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Recombinant H5 hemagglutinin adjuvanted with nanoemulsion protects ferrets against pathogenic avian influenza virus challenge

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ABSTRACT

Background: Highly pathogenic H5N1 influenza viruses remain a pandemic risk to the world population. Although vaccines are the best solution to prevent this threat, a more effective vaccine for H5 strains of influenza has yet to be developed. All existing vaccines target only serum antibody against influenza as the primary outcome, while mucosal immunity has not been addressed. To address these shortcomings we have used an effective mucosal adjuvant system to produce a prototype vaccine that provides antibody, cellular and mucosal immunity to multiple serotypes of H5.

Methods: Plant-derived recombinant H5 (rH5) antigen was mixed with a novel nanoemulsion NE01 adjuvant. The rH5-NE01 vaccine was administered intranasally to CD-1 mice and ferrets. Immunogenicity of this immunization was evaluated through rH5-specific antibody and cellular immune responses. Hemagglutination inhibition (HI) and virus neutralization (VN) assays were performed. Protection against H5N1 virus challenge was evaluated in ferrets.

Results: Intranasal immunization with rH5-NE01vaccine induced high titers (>10^6) of rH5-specific IgG in mice. In mice and ferrets this vaccine also achieved titers of ≥40 for both HI and VN. Additionally, the levels of rH5-specific IgA were significantly increased in bronchial secretions in these animals. The rH5-NE01 vaccine enhanced rH5-specific cellular immune responses including IFN-γ and IL-17. Ten-day survival post challenge was 100% in ferrets that received rH5-NE01compared to 12.5% in the PBS group. Furthermore, this vaccine prevented weight loss and increases in body temperature after H5N1 challenge as compared to the controls. Moreover, H5N1 virus in nasal wash of rH5-NE01-vaccinated ferrets was significantly decreased compared to controls.

Conclusion: Intranasal immunization with rH5 antigen formulated with NE01 adjuvant elicited strong, broad and balanced immune responses that effectively protect against H5N1 influenza virus infection in the ferret model. The ease of formulation of rH5-NE01 makes this novel combination a promising mucosal vaccine candidate for pandemic influenza.

1. Introduction

Influenza A virus hemagglutinin (HA) and neuraminidase (NA) coat glycoproteins are critical to viral antigenicity, host susceptibility and pathogenicity. In particular, HA has been shown to be a primary target for eliciting neutralizing immunity to the virus and has been the focus of most vaccine efforts [1,2]. H5 serotype strains involve highly pathogenic avian influenza (HPAI) viruses that can be lethal to birds and humans [1,2]. Vaccination is the most effective intervention to prevent HPAI virus infection, however there are several problems with this approach. H5 strains show high variability and constantly evolve in birds resulting in immunologically novel viruses. This evolution enables the virus to escape immunity generated by the vaccines [1]. Even small differences in H5, especially adjacent to the receptor-binding site, can result in immune escape [3,4]. Additionally, while HA similarity between

Abbreviations: BAL, bronchoalveolar lavage fluid; ELISpot, enzyme-linked immunospot; HI, hemagglutination inhibition; HA, hemagglutinin; HPAI, highly pathogenic avian influenza; Ig, immunoglobulins; MDCK, Madin-Darby canine kidney; NE01, nanoemulsion $W_{80}5EC$; NEs, nanoemulsions; rH5, recombinant H5; SFC, spot-forming cells; $TCID_{50}$, 50% tissue culture infective dose; VN, virus neutralization.

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vaccine and circulating virus strains is associated with higher vaccine efficacy [5], the constant evolution of H5 makes any vaccine strain unlikely to retain close identity with a pandemic H5 HPAI.

Methods to improve the breath of influenza vaccine coverage have involved the use of adjuvants, higher vaccine antigen doses, and the use of multiple vaccinations against heterologous HA strains. While none of these approaches has been developed for H5 viral serotypes, enhancing strategies are particularly important for H5 because it is a weak antigen [1,6,7]. In addition, the rapid production of an H5 vaccine would be crucial in a pandemic situation. In this regard, rapidly produced plant-derived recombinant H5 (rH5) has proven to be a safe and immunogenic approach to a pandemic vaccine; however, its immunogenicity was inadequate when it was administered intramuscularly either alone or with Alhydrogel® [8].

An attractive approach to enhance H5 vaccines would be to develop a highly effective mucosal adjuvant system. Mucosal vaccination could prove highly effective as mucosal immunity could not only prevent respiratory infections but could also reduce viral shedding curtailing pandemic spread. While few mucosal adjuvants exist, a nanoemulsion (NE01) adjuvant has been developed for intranasal vaccines and it has been shown to induce protective systemic and mucosal immunity against a number of organisms in animal models [9–13]. It is unclear, however, whether NE01 can enhance the immunogenicity of rH5 antigenic hemagglutinin protein, especially when derived from a plant source [1,6,7]. The current study evaluated systemic and mucosal immunity and protective effects of a nanoemulsion-formulated, plant derived rH5 antigen. We found that the resulting vaccine provided highly effective protective immunity in both mice and ferrets, and did prevent viral shedding. This appears to be a promising vaccine approach for pandemic influenza and may provide a pathway for improvement of other flu vaccines including seasonal influenza.

2. Materials and Methods

2.1. Nanoemulsion adjuvant

60%NE01 (formerly identified as W805EC) was used to prepare the intranasal nanoemulsion-based vaccines. The nanoemulsion vaccines were prepared by simple mixing of one volume 60% NE01 with various concentrations of rH5 antigen in twice the volume of buffer (1:2 ratio) to yield the final rH5 dose in 20%NE01. This formulation was designated as rH5-NE01.

2.2. Engineering and purification of recombinant influenza H5 HA

rH5 from the Indonesia/05/05 strain of influenza virus was engineered by Fraunhofer CMB [14]. Resultant plant-derived protein extracts were purified by chromatography. Before evaluation of immunogenicity, purified recombinant rH5 was characterized for purity based on SDS-PAGE and reverse-phase chromatography and for identity by Western blot analysis [8,15]. rH7 (A/Anhui/01/2013) subtype H7N9 recombinant antigen produced using the baculovirus expression system was purchased from Protein Sciences Inc, (Meriden, CT).

2.3. Animal immunization with rH5 vaccine

Two types of animals, mice and ferrets, were used. Mouse protocols were approved by the University of Michigan Institutional Animal Care and Use Committee (IACUC). CD-1 outbred female mice (6–8 week old) were purchased from Charles River Labs, Raleigh, NC. Groups of 8–10 mice were immunized intranasally using rH5-NE01 (6 μl per nare) at 4-week intervals on days 0, 28, and 56. Four different antigen doses were tested: 3, 10, 20 and 30 μg. Mice were euthanized at 2 or 4 weeks after the last immunization. Ferret studies were conducted at Southern Research (SR), Birmingham AL. Ferret protocols were approved by the SR IACUC. Castrated male Fitch ferrets, 5–8 months of age, were supplied by Triple F Farms (Sayre, PA). The ferrets were confirmed as seronegative for circulating human influenza A, B and H5N1 strains. To obtain groups that are comparable by body weight, all ferrets were assigned to their respective treatment groups using a computer-generated randomization procedure. The ferrets were immunized intranasally by a series of three vaccinations (0.5 mL rH5-NE01 at four-week intervals) and then challenged by H5N1 virus 4 weeks' post-vaccination.

2.4. H5N1 virus challenge of ferrets

Stock viruses of H5N1 Strain A/Indonesia/5/2005 (A/Indo/5/05) (clade 2.1.3.2) were received from the Centers for Disease Control and Prevention. The stock virus was amplified in embryonated hen's eggs according to standard protocols. The 50% tissue culture infectious dose ($TCID_{50}$) of the newly prepared stock virus was determined in naïve ferrets using a range of viral doses. The $TCID_{50}$ was found to be at 5×10^5. Ferrets were challenged with a target dose of 10X of the TCID50 i.e 5×10^6 $TCID_{50}$ in the current study. Groups of ferrets (n = 8 animals per groups) received a single intranasal challenge target dose of 5×10^6 $TCID_{50}$ per animal on study day 84 (four weeks' post-vaccination). The final inoculation volume of 1.0 mL per animal was administered by the intranasal route (0.5 mL per nare) using a pipette. The animals were evaluated post-challenge to assess clinical signs of infection, activity, body temperature, body weight, H5N1-viral shedding, and survival. Nasal washes were collected at day1, day 3 and day 5 post-challenge. H5N1-viral shedding in nasal washes was titrated on Madin-Darby canine kidney (MDCK) cells and reported as $TCID_{50}$ per ml.

2.5. Determination of serum IgG and bronchoalveolar lavage fluid IgA

The serum IgG and IgG-subtype responses were serially diluted and measured via ELISA with 96-well plates coated with 2 μg/ml of rH5 protein with antibody detection using anti-mouse IgG conjugated with HRP (Jackson ImmunoResearch Laboratories, Inc.). For ferret antibody analysis, Anti-ferret IgG (Abcam) or Anti-ferret IgA (Sigma) were used as secondary antibodies. ELISA units were also calculated from optical density values determined over a range of reference serum dilutions starting at 1:1000, and denoted as 100 units based on measurement of area under the curve. Sample ELISA units are determined by calculation of area under the curve over a range of sample serum dilutions when compared to the reference serum control. The bronchoalveolar lavage fluid (BAL) IgA responses were measured via ELISA with 96-well plates coated with rH5 protein with antibody detection using anti-mouse IgA conjugated with alkaline phosphatase (Rockland Immunochemicals Inc.) Samples were diluted 1:5 and assessed in duplicate.

2.6. rH5-specific cytokine analysis

The frequency of cytokine-secreting cells was measured by ELISpot assay (MABTECH, Cincinnati, OH). Mouse splenocyte suspensions or ferret PBMCs were added with either 2 μg/mL rH5 or medium to the plate immobilized with cytokine-specific monoclonal capture antibodies. After 48 h of incubation, specific cytokine detection antibodies were added and plates were visualized. The plate was evaluated using an AID ELISpot reader system. Data are expressed as spot-forming cells (SFC) per million cells.

The production of cytokines in response to antigen was also determined in the cultured supernatants using multiplex cytokine analysis kits. On a 96-well plate, 100 μl/well of splenocyte suspensions, and 2 μg/ml rH5 or medium were added. After 72 h of incubation, supernatants were harvested and frozen at −80 °C prior to analysis using murine multiplex magnetic bead kits (EMD Millipore Corp, Billerica, MA).

2.7. Hemagglutination inhibition (HAI) and virus neutralization (VN) assays

Serum samples were treated with receptor-destroying enzyme and an HAI assay was performed as described previously [14,16]. The micro-neutralization assay was performed using MDCK cells and re-assortant Indonesia/05/05[14]. MDCK cells were plated in 96-well plates and incubated for 18-hours. In parallel, RDE-treated serum samples were serially diluted and mixed with an equal volume of 4×10^3 $TCID_{50}$/ml of A/Indonesia/05/05 influenza virus. Following 1-hour incubation, the serum-virus mixtures were added to the plated MDCK cells. The neutralizing endpoint titer of each sample was determined by ELISA [17].

2.8. Statistical analysis

Group means and percent loss/change from baseline were calculated for body weights, temperatures, and nasal wash titers. Geometric mean titers were calculated for HAI and VN. Student *t* test with Welch's correction, one-way ANOVA and Mann-Whitney analysis was utilized to detect statistical differences. P values less than 0.05 were considered significant. Comparison of survival curves was evaluated using the Log-rank (Mantel-Cox) test.

3. Results

3.1. Immunogenicity of the rH5-NE01 vaccine in mice

Reagents for evaluation of cellular, humoral and mucosal immune responses are widely available for mice. Therefore, immune responses were initially characterized in an outbred mouse model as a prelude to H5N1 challenge studies in ferrets; which are the influenza animal model that best recapitulates the human pattern of infection, pathology and acute respiratory disease but lacks many reagents.

The murine immunogenicity evaluation of the rH5-NE01vaccine included serum and BAL rH5-specific antibodies, functional rH5 antibodies and Th1, Th2 or Th17 cell-mediated immune responses (Fig. 1, panels a–d). Initial dose ranging was performed in CD-1 mice that received three intranasal immunizations using 3, 10 or 30 μg of rH5 antigen, with or without NE01 adjuvant. Immunogenicity of the rH5 in the NE01 adjuvant appeared to near optimal dose at 10 μg and did not improved at 30 μg (Fig. 1a, right panel). Since the optimal dose appear to occur between 10 μg and 30 μg of rH5 antigen studies were repeated with 20 μg rH5 in NE01 adjuvant (Fig. 2). This demonstrated that 20 μg rH5-NE01 was as effective as 30 μg rH5-NE01 in inducing the production of rH5-specific serum IgG levels (Fig. 2a) as well as producing rH5-specific IgA in BAL (Fig. 2b), which is potentially important for mucosal protection [18].

The capability of immunization with 20 μg rH5-NE01 to induce serum antibodies to block H5N1 A/Indonesia influenza virus HA receptor-binding sites and neutralize virus infectivity was assessed by determination of HAI and VN antibody titers. An HAI titer ≥ 40 is considered the minimum threshold for protection against influenza infection [21]. 90% of CD-1 mice vaccinated intranasally using 20 μg rH5-NE01 achieved serum titers of ≥40 for both HAI and VN, which were significantly higher than control mice (Fig. 2d). The HAI and VN titers also correlated directly with the overall rH5-specific IgG titers (Fig. 2a).

The type and degree of cell-mediated immune responses induced after intranasal immunization using the 20 μg rH5-NE01 vaccine was evaluated by ELISpot analysis of splenocytes from immunized mice. This is important as it is thought that cellular immunity can enhance protection against influenza or other viral infections [19,20]. Antigen-specific IFN-γ, IL-5 and IL-17 are associated with Th1, Th2 and Th17-type immune responses, respectively, and therefore antigen specific production of these cytokines was used to characterize cellular immunity. The frequencies of rH5-specific cytokine-producing cells for all three of these cytokines were increased significantly in mice receiving rH5-NE01 as compared with the control mice (Fig. 2c). This rH5-specific increase in cytokine production was confirmed by multiplex cytokine analysis of supernatants from *in vitro* antigen re-stimulated spleen cells (Luminex Corporation, data not shown). Thus, the combination of 20 μg rH5 antigen with NE01 adjuvant was deemed to be optimal for the influenza challenge studies in ferrets.

3.2. rH5-NE01 enhances systemic and mucosal rH5-specific antibody responses in ferrets

To evaluate immune responses induced by intranasal administration of the rH5-NE01 vaccine in ferrets, the titer of rH5-specific serum IgG was analyzed (Fig. 3). rH5-specific IgG was evaluated in serum samples from ferrets at 2 weeks following a third intranasal immunization using 20 μg of rH5 antigen, with or without NE01 adjuvant. The level of rH5-specific IgG was much higher in ferrets that received rH5-NE01 than the controls (Fig. 3a) and was similar to the data obtained in the mouse model (Fig. 2a). Furthermore, rH5-specific IgA and IgG antibody levels were significantly higher in nasal wash from rH5-NE01 vaccinated ferrets than that from ferrets immunized using 20 μg of rH5 alone (Fig. 3b), indicating the induction of a mucosal immune response, thought to be important for protection against virus infection [18]. Similar to the experimental mouse data, the rH5-NE01 mucosal vaccine also produced robust HAI and VN serum antibody responses in the ferret when compared to the control animals or ferrets immunized with rH5 alones (Fig. 3c and 3d). Overall, the intranasal NE01-adjuvanted rH5 vaccine achieved over 90% seroconversion (titer ≥ 1:40 cutoff) of HAI and VN antibody responses.

3.3. rH5-NE01 vaccine activates cell-mediated immune responses in ferrets

Cell-mediated immunity was evaluated in ferrets immunized with 20ug rH5 in NE01 using a ferret IFN-γ ELISpot analysis of peripheral blood mononuclear cells (PBMC) obtained one week following the third intranasal vaccination. rH5-specific IFN-γ producing T-cells were detectable in a high proportion (>75–100%) of rH5-NE01 nasal immunized ferrets when compared to ferrets immunized with antigen alone (Fig. 4). This indicated that the nasal rH5-NE01vaccine induced systemic rH5-specific Th1-cell responses in the peripheral blood.

3.4. The intranasal rH5-NE01 vaccine fully protects ferrets against morbidity and mortality following H5N1 virus challenge

Ferrets were challenged intranasal with a 10X LD_{50} dose of H5N1 A/Indonesia/5/2005, four weeks following a third intranasal vaccination. Survival data for animals in the intranasal rH5-NE01 vaccine and control groups over a 10-day observation period post-challenge is shown in Fig. 5a. Complete protection from chal-

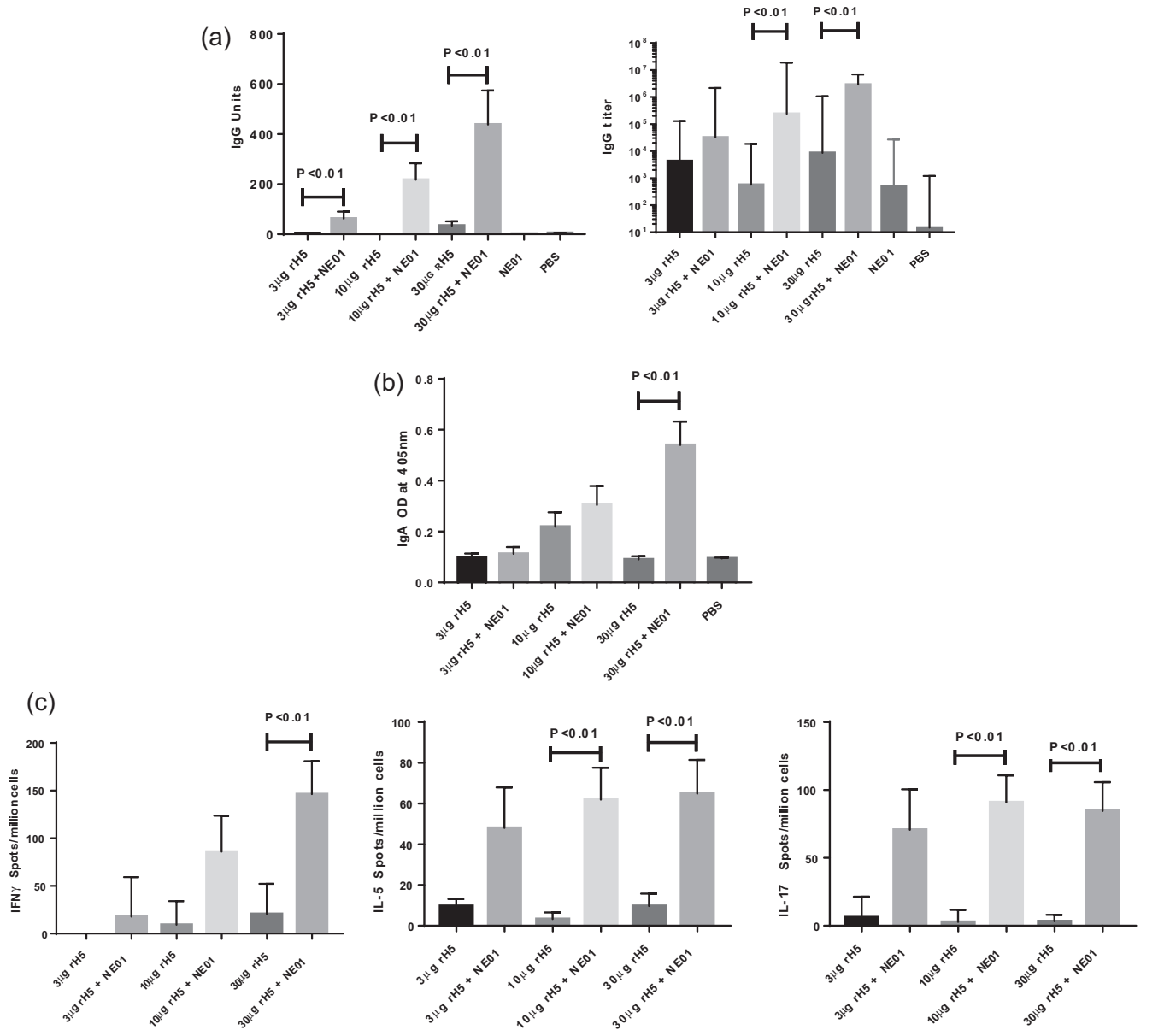

Fig. 1. rH5-NE01 immunization induces r5H-specific serum IgG, mucosal IgA and cellular immune responses suggestive of a Th1 and Th17 phenotype. rH5-specific IgG in sera (Fig. 1a) and rH5-specific IgA in BAL (Fig. 1b) were determined by ELISA and showed significant increases in antibody titers compared to animals immunized with rH5 antigen alone. Responses plateaued in the 10–30 ug/immunization range. The production of rH3 specific IFN-γ, IL-6 and IL-17 (Fig. 1c) in splenocytes was measured by ELISpot using different doses of rH5 or rH5-NE01 and again showed significant increases in r5H-specific cytokines as compared to animals immunized with antigen alone. Responses again plateaued in the 10–30 ug/immunization range Serum and spleens were collected from mice 4 weeks after the third intranasal immunization and all data are shown as mean and standard deviation for 10 mice in each group.

lenge (100% survival) was observed for all animals (8/8) receiving the intranasal rH5-NE01 mucosal vaccine. In contrast, all animals in the PBS control group were moribund beginning on day 6 post-challenge with the majority of animals (7 of 8, 12.5% survival) succumbing to disease by day 8 post-challenge. While all animals immunized with rH5 alone appeared ill, only 2 of 8 died (75% survival).

Ferrets immunized using the 20ug rH5-NE01 vaccine also demonstrated other parameters suggesting complete protection from infection as they maintained normal body temperature and body weight. Importantly these animals also displayed no H5N1 virus shedding in the nasal wash at any time point post-challenge (Fig. 5d). In contrast, ferrets in the PBS control group showed a significant loss of body weight (>18% mean weigh loss over 10 days, Fig. 5b) associated with a rapid rise in body temperature peaking at 2–3 degrees above baseline within 24 h post-challenge (Fig. 5c). In unimmunized animals there also was virus shedding in nasal wash for a least 5 days post-challenge (Fig. 5d). Although animals vaccinated with rH5 antigen alone showed some protection from infection based on survival (Fig. 5a), these animals still demonstrated a rapid spike in body temperature, significant weight loss (>10% mean weigh loss over 10 days), and prolonged viral shedding in nasal washes for >3 days post-challenge (Fig. 5b–d). Furthermore, ferrets that received three intranasal vaccinations using rH7-NE01 as an irrelevant antigen control vaccine showed no protection against challenge based on survival, weight loss, rise in body temperature and viral shedding post-challenge (data not shown).

Finally, ferrets immunized using the rH5-NE01 mucosal vaccine showed normal activity scores during observation over the 10 days

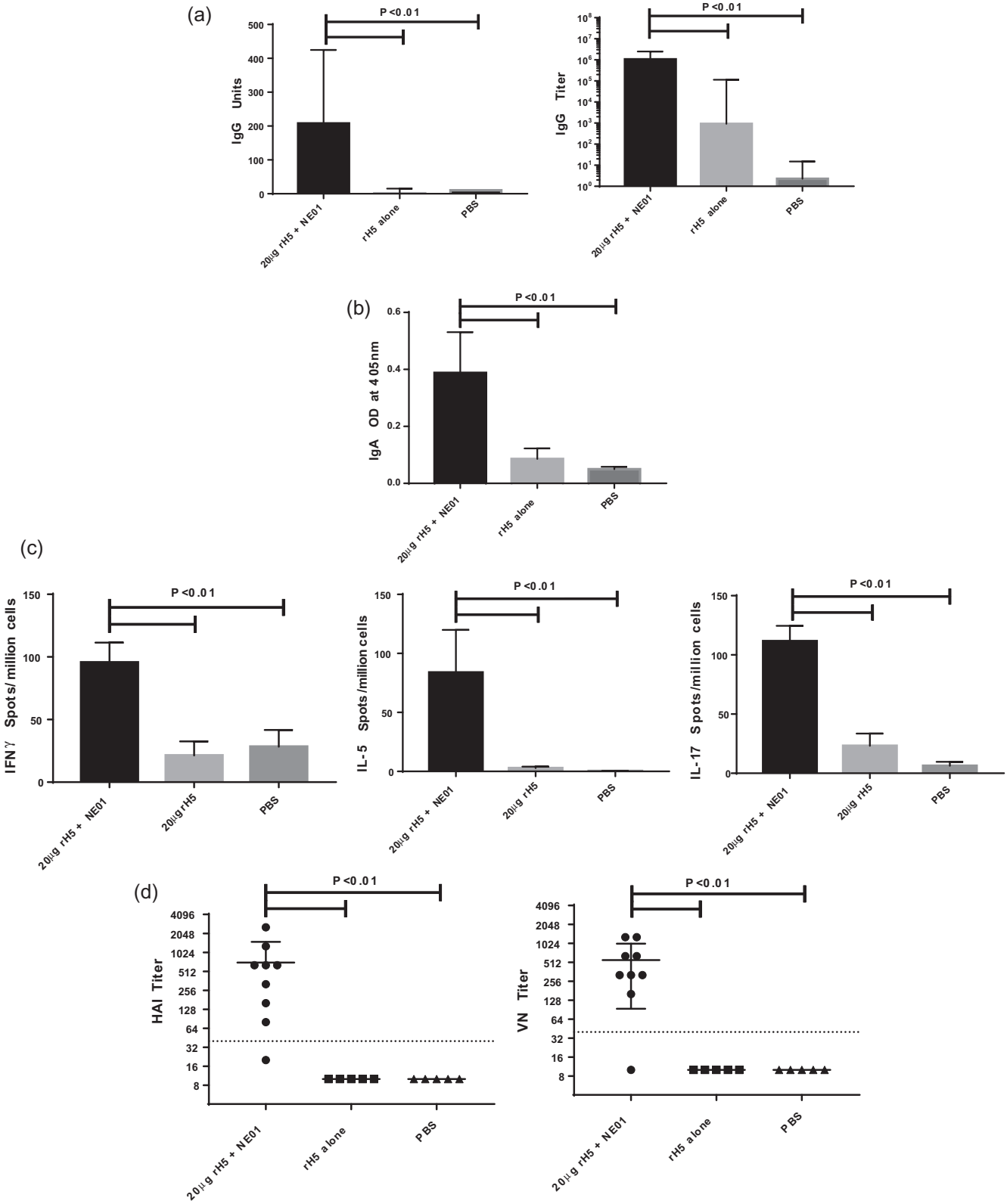

Fig. 2. Immune responses observed with a 20ug rH5-NE01 vaccine antigen dose bracketed by the results in Fig. 1. rH5-specific serum IgG (Fig. 2a) and BAL IgA (Fig. 2b), rH5-specific hemeagglutination inhibition (HAI) and virus neutralization (VN) serum antibodies (Fig. 2d) and ELISpot profile of cell-mediated immunity (Fig. 2c), were all significantly increased to levels similar to the results seen in Fig. 1. Ten mice were evaluated in each treatment group. From these studies 20 μg per immunization was chosen as the optimal dose for immunization and used in all further studies.

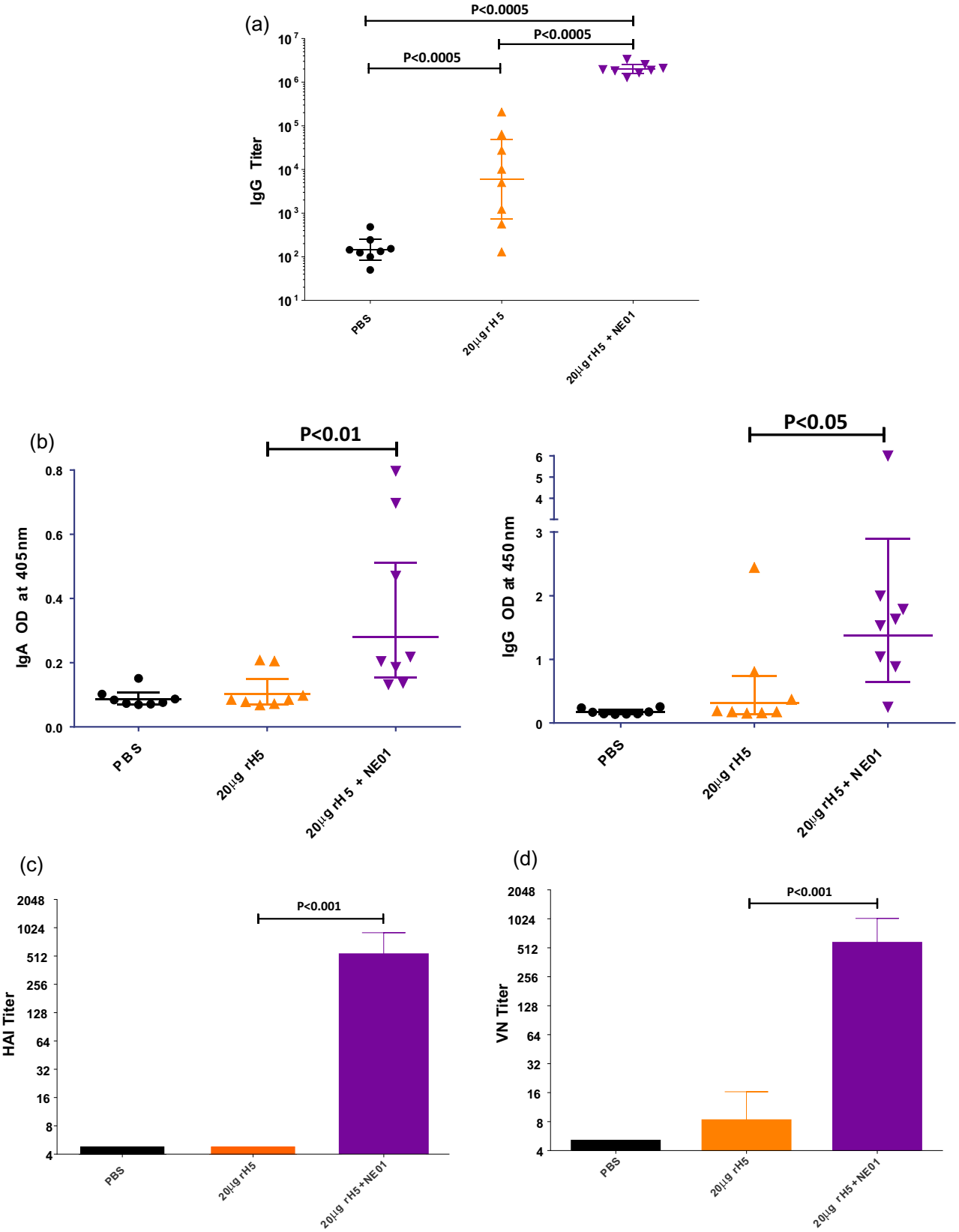

Fig. 3. 20 μg rH5-NE01 induces significant rH5-specific antibody responses in ferrets after nasal immunization. Serum rH5-specific IgG responses (Fig. 3a), and nasal wash rH5-specific IgA (left panel Fig. 3b) and IgG (right panel Fig. 3b) antibody responses all showed significant increases when compare to animals immunized with antigen alone or unimmunized controls. In addition, serum HAI (Fig. 3c) and VN (Fig. 3d) antibody titers were also significantly elevated compared to controls. Sera and nasal washes were obtained two weeks following the third intranasal vaccination with 20 μg rH5-NE01. Data in all panels are expressed as Geometric mean antibody titer with 95% confidence interval with 8 animals per group.

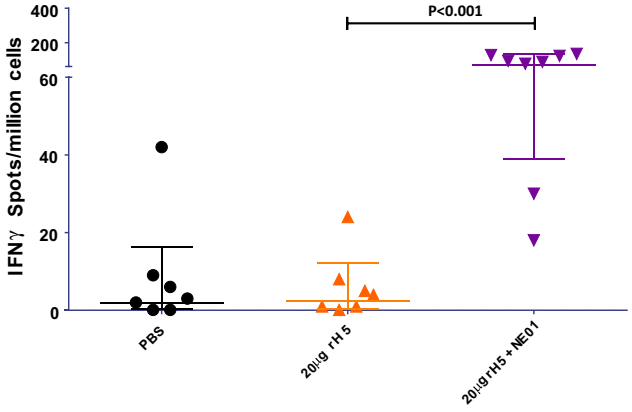

Fig. 4. rH5-NE01 vaccine induces rH5 specific production of IFN-γ by PBMC in ferrets. The rH5-NE01 immunized animals had significant production of antigen-specific IFN-γ by PBMC as compared to animals immunized with either rH5 alone or control, non-immunized animals. The PBMC were obtained from ferrets one week after the third intranasal vaccination and ELISpot was used to analyze rH5-specific IFN-γ.

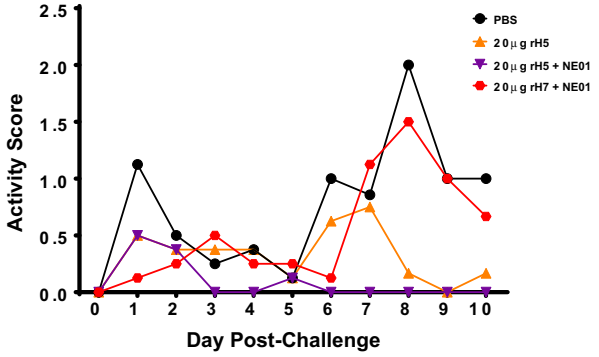

Fig. 6. rH5-NE01 mucosal vaccination normalized the activity scores of ferrets after H5N1 virus challenge. Ferrets showed normal activity if vaccinated with rH5-NE01 vaccine before viral challenge. The activity was quantitated using the following activity scoring system: 0 = alert and playful; 1 = alert but playful only when stimulated; 2 = alert but not playful when stimulated; 3 = neither alert nor playful when stimulated. Animals vaccinated with antigen alone or unvaccinated animals had significant decreases in their activity scores.

following H5N1 virus challenge when compared to groups of animals that received PBS, rH5 alone or rH7-NE01 (Fig. 6). In particular, animals in the control groups that received either PBS or rH7-NE01 presented with lethargy/hypoactivity, emaciation, dehydration, poor grooming, and were cold-to-the-touch upon removal from the study. Furthermore, recumbence, non-responsiveness to stimuli, ataxia and other neurological deficits were observed in the moribund animals. Similarly, animals that received rH5 alone presented with lethargy/hypoactive, emaciation, dehydration and poor grooming at the time they were removal from the study. One animal in this latter group also exhibited neurological deficits reminiscent of central nervous system influenza infection.

4. Discussion

The global emergence of highly pathogenic H5N1 avian influenza in combination with the genetic re-assortment, mutation,

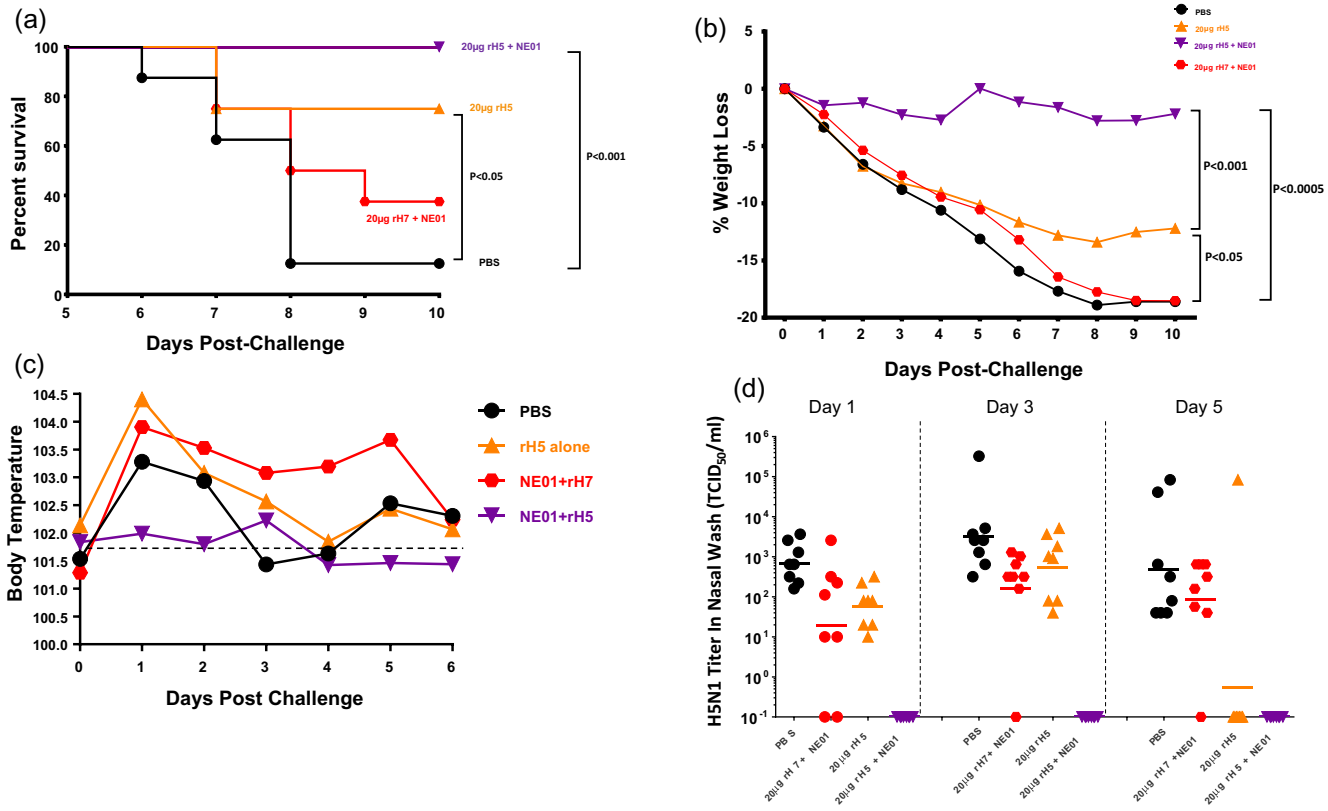

Fig. 5. rH5-NE01 mucosal vaccine provides full protection against morbidity and mortality following H5N1 virus challenge in ferrets. Ferrets challenged with a 10 × $TCLD_{50}$ dose of H5N1/A/Indonesia/5/2005 virus subsequent to three doses of rH5-NE01 vaccine demonstrated complete protection against viral infection. This was confirmed by complete animal survival (Fig. 5a), maintenance of body weight (Fig. 5b) stability of body temperature (Fig. 5c), and the absence of H5N1 virus shedding in nasal washes (Fig. 5d).

and pathogenic diversification of H5 influenza virus strains is a major public health issue [22]. The threat of these viruses necessitates global pandemic preparedness in the event that strains acquire the ability for bird-to-human and/or human-to-human respiratory transmission. Inactivated vaccines for seasonal influenza have been a cost-effective way to reduce influenza morbidity and mortality for over 70 years; however, these vaccines would not be adequate for use in an H5 pandemic. Vaccines against H5N1 influenza that use whole or split and inactivated H5N1 virus cannot be safely produced given this virus' potential hazards [6,23–25]. Viral production, either in eggs or tissue culture, cannot achieve the safe, rapid and efficient large-scale vaccine production necessary for distribution during a global pandemic. Intramuscular administration of H5N1 vaccines would also require sterile needles and trained personnel. Therefore, public health initiatives have focused on identifying an easily administered, mucosal influenza vaccine. In this regard, the potential of the H5 mucosal influenza vaccine has been demonstrated using a plant-based rH5 adjuvanted with (3′,5′)-cyclic dimeric guanylic acid (c-di-GMP) as the adjuvant in animals [34]. But the utility of this approach for humans remains unclear.

Recombinant H5 proteins from multiple sources can help resolve the production issues for H5N1 vaccines and antigen produced by plant-based technology is a cost-effective method that can be readily engineered to match genetic changes that occur in H5 [14,17]. Plant-derived rH5 appears stable for a long period of time and was safe in humans when administered intramuscularly either alone or with Alhydrogel® adjuvant [8]. However, despite high antigen concentrations the alum-adjuvanted H5 intramuscular vaccines demonstrated insufficient immunogenicity for significant protection in humans using either plant derived rH5 or inactivated whole or split H5N1 virus [8,26,27]. To overcome the weak antigenicity of H5N1 proteins, several approaches have used adjuvants with multiple, pro-inflammatory components [31–36]. Unfortunately, these approaches have been marginally successful in producing high titer influenza antibodies [31–37]. Therefore, alternative vaccine formulations are necessary.

NEs are adjuvants manufactured by high-speed emulsification of ionic and nonionic surfactants, ethanol, soybean oil and purified water. We have generated a series of NEs with combinations of ionic and nonionic surfactants at different ratios [28,29]. Among these NEs, the NE01 formulation has been shown to induce strong systemic and mucosal immunity as an intranasal adjuvant for seasonal influenza and several other vaccines in animal models [9–12,30]. This adjuvant can be readily manufactured at large scale and has shown excellent thermostability over a range of temperatures for at least two years [10,11,13]. These features would enable a NE01-adjuvanted recombinant rH5 vaccine to be rapidly produced on a large scale or stockpiled for pandemic preparedness.

Given these advantages the current study evaluated the immunogenicity and immune response attributes of rH5-NE01 nasal vaccine in mice and the protective efficacy of the vaccine in the ferret H5N1 lethal challenge model. The rH5-NE01 vaccine elicited strong systemic and mucosal immunity in both mice and ferrets. In the mouse model, examination of specific cellular immunity induced by the rH5-NE01 nasal vaccine shows systemic Th1 and mucosal Th17-type responses, as reflected by the increase of the antigen-specific IFN-γ and IL-17 production. It also demonstrated mucosal production of IgA, which has been reported to enhance host defenses against influenza [19,20]. Robust rH5-specific humoral immune responses are induced by the rH5-NE01 vaccine as evident from the dose-dependent rise of rH5-specific serum IgG titers. After intranasal immunization with the rH5-NE01 vaccine, both HAI and VN were observed in 90–100% of the mice and ferrets tested (titers of $\geq$40, the level of which is considered the minimum threshold for protection against influenza infection) [21]. This indicates that the rH5-NE01 nasal vaccine can induce a wide range of systemic and mucosal antibodies, and these antibodies can serve to neutralize virus, inhibit virus entry into cells and suppress viral replication. These immune responses were clearly dependent on the adjuvant, as they were not observed in animals immunized with antigen alone.

The H5N1 virus challenge study in ferrets demonstrated the efficacy of the mucosal vaccine as 100% of animals that were immunized with 20ug rH5-NE01 survived as compared to only 12.5% of non-immunized, control animals. No symptoms, fever, weight loss, or viral shedding in nasal washes were noted in the rH5-NE01 immunized animals. Prevention of colonization and shedding is indicative of mucosal immunity especially localized IgG and IgA-secreting B-cells and Th17-type responses. While intranasal administration of rH5 antigen alone did elicit weak local and systemic immunity (including low levels of serum IgG antibody with GMT $<5 \times 10^3$) that likely contributed to the survival of 75% of animals, this did not prevent colonization, shedding, and illness. As the combination of rH5 with NE01 enhanced mucosal and humoral immunity that completely prevented colonization, shedding, and illness, this type of protection could play a crucial role in preventing transmission in a pandemic situation.

Although protective antibody levels for avian influenza have not been determined, an HAI titer of 40 was used as a benchmark in clinical trials to evaluate H5N1 vaccines [38,39]. Recently, Wong et al reported that animals that had low HAI titer were protected against viral challenge in the ferret model [40]. They proposed that the protection observed in these vaccinated animals can be attributed to non-HAI IgG and to high levels of $CD8^+$T-cells producing IFN-γ in the airways. Our intranasal vaccine generated high levels of IgG antibodies and HAI activity in ferrets with 100% survival, similar to studies using the AS03-adjuvanted intramuscular H5 vaccine as reported by Wong et al. [40]. However, animals in the AS03 vaccinated groups showed continued H5N1 virus shedding in nasal washes through day 5 post-challenge [40]. In contrast, ferrets vaccinated using the mucosal rH5-NE01 vaccine had no detectable virus in nasal washes on any day post-challenge. This striking difference viral shedding from the respiratory and nasal compartments may be attributed to long-lasting mucosal immune responses including local Th1, Th17 and IgA-producing B-cells induced by intranasal vaccination using the rH5-NE01 adjuvanted vaccine.

One of the most unique aspects of rH5-NE01 immunization is the production of antigen-specific, mucosal Th17 immunity. Th17 cells enhance the secretion of antimicrobial peptides and trafficking of lymphocytes and neutrophils to mucosal sites of infection, and increase polymeric immunoglobulin receptors in the mucosal epithelium promoting delivery of IgA to mucosal surfaces [41]. IL-17 may also contribute to B-cell-recruitment to the respiratory mucosa aiding in immune protection against H5N1-infection. Of interest, IL-17 knockout mice infected with H5N1 had significant reductions of B cells in the lung, greater weight loss, increased lung immunopathology, and significantly reduced influenza survival compared to wild-type controls [42]. Also, murine, Th17-polarized $CD4^+$ T-cells can transfer protection against lethal influenza virus challenge in naïve mice [43]. Moreover, IL-17 promotes both Th1 and Th2-type specific immunity [44,45], This is interesting as an increase in the level of antigen-specific bronchoalveolar IgA has been correlated with protection against viral challenge [46,47] and increased rH5-specific IgA and IgG was found in the airway of mice and ferrets with the rH5-NE01 vaccine. Therefore, Th17 immunity induced by rH5-NE01 may enhance protection from H5 infection.

In summary, our data demonstrate that the intranasal administration of plant-derived rH5 formulated in NE01 can generate specific systemic and mucosal immunity, thereby efficiently pre-

venting avian influenza infection, colonization and shedding. In addition, intranasal immunization with this adjuvant is able to generate a balanced, antigen specific Th1/Th2/Th17 cellular immune response. Given these attributes, this novel approach has significant potential for mass immunization programs and vaccine stockpiling to protect against pandemic influenza.

Conflicts of interest

Dr. Baker holds stock in BlueWillow Biologics and is the inventor of vaccine technologies that the University has licensed to BlueWillow Biologics. DS, AF, are employees of BlueWillow Biologics. JAC, VY and SJS are employees of Fraunhofer COI.

Author contributions

SHW, JB, DS and AF contributed to concept and protocol designs. SHW, JB, ZC, JC, DS, HA, and SS contributed to data analysis, collection and data interpretation. DS, AF, VY and SS contributed to manufacture of key compounds used in this study. SHW, JB, DS and AF contributed to the writing of the manuscript and approved the final version of the manuscript.

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