4 weeks following the second vaccination as described in the legend of Fig. 5. **A.** Body Temperature is shown as change in body temperature relative to Day 0 (Mean, n = 8 animals per group). **B.** Activity Scores (Mean) were determined as described in the Materials and Methods section. *P < 0.05; **P < 0.01; ***P < 0.001 (Mann-Whitney).

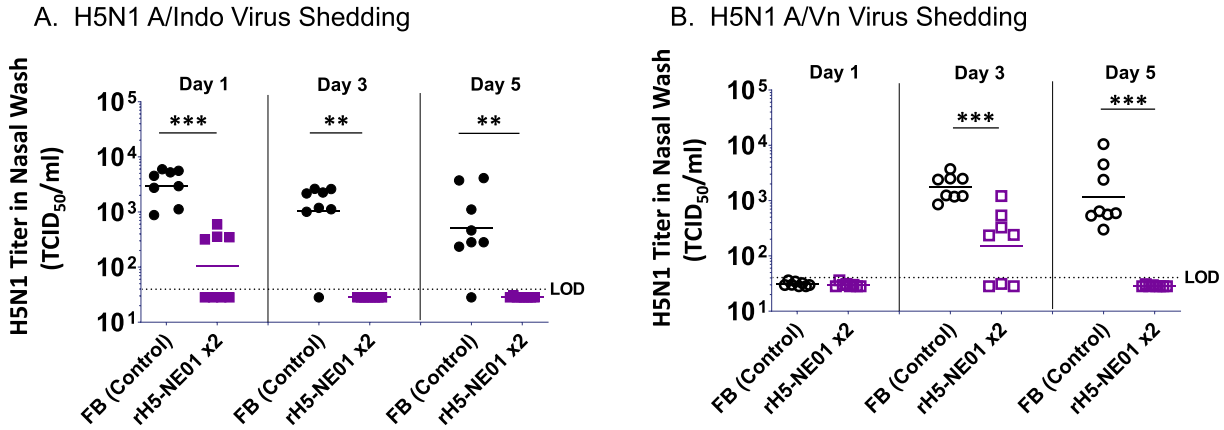

Fig. 7. Viral shedding in nasal wash post-challenge using homologous (A/Indo) or heterologous (A/Vn) H5N1 influenza virus following two intranasal vaccinations. Ferrets were immunized using 45 μg rH5-NE01 or formulation buffer (FB) and challenged with either H5N1 A/Indo or A/Vn strains of influenza virus (8 animals per group) 4 weeks following the second vaccination as described in the legend of Fig. 5. Data are shown as Tissue culture infectious dose per ml ($TCID_{50}$/ml) for each animal and geometric mean (bar) for each group. *P < 0.05; **P < 0.01; ***P < 0.001 (Mann-Whitney); LOD: Limit of detection.

tional parenteral vaccines do not efficiently activate mucosal mechanisms of protection [39,40]. Interestingly, early studies (2001) using an enterotoxin-based nasal adjuvant in a murine heterologous influenza virus challenge model demonstrated striking advantages for cross-protection against H5N1 virus when comparing mucosal versus parenteral routes of vaccine administration [41]. Animals that intranasally received 3 vaccinations using an enterotoxin-based adjuvanted H3N2 inactivated whole virus vaccine were protected fully against heterologous challenge using highly pathogenic H5N1 virus as shown by 100% survival and reduced virus shedding in the respiratory tract compared to control animals. In contrast, animals vaccinated via the subcutaneous route were not protected and showed high levels of H5N1 virus shedding in the respiratory tract and tissues following challenge.

Furthermore, protected animals did not produce neutralizing antibodies against H5N1 virus; however, cross-reactive HA-specific non-neutralizing IgG antibodies were detected only in animals that received the mucosal vaccine. These results are highly consistent with the cross-clade protection and reduced H5N1 viral shedding in nasal washes even in the absence of neutralizing antibody following mucosal rH5-NE01 vaccination observed in the current ferret study. Taken together these studies emphasize the potential advantages of mucosal adjuvants for induction of broadly protective immunity against respiratory pathogens such as highly pathogenic influenza. The nanoemulsion adjuvant already has proven to be safe and effective as a nasal mucosal adjuvant for seasonal influenza in Phase 1 clinical trials [17] establishing a foundation for advancement of the rH5-NE01 intranasal vaccine toward testing in humans.

We identified few limitations of the current study will be addressed in future studies including: i. the wider extent of cross clade protection (for example beyond clades 1 and 2) and the specific effectors contributing to the observed protection; ii. the contribution of HA stem reactive antibodies to protection; and iii. specific contributions of the NE adjuvant and route of administration. The contribution of mucosal immunity was not fully investigated due mainly to limited availability of ferret specific reagents.

In summary, the current studies conducted in the pivotal ferret H5N1 lethal challenge model provide definitive proof-of-concept for the efficacy of the rH5-NE01 mucosal vaccine utilizing the intranasal route of administration to generate mucosal as well as systemic immunity. Although a single vaccination using the rH5-NE01 vaccine may provide considerable protection against virus exposure, it is likely that multiple intranasal vaccinations will be necessary in a human population generally naïve to the H5 antigen. Efficient priming of non-circulating mucosal tissue-resident effector memory cells (T_{RM}) [39] at sites of viral entry in the nasal mucosal, respiratory tract, and lungs would enable an immediate response to infection (Wave 1) with rapid recruitment of systemic immunity (Wave 2) (reviewed in [42]). These features are uniquely elicited by mucosal immunization and are enhanced by the formulation with the nanoemulsion NE01 adjuvant delivery system [19,33,37,43].

Our findings warrant clinical evaluation of this intranasal rH5-NE01 vaccine to assess systemic and mucosal antigen-specific immune responses. That includes analysis of non-HAI antibodies that mediate functions such as antibody-dependent cell-mediated cytotoxicity (ADCC) and assessment of cell-mediated immunity including Th1 and Th17 responses systemically and mucosally in nasal washes. An effective intranasal rH5-NE01 vaccine for rapid elicitation of complete protective immunity after two immunization, and that can be readily manufactured, stockpiled and distributed for self-administration on a global scale will enhance pandemic preparedness against highly pathogenic influenza viruses.

Author contributions

DS and AF contributed to concept and protocol designs. DS, AF, HA and SG contributed to data analysis, collection and data interpretation. DS, AF and SG contributed to manufacture of key compounds used in the study. DS, AF and SG contributed to the writing of the manuscript and approved the final version of the manuscript.

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: DS, HA, SG and AF are employees of Blue Willow Biologics. SJS is employee of Fraunhofer COI.

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