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Intranasal Antibody Gene Transfer in Mice and Ferrets Elicits Broad Protection Against Pandemic Influenza

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Abstract

The emergence of a new influenza pandemic remains a threat that could result in a substantial loss of life and economic disruption worldwide. Advances in human antibody isolation have led to the discovery of monoclonal antibodies (mAbs) that have broad neutralizing activity against various influenza strains, although their direct use for prophylaxis is impractical. To overcome this limitation, our approach is to deliver antibody via adeno-associated virus (AAV) vectors to the site of initial infection, which, for respiratory viruses such as influenza, is the nasopharyngeal mucosa. AAV vectors based on serotype 9 were engineered to express a modified version of the previously isolated broadly neutralizing mAb to influenza A, FI6. We demonstrate that intranasal delivery of AAV9.FI6 into mice afforded complete protection and log reductions in viral load to 100 LD_{50} (median lethal dose) of three clinical isolates of H5N1 and two clinical isolates of H1N1, all of which have been associated with historic human pandemics (including H1N1 1918). Similarly, complete protection was achieved in ferrets challenged with lethal doses of H5N1 and H1N1. This approach serves as a platform for the prevention of natural or deliberate respiratory diseases for which a protective antibody is available.

INTRODUCTION

Influenza infections are the seventh leading cause of death in the United States and result in almost 500,000 deaths worldwide per year (1). Several aspects of the influenza virus and the response of the human host to an influenza infection conspire against a simple remedy. Key targets of the adaptive immune response such as the hemagglutinin (HA) protein of the virus evolve rapidly, rendering immune memory responses partially protective to new infections (2). The response of humans to a

natural infection or a traditional vaccine is usually limited in breadth, providing protection only against closely related subtypes. This has led to annual vaccinations against seasonal strains of influenza viruses that are predicted to emerge during the upcoming season. It is believed that the repertoire of immune memory generated from previous influenza infections and vaccinations helps to blunt the sequelae of a new infection and augments the efficacy of a vaccine. This is not the case when an influenza virus residing in animal reservoirs acquires a human respiratory tropism and is transmitted to humans (3). These zoonotic strains are quite distinct from those that normally circulate in humans, can lead to pandemics with lethal consequences, and are not effectively controlled by vaccines developed to human strains of the virus. As was learned from the 2009 H1N1 pandemic, the vaccine development time is not fast enough to support vaccination in response to an emerging pandemic (4).

One approach for confronting influenza pandemics is to have available a vaccine that elicits a broad neutralizing response, which, until recently, was not thought possible. The ability to clone and characterize monoclonal antibodies (mAbs) from single human B cells has provided insights into molecular mechanisms of immunity that are important to vaccine development. For influenza, it was possible to isolate high-affinity human mAbs against highly conserved regions of the HA protein that had an unexpected advantage of broad neutralization (5–8). The most compelling results were observed with mAbs directed against the stem region of HA that show neutralizing activity against a broad array of group 1 and 2 influenza A viruses including many seasonal strains and most pandemic strains (5). Although this knowledge has not yet translated into improved immunogen design in active vaccine regimens, broadly neutralizing mAbs could potentially be developed as protein therapeutics in passive transfer products. However, the requirement of repeated parenteral administration of the mAb in at-risk populations is impractical to administer and too expensive to be considered at any scale.

We propose to use adeno-associated virus (AAV) vectors to deliver broadly neutralizing mAbs against conserved regions of HA as a practical and affordable way to confer broad-based protection against pandemic strains of influenza. This strategy has been suggested as an approach to treat and potentially prevent HIV with some level of protection achieved after intramuscular injection of vector into humanized mice (9) and nonhuman primates for simian immunodeficiency virus (10); in these studies, the goal was to systemically produce stable levels of broadly neutralizing anti-HIV mAbs and prevent infection at the primary sites of transmission, which, for most HIV infections, is the rectal and vaginal mucosa. We reasoned that a more effective and safer way to express protective levels of the influenza mAb is to localize its expression to the portal of entry, which, in the case of respiratory-transmitted pathogens such as influenza, is predominantly the nasopharyngeal mucosa. AAV9 transduction of respiratory epithelial cells is indeed possible, and here, we demonstrate the protective efficacy of expression of the broadly neutralizing mAb in the airway surface liquid layer, which is the site where the influenza virus initially replicates.

RESULTS

AAV9 vectors effectively target murine respiratory epithelial cells

Successful expression of a broadly neutralizing mAb in the airway requires a gene delivery vehicle that is safe and effective. Our previous studies of cystic fibrosis gene therapy identified vectors based on a natural variant of AAV, called AAV9, that efficiently transduced proximal and distal airway epithelial cells in mice after nasal delivery (11). Transgene expression is durable, lasting at least 9 months, and AAV9 vectors can be readministered without loss of efficiency (11). AAV9 vectors expressing LacZ or ffluciferase (ffLuc) were administered via intranasal aerosolization to more thoroughly evaluate transduction profiles. Mice were dosed with two different volumes (20 or 50 μl) at a fixed quantity of vector [10^{11} genome copies (GC)]. Vector dosed in 20 μl demonstrated high-level transduction of nasal ciliated epithelial cells (219 ± 128 LacZ-positive cells per high-power field, $n = 5$; Fig. 1, A and B)

with low transduction of lung epithelial cells (7 ± 5 LacZ-positive cells per high-power field, $n = 5$; Fig. 1C). Expression of ffLuc was primarily localized to the nose, where expression was 200- to 250-fold above background, with less ffLuc found in lung, where it was 17-fold above background (Fig. 1, D and E). Similarly, high levels of nasal ciliated epithelial cell transduction were observed at the higher dosing volume as measured by LacZ expression (207 ± 150 LacZ-positive cells per high-power field, $n = 5$; Fig. 1, F and G) and ffLuc expression (175- to 200-fold ffLuc expression over background, $n = 5$; Fig. 1, I and J). Expression of transgene in lung was higher with the larger volume: 168 ± 45 LacZ-positive cells per high-power field ($n = 5$; Fig. 1H) and 50-fold ffLuc expression over background ($n = 5$; Fig. 1, I and J). The kinetics of AAV9 transgene expression was rapid, with 90% of steady-state levels of ffLuc in the nose achieved within 5 days (Fig. 1, E and J).

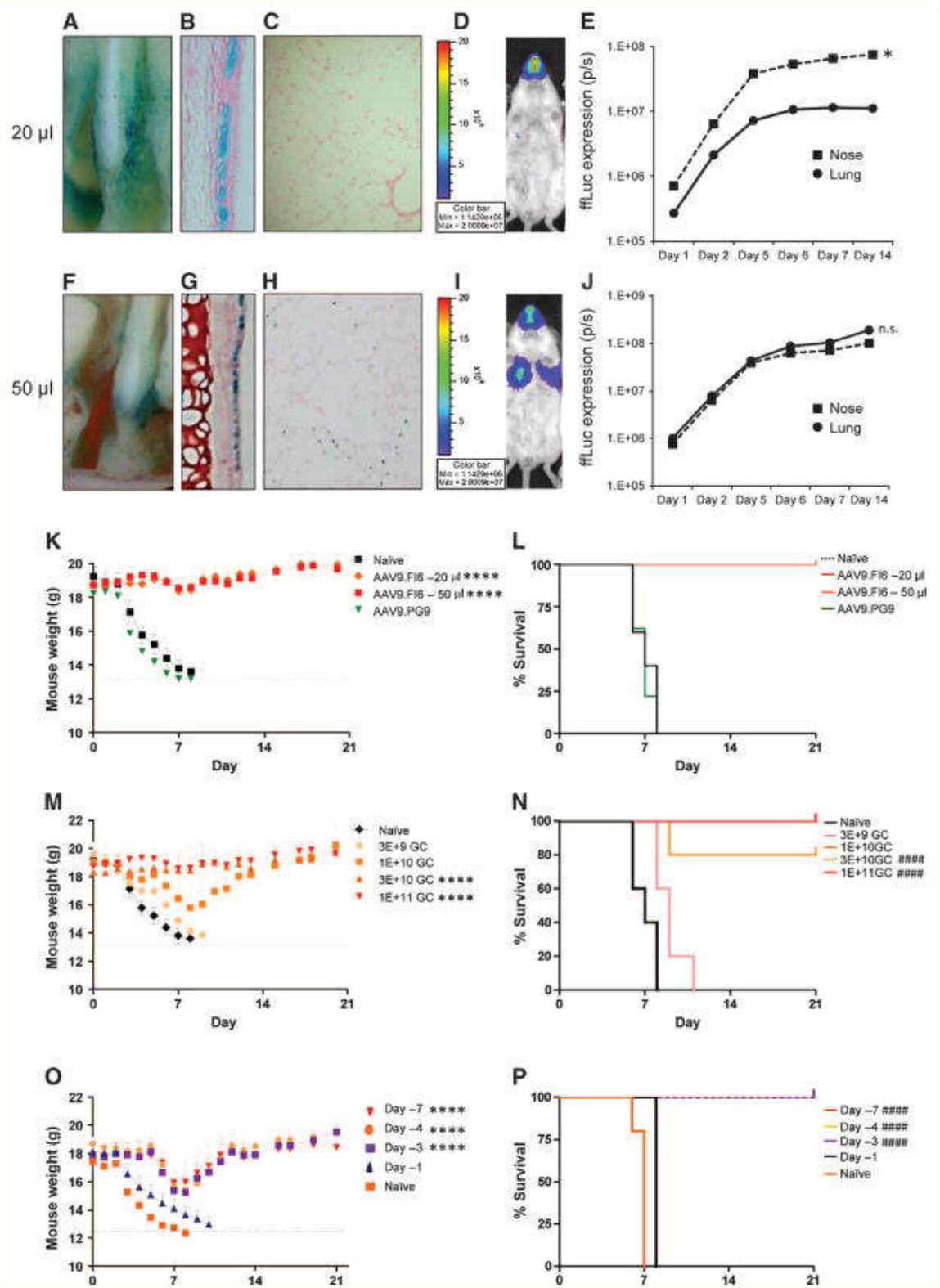

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Fig. 1

AAV9 transduction of airway epithelia and protection against a mouse-adapted strain of H1N1

BALB/c mice were dosed with AAV9 vectors expressing either LacZ or ffLuc and subjected to histological and imaging analyses. (**A** to **J**) The vector was administered in either 20 µl (A to E) or 50 µl (F to J), and analyses of LacZ expression were performed 14 days later including en face of nasal septum

(A and F) and histological sections of nasal septum (B and G) and lung (C and H). Mice were imaged for ffLuc expression at multiple days with a representative image at day 14 shown in (D) and (I) and a time course shown in (E) and (J). Background luminescence was about 4×10^5 photons/s. (**K**, **L**, **O**, and **P**) BALB/c mice ($n = 5$ per group) were dosed intranasally with AAV9.FI6 and subsequently challenged with 10 LD_{50} of A/PR8 delivered intranasally. Animals were followed for weights and survival as depicted in the Kaplan-Meier plots. (K) and (L) present mice treated with neuraminidase (NA) and dosed with 10^{11} GC/50 μl of a control AAV9 vector expressing an irrelevant human IA (PG9), as well as those dosed with 10^{11} GC of AAV9.FI6 in either 20 or 50 μl. (**M** and **N**). BALB/c mice ($n = 5$ per group) were treated with NA as above and then dosed with increasing amounts (3×10^9, 10^{10}, 3×10^{10}, and 10^{11} GC per mouse) of AAV9.FI6 vector to determine the minimal protective dose. Fourteen days later, vector-treated and naïve mice were challenged with 10 LD_{50} of A/PR8 delivered intranasally in 50 μl. The weight of challenged mice was monitored daily. (O) and (P) present animals dosed with 10^{11} GC of AAV9.FI6 in 50 μl and challenged 1, 3, 4, or 7 days after vector dosing; naïve mice were dosed with phosphate-buffered saline (PBS). A potentiator of AAV9-airway transduction, NA, did not enhance protection in this model (fig. S3) and was therefore excluded from subsequent experiments. $^{*}P \leq 0.05$, Student's t test; $^{\#\#\#\#}P \leq 0.0001$, Mantel-Cox test; $^{****}P \leq 0.0001$, Dunnett's test. n.s., not significant. Dotted lines denote 30% weight loss.

Vector expression of the broadly neutralizing antibody FI6 protects against a broad array of pandemic influenza strains in mice

To evaluate the efficacy of this approach in animal models of influenza infection, we cloned into AAV9 vectors a gene for a modified antibody [immunoadhesin (IA)] derived from a previously isolated mAb to influenza, called FI6 (5), under the control of a hybrid cytomegalovirus (CMV) enhancer chicken β-actin promoter. This mAb was isolated from human immortalized B cells and was found to bind to and neutralize a wide spectrum of group 1 and 2 influenza A viruses including most of those assessed to be a potential pandemic-causing risk (5). Corti and colleagues showed that passive transfer of FI6 into mice resulted in protection against H1, H3, and H5 viruses (5). AAV9.FI6 was intranasally administered to mice, and 14 days later, the nose [nasal lavage fluid (NLF)] and lungs [bronchoalveolar lavage fluid (BALF)] were lavaged. FI6 protein was detected in the NLF and BALF by Western blot and enzyme-linked immunosorbent assay (ELISA). In each case, the IA was easily detected, although it was difficult to accurately determine concentrations because of uncertainties of recovery and dilution of the lavage (fig. S1); estimates based on projected dilutions are about 0.5 μg/ml in the nose and 2.0 μg/ml in the lung. Low levels of FI6 were detected in blood after intra-nasal administration (about 120 ng/ml).

The efficacy of nasal delivery of AAV9. FI6 was first evaluated in mice that were challenged 14 days later with 10 LD_{50} (median lethal dose) of influenza A/Puerto Rico/8/34 (A/PR8), which has been adapted to be lethal in mice. Animals showing substantial distress as defined by the respective Institutional Animal Care University Guidelines or by ≥30% weight loss were euthanized; surviving mice were sacrificed 21 days after challenge, and tissue samples were harvested for analysis. Animals treated with 10^{11} GC of an AAV9 vector expressing an irrelevant human IA rapidly lost weight and had to be euthanized within 8 days (Fig. 1, K and L), at which time the lungs showed perivascular infiltrates and alveolar mononuclear inflammation (fig. S2). Treatment with an identical dose of AAV9.FI6 in either 20 or 50 μl resulted in complete protection as evidenced by 100% survival and no loss of weight (Fig. 1, K and L); lungs of mice treated with 50 μl of vector and analyzed 21 days after challenge showed no pathology (fig. S2). The impact of vector dose on efficacy was also evaluated.

Complete protection was achieved at 10^{11} GC and 3×10^{10} GC, whereas 3×10^{9} GC showed no benefit (Fig. 1, M and N). Virtually, all animals treated with the intermediate vector dose of 3×10^{10} GC did lose weight (that is, 90%), although they eventually recovered and survived the challenge.

The rapid onset of efficacy is beneficial when dealing with an outbreak that requires fast response times. Hence, we next varied the interval between AAV9. FI6 treatment and A/PR8 challenge to determine how quickly protection could be achieved. Animals challenged 3, 4, or 7 days after treatment lost some weight but completely recovered. All animals had to be euthanized when the interval was only 1 day, although the kinetics of weight loss was delayed by 1 day (Fig. 1, O and P).

To assess the potential of AAV9.FI6 as a vaccine for pandemic influenza, we conducted similar challenge studies with a number of clinical isolates of influenza that have been associated with worldwide pandemics, all of which are lethal in mice (Fig. 2). The stringency of these studies was enhanced by using a higher dose (100 LD_{50}) of highly virulent influenza strains. Some animals were necropsied 6 days after challenge, and lung homogenates were evaluated for infectious titers of influenza. Three different strains of pandemic H5N1 were evaluated, which, in the absence of AAV9.FI6, led to rapid weight loss and euthanasia of all animals within 10 days (Fig. 2, A, B, D, E, G, and H). AAV9.FI6 provided protection against all three H5N1 pandemic strains with no evidence of residual virus at the conclusion of the study at day 28 (Fig. 2, C, F, and I). Protection ranged from 100% survival and 6-log decrease in virus loads at day 6 for H5N1 A/Indonesia/5/05 (Fig. 2, A to C), 90% survival and 5-log decrease in virus load for H5N1 A/Vietnam/1203/04 (Fig. 2, D to F), and 50% survival and 3-log reduction in virus load for H5N1 A/Hong Kong/483/97 (Fig. 2, G to I). Similar studies were performed with two isolates of H1N1, both of which were associated with substantial replication of virus in the lungs and complete mortality in the absence of treatment. AAV9.FI6 yielded complete protection against a clinical isolate of H1N1 2009 that caused the most recent human pandemic and a 3-log reduction in virus load at day 6 (Fig. 2, J to L). A particularly noteworthy finding was that AAV9.FI6 fully protected animals against challenge with a virus reconstructed from clinical material associated with the H1N1 1918 pandemic, and no virus was detected at day 6 (Fig. 2, M to O).

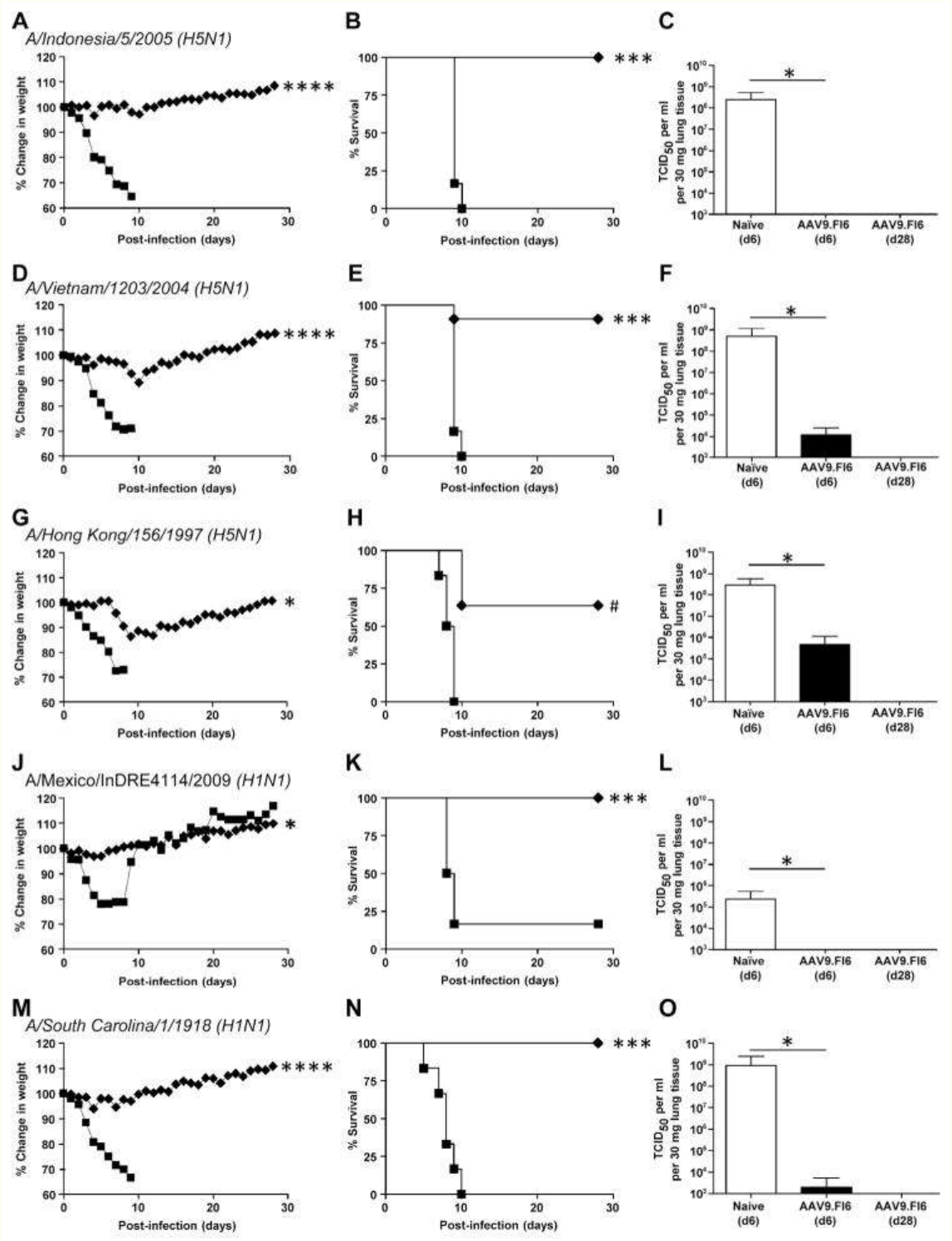

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Fig. 2

AAV9.FI6 protects mice against pandemic strains of H5N1 and H1N1

BALB/c mice were given 10^{11} GC of AAV9.FI6 vector in 50 μl intranasally (n = 15 per group). (**A** to **O**) Fourteen days later, vector-treated and naïve (n = 10) mice were challenged intranasally with 100 LD_{50} of three different strains of H5N1 [A/Indonesia/5/2005 (A to C), A/Vietnam/1203/2004 (D to F), and A/Hong Kong/156/1997 (G to I)] and two different strains of H1N1 [A/Mexico/InDRE4114/2009 (J to L) and A/South Carolina/1/1918 (M to O)]. The weights of the animals were followed daily (first column), and

mice were euthanized when they appeared in distress or their body weight declined >30% as depicted in the Kaplan-Meier plots (second column). Four mice from the naïve and vector-treated groups were necropsied at day 6 to quantify viral load in the lung (right column). The viral load was also quantified at the conclusion of the experiments in surviving animals (day 28). Diamonds and squares represent AAV9.FI6 vector–treated and naïve mice, respectively. *$P \leq 0.05$, ****$P \leq 0.0001$, Mann-Whitney test; $^{\#}P \leq 0.05$, ***$P \leq 0.001$, Mantel-Cox test.

Intranasal AAV9.FI6 vector delivery affords protection against pandemic influenza in ferrets

Further evidence for the utility of this approach was obtained in ferrets, an accepted animal model for the assessment of influenza vaccines (12). Before conducting challenge experiments, we evaluated AAV9 transduction after intranasal delivery using a variety of reporter genes. Noninvasive imaging of animals 7 days after intranasal delivery of AAV9.ffLuc showed signal specifically over the nose on the side where the vector was administered (Fig. 3A). Animals administered AAV9.nLacZ were sacrificed 7 days later, and nasal turbinates were harvested and stained en bloc with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside); high levels of transduced cells were observed (Fig. 3B). Finally, animals were dosed bilaterally in the nose with AAV9 expressing secreted rhesus α-fetoprotein (rhAFP). Analyses of NLFs revealed rhAFP expression 7 days after vector delivery (Fig. 3C).

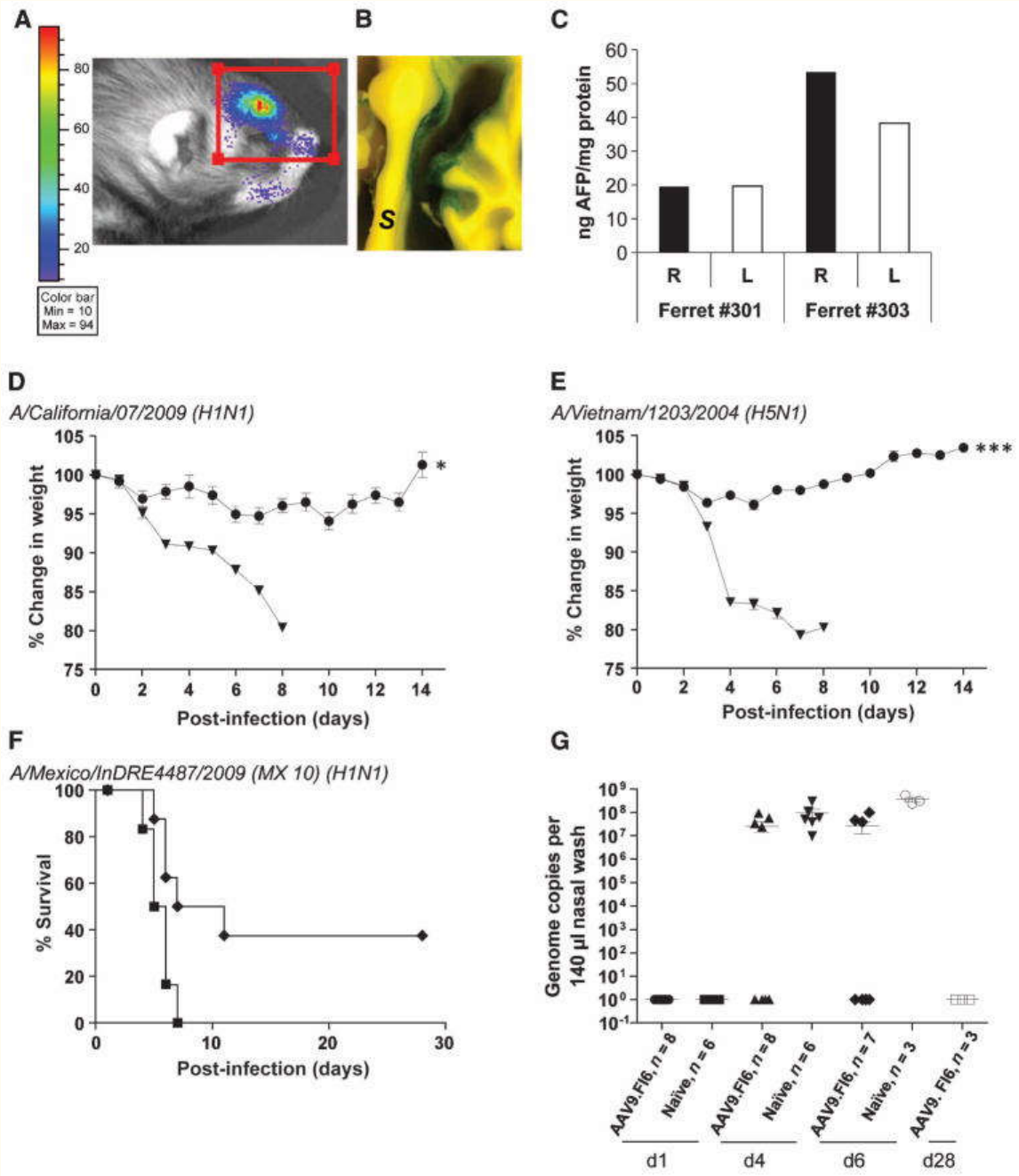

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Fig. 3

AAV9-mediated transduction of the ferret nasal airway

(**A**) Distribution of luminescence in the nasal cavity of a representative ferret 7 days after intranasal delivery of 10^{12} GC of AAV9.ffLuc. (**B**) En face view of the nasal septum (S) of a ferret administered 10^{12} GC of AAV9.nLacZ. (**C**) rhAFP expression in the NLF of ferrets collected 7 days after the intranasal administration of 10^{12} GC of AAV9.rhAFP. For the influenza challenge experiments, ferrets were dosed with 10^{12} GC of AAV9.FI6 vector given intranasally in 400 µl (200 µl per nare) to target the nasopharynx. (**D** and **E**) Seven days later, the ferrets were challenged with a lethal dose of either A/California/07/2009 (H1N1) (D) given under ABSL2 conditions or A/Vietnam/1203/2004 (H5N1) (E) given under ABSL3 conditions. The challenged animals were followed for weight loss and signs of respiratory distresses per guidelines. Circles, treated; triangles, naïve. (**F**) Fitch ferrets (n = 8 for the AAV9.FI6 group, n = 6 for

naïve controls) were administered intranasally with 10^{12} GC AAV.FI6 and challenged intranasally with 100 LD_{50} (10^6 PFU) of Mx10 7 days after treatment, and survival was monitored. Diamonds, treated; squares, naïve. (**G**) Viral load in nasal washes was monitored after challenge. Each data point represents viral load from an individual animal. Closed circles and triangles depict vector-treated and naïve ferrets, respectively. *$P \leq 0.05$, ***$P \leq 0.001$, Mann-Whitney test.

In experiments performed at the University of Pittsburgh, ferrets were given AAV9.FI6 (10^{12} GC in 400 μl, intranasally) and, 7 days later, challenged with a pandemic H5N1 strain, A/Vietnam/1203/2004, and an H1N1 clinical isolate, A/California/07/2009, in volumes of 1 ml. The naïve animals that did not receive AAV9.FI6 presented with marked weight loss, exhibited signs of respiratory distress, and had to be euthanized within 8 days of the challenge. The AAV9.FI6 treatment groups showed only minor and transient weight loss, and all fully recovered (Fig. 3, D and E). Additional ferret experiments were conducted at the National Microbiology Laboratory (NML) of the Public Health Agency of Canada (PHAC). Animals treated with AAV9.FI6 vector were challenged 7 days later with a pandemic H1N1 strain that is very virulent in ferrets [A/Mexico/InDRE4487/2009 (Mx10); Fig. 3F]. Control animals that did not receive AAV9.FI6 developed high fevers, severe neurological symptoms including hindlimb paralysis, and marked decreases in body temperature before death, and all had to be euthanized within 7 days after challenge (Fig. 3F). Four of the eight AAV9. FI6-treated animals demonstrated some level of efficacy, with one animal exhibiting a delayed time to death and three animals surviving with minimal symptoms. Real-time reverse transcription polymerase chain reaction results of nasal washes collected on days 1, 4, 6, and 28 showed that for untreated moribund animals, virus could be detected at very high levels in the nasal wash 4 days after infection and persisted until death (Fig. 3G). Replication of influenza virus in vector-treated animals was segregated into two different groups: high viral loads (>10^8 GC per nasal lavage) were found in all animals that were euthanized, whereas virtually no virus (below limit of detection) was detected in nasal lavage of treated animals that survived (Fig. 3G). The virologic data raise concerns that vector was not consistently delivered in this experiment.

AAV9 provides prolonged and robust transgene expression in nasal epithelia of rhesus macaques

Pilot studies were performed in rhesus macaques to assess the feasibility of translating this delivery strategy into primates. An AAV9 vector expressing the secreted reporter rhAFP was administered intranasally into macaques that were followed for expression of the trans-gene by ELISA of NLF; data from two animals are shown in Fig. 4. The use of a rhesus-derived version of AFP allowed for evaluation of the kinetics of expression without confounding adaptive immune responses. Onset of expression was fast with near peak levels noted at the first time point, which was 14 days, and lasted to more than 100 days. Expression at peak was higher than the level of rhAFP obtained in ferrets at doses of vector that demonstrated complete protection against both H1N1 and H5N1. This suggests that AAV9 should be capable of achieving levels of FI6 in the primate nose that can protect against pandemic influenza.

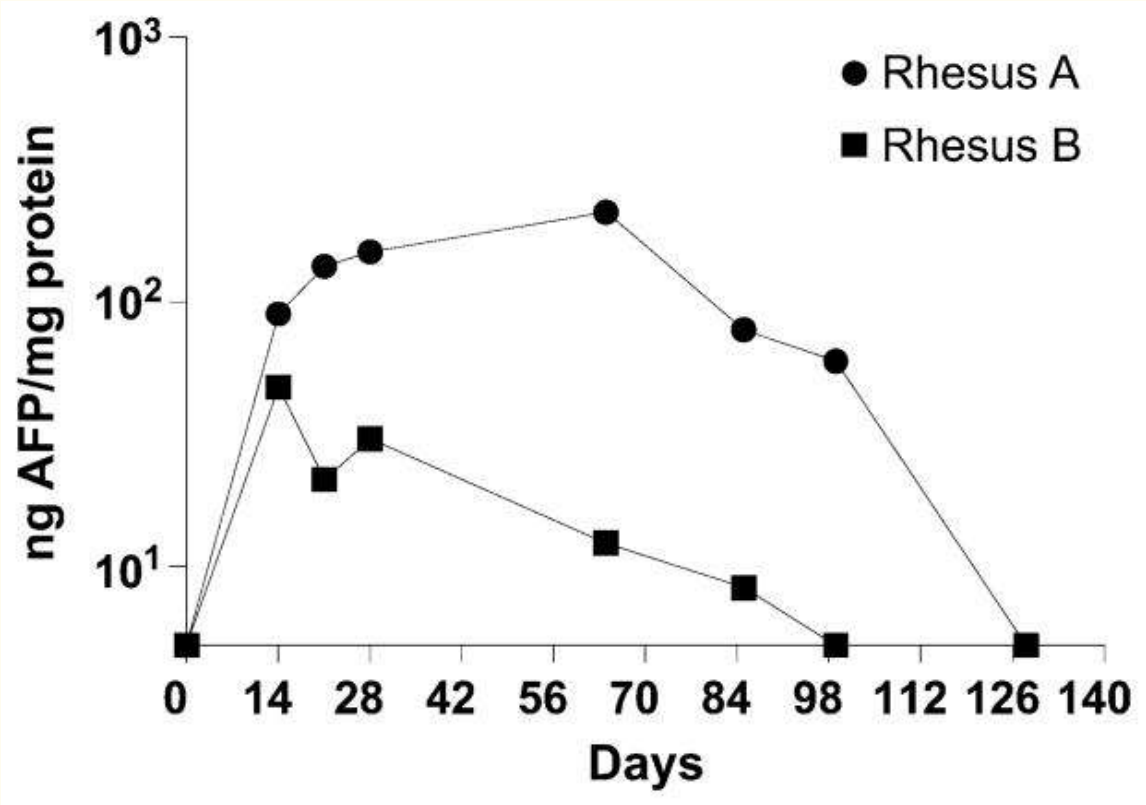

Fig. 4

AAV9-mediated transduction of the rhesus macaque nasal airway

An AAV9 vector (10^{13} GC) expressing rhesus-derived AFP was administered into the left nostril of two rhesus macaques. Sequential nasal lavage samples were harvested and analyzed for total protein and rhAFP.

DISCUSSION

The goal of the study was to evaluate the potential of vector-mediated expression of a broadly neutralizing antibody in the nasal cavity in affording protection against strains of pandemic influenza. Localized, durable, and high-level expression of the broadly neutralizing influenza antibody FI6 was achieved in nasal epithelia of mice and ferrets with AAV9 vectors. This treatment provided virtually complete protection against a wide range of clinical pandemic influenza isolates in both animal species.

Two near-term applications of this platform for influenza infections should be considered. The first is in the setting of an emerging influenza pandemic, which, in most cases, may be controlled with the FI6-expressing AAV9 vector that is effective against a broad range of group 1 and 2 influenza A viruses. The product can be stockpiled and rapidly deployed, with efficacy realized only days after dosing. A second application of the AAV platform could be in the context of seasonal influenza infections in high-risk individuals. The vector can be engineered to express two IAs to allow for a second antibody with broad activity against influenza B strains to complement the activity of FI6, which is effective against influenza A strains (5, 6). The 2012/2013 influenza season illustrated the limitations of the current approach to active immunization, which was active in only 9% of those more

than 65 years old (http://www.cdc.gov/mmwr/). Administration of an AAV9 vector expressing antibodies that cover both influenza A and B viruses at the beginning of the season would provide rapid onset and broad coverage in this high-risk group during the period of high seasonal infection activity.

Intranasal delivery of AAV9 could be considered for other respiratory pathogens for which mAbs are available or could be rapidly identified and optimized. The system as configured is a platform in that all products share the exact same manufacturing processes and any mAb gene, or a combination of mAb genes, can be inserted into the vector genome. One potential application of this technology is as a generic countermeasure against a wide range of naturally occurring pathogens or intentionally released bioweapons. The very rapid onset of activity that this system provides is attractive in the setting of the release of a bioweapon or a rapidly expanding pandemic to a newly emerging pathogen.

Clinical development of vector-mediated antibody expression for infectious diseases must carefully consider the selection of antibodies so as to avoid immunological escape of the pathogen. Broadly neutralizing antibodies such as FI6 are generally directed against invariant domains of pathogen proteins that are essential for their growth and are intolerant to structural changes. However, broad-scale distribution of a vector expressing an antibody will impose substantial immunologic pressure on a diverse flora of variants across large populations of humans, which could predispose to viral escape. One could potentially mitigate this problem by coexpressing broadly neutralizing antibodies directed to different invariant domains of the pathogen.

The broad spectrum of efficacy in two relevant animal models of influenza suggests that intranasal delivery of AAV9.FI6 should be seriously considered for further development as a rapid and effective prophylaxis for influenza. The safety profile of the AAV vector system in humans has been encouraging in clinical trials (13–16). An advantage of nasal delivery is that the AAV vector and its transgene product are localized to nasal epithelia and not widely disseminated. Furthermore, vector engraftment and IA expression are not permanent because the vector genome does not integrate and the airway cells turnover with time (11). Vector-encoded transgene expression, however, is sufficiently durable to provide a prolonged period of protection before readministration is necessary, which, for this vector in the airway, is indeed possible as shown in mouse gene transfer studies and nonhuman primate vaccine experiments (11, 17). Once safety of the product is discharged in phase 1 human studies, it may be possible to evaluate efficacy in human influenza challenge experiments (18).

MATERIALS AND METHODS

Rationale

AAV9 vector applied directly onto the airway has been shown to efficiently target ciliated cells. Our approach focuses on topical airway application of AAV9 for expression of broadly neutralizing antibodies against influenza. These antibodies should neutralize influenza virus, minimizing or preventing severe symptoms that include death. The size of animal groups was determined as the minimum number of animals necessary to allow for statistical analysis. Sample size for all ABSL2 mouse studies was five mice per group. For ABSL4 mouse studies, there were 15 mice per group; 4 mice were removed for viral load determination at day 6. For the ABSL2/3 ferret studies, six naïve and six to eight vector-treated animals were used. All animal studies were conducted in a controlled and nonblinded manner.

Vector design

Expression cassettes were constructed encoding the variable regions of FI6 [Protein Data Bank (PDB): AEL31310.1, GenBank identifiers (GIs): 342674599 and 342674581], linked to the constant (CH2 and CH3) domains of human immunoglobulin G1 (IgG1), generating an antibody architecture known as an IA. A human IgG1 signal peptide was used to ensure cellular secretion of the IA. Protein sequences

were backtranslated, codon-optimized for mammalian expression, and synthesized de novo (GeneArt, Life Technologies). The FI6-IA sequences were cloned downstream of the hybrid CMV enhancer chicken β-actin promoter into the vector plasmids used to produce AAV9 vector stocks as previously described (19). As controls, vector expressing either the variable domains from the anti-HIV antibody PG9 (PDB: 3U36_L and 3U36_H, GIs: 358439909 and 358439908) using the IA design, nLacZ, or ffLuc was constructed and also cloned into the AAV-CB7 backbone, and AAV9 vector was produced.

AAV9 vector dosing in mice

Female BALB/c mice (6 to 8 weeks old) were purchased from Charles River Laboratories and housed at the Animal Facility of the Translational Research Laboratories at the University of Pennsylvania. Intranasal vector delivery was performed in mice anesthetized by intraperitoneal injection of ketamine/xylazine. For vector delivery targeting the lungs, mice were suspended by their dorsal incisors and received vector diluted in PBS to a total volume of 50 μl. For vector delivery targeting the nose, anesthetized mice received the vector dose in a total volume of 20 μl while lying on their side. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

AAV9 vector dosing in rhesus macaques

Two healthy adult rhesus male macaques, weighing 8.2 kg (rhesus A) and 5.05 kg (rhesus B), respectively, which were seronegative for AAV9-specific neutralizing antibody, were anesthetized with a mixture of ketamine (10 to 15 mg/kg) and dexmedetomidine (0.5 to 1.0 mg/kg) injected intramuscularly and intubated with an endotracheal tube to maintain a patent airway. A total of 10^{13} GC of AAV9.CB7.rhAFP diluted in 1 ml of PBS was delivered to the left nostril as previously described (20). The macaques were observed and monitored daily. NLF was collected as described (20), and secreted AFP was detected with the Human alpha-Fetoprotein Quantikine ELISA Kit (R&D Systems Inc.). All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Gene expression studies

Analysis of LacZ expression in the airways was performed as previously reported (11). For ffLuc expression, mice (~20 g) were anesthetized and injected intranasally with 50 μl of D-luciferin (15 mg/ml) (Caliper). After 5 min, mice were imaged with the IVIS Xenogen imaging system. Quantitation of signal was calculated with the Living Image 3.0 software.

For nLacZ gene expression, the lungs were processed as previously described (11). Transduction efficiency was estimated by examining five high-power fields (200×) from one cryosection and presented as LacZ-expressing cells per field. For assessment of gene transfer to the nasal airway epithelium, the heads were processed as previously described (11).

Influenza challenge experiments in mice—ABSL2

All mouse challenge studies occurred 14 days after AAV9 vector administration. Mice were anesthetized by an intraperitoneal injection of ketamine/xylazine, hung by their dorsal incisors, challenged with 10 LD_{50} influenza A/Puerto Rico/8/34 in a total volume of 50 μl, and weighed twice daily for the first 14 days. Any mice showing signs of distress or ≥30% weight loss were euthanized. Surviving mice were sacrificed 21 days after challenge, and tissue samples were harvested for analysis.

BALF and NLF collection

BALF was collected as previously described (11). For the NLF collection, mice were decapitated, a cannula attached to a 1-ml syringe was placed into the tracheal remnant, and 200 µl of sterile PBS was flushed through the nasal passages. The fluid was collected into a sterile tube, and the recovered fluid was used to flush the nasal cavity for a total of five times.

Influenza challenge experiments in mice—ABSL4

Mice were anesthetized with inhalational isoflurane (Baxter Healthcare), hung by their dorsal incisors, and challenged with 100 LD_{50} of live influenza H5N1 A/Hong Kong/483/97 [450 plaque-forming units (PFU)], H5N1 A/Vietnam/1203/04 (100 PFU), H5N1 A/Indonesia/5/05 (100 PFU), H1N1 A/South Carolina/1/18 [an engineered isolate from a discovered sequence (1.58×10^4 PFU = 10 LD_{50})], and H1N1 A/Mexico/2/2009 [10^5 $TCID_{50}$ (tissue culture infectious dose 50)] in 50 µl of Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 2% fetal bovine serum (HyClone, Thermo Fisher Scientific). Body weight, temperature, clinical signs, and mortality were recorded daily for 28 days after challenge. On day 6 after challenge, four naïve and four vector-treated mice for each challenge virus were sacrificed and the lungs were harvested for titration of virus. On day 28 after challenge, mice were sacrificed and the lungs were harvested for titration of virus. All animal procedures and scoring sheets were approved by the Institutional Animal Care Committee at the NML of the PHAC according to the guidelines of the Canadian Council on Animal Care. All infectious work was performed in the Biosafety Level 4 facility at NML, PHAC.

Harvested lung tissues from mice were frozen at −80°C. Viral load was determined by the $TCID_{50}$ assay, which results in cytopathic effects (CPEs) in 50% of cells. $TCID_{50}$ assay was performed by adding serial dilutions of homogenized lung tissue onto Madin-Darby canine kidney cells and monitoring for the presence of CPEs after 48 hours. The $TCID_{50}$ titer was calculated with the Muench and Reed method (21) and normalized per 30 mg of lung tissue.

Influenza challenge experiments in ferrets—ABSL2/3

Fitch ferrets (*Mustela putorius furo*, female, 4 to 6 months), which were determined to be negative for antibody to circulating influenza A (H1N1, H3N2) and influenza B viruses, were descented and purchased from Marshall Farms. Ferrets were pair-housed in stainless steel cages (Shor-Line) containing Sani-Chips Laboratory Animal Bedding (P.J. Murphy Forest Products). Ferrets were lightly anesthetized by inhalational isoflurane, and the vector was delivered as 200-µl boluses in each nare for a total dose of 10^{12} GC. The ferrets were returned to their cage and, at day 7, were challenged intranasally with 10^6 PFU of the novel 2009 H1N1 virus A/California/07/2009 or the H5N1 virus A/Vietnam/1203/2004 in a volume of 0.5 ml in each nostril for a total infection volume of 1 ml. After infection, the ferrets were monitored daily for weight loss and signs of disease. Individual body weights, sickness scores, and death were recorded for each group on each day after inoculation. Experimental endpoints were defined as >20% weight loss, development of neurological disease, or an activity score of 3 (neither active nor alert after stimulation). All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

Influenza challenge experiments in ferrets—ABSL4

Fitch ferrets (as above), which were determined to be negative for antibody to circulating influenza A and B viruses, were purchased from Marshall Farms. Ferrets were anesthetized by an intramuscular injection of ketamine/xylazine, and the vector was delivered as 200-µl boluses in each nare for a total dose of 10^{12} GC. The ferrets were challenged intranasally with 100 LD_{50} (10^6 PFU) of A/Mexico/InDRE4487/2009 (H1N1) in a volume of 0.5 ml in each nostril for a total infection volume of 1 ml. After infection, the ferrets were monitored daily for weight loss, body temperature, and signs

of disease. All animal procedures and scoring sheets were approved by the Institutional Animal Care Committee at NML, PHAC, according to the guidelines of the Canadian Council on Animal Care. All infectious work was performed in the Biosafety Level 4 facility at NML, PHAC.

Characterization of IAs by Western blot

BALF or NLF was run under nonreducing conditions on a 4 to 12% SDS–polyacrylamide gel (Invitrogen), and proteins were transferred to a polyvinylidene difluoride membrane. After blocking with 50 μM tris-hydrochloride, 150 μM sodium, 50 μM EDTA, 0.05% Triton X-100, and 2% gelatin, IAs were detected with a cocktail of goat anti-human IgG biotin-conjugated antibody (ab7152 and ab97168, Abcam) and a secondary streptavidin–horseradish peroxidase conjugate (ab7403, Abcam). The proteins were visualized by ECL detection (Thermo Scientific).

Statistical analysis

Analysis was performed with GraphPad Prism version 5.00 for Windows (GraphPad Software). The Mantel-Cox test was used to test the survival distributions for differences. The Student's *t* test or the Mann-Whitney test was used to determine differences between two groups. The Dunnett's test was used to compare a number of variables with a single control.

Supplementary Material

Supplemental Figures

Click here to view. (1009K, pdf)

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Footnotes

SUPPLEMENTARY MATERIALS

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Fig. S1. Expression of FI6-IA after intranasal delivery of AAV9 vector in mouse airway.

Fig. S2. Lung pathology after influenza challenge.

Fig. S3. Effect of neuraminidase pretreatment and vector dose on protection.

Author contributions: M.P.L. provided direction for conduct of all studies performed at Penn and helped write the first draft of the manuscript. She also performed the ferret and nonhuman primate reporter gene studies. V.S.A. conducted mouse experiments at Penn and helped clone the constructs. G.W. conducted the mouse challenge studies with pandemic isolates in BSL4. J.G. conducted the ferret challenge studies in BSL3 in Winnipeg. D.K. reconstructed the 1918 pandemic virus. T.M.R. was responsible for the ferret challenge studies at Pittsburgh. G.P.K. provided overall direction for all challenge studies in Winnipeg and helped develop

strategy for overall conduct of the program. A.T. provided advice regarding the design of the antibody gene cassettes. J.M.W. provided overall direction of the program and was the architect of the initial concept. He also worked with M.P.L. on the writing of the paper.

Competing interests: J.M.W. is a consultant to ReGenX Holdings and is a founder of, holds equity in, and receives a grant from affiliates of ReGenX Holdings; in addition, relevant to this work, he is an inventor on patents licensed to various biopharmaceutical companies, including affiliates of ReGenX Holdings. The other authors declare no competing interests. J.M.W. holds a patent on adeno-associated virus (AAV) clades (U.S. Patent 7,906,111B2) with pending continuation (U.S. Patent 13/023,918). J.M.W. and M.P.L. have a pending application on AAV-mediated passive immunization of airborne pathogens (PCT/US2012/034355).

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