Single-Dose Mucosal Immunization with a Candidate Universal Influenza Vaccine Provides Rapid Protection from Virulent H5N1, H3N2 and H1N1 Viruses

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Abstract

**Background:** The sudden emergence of novel influenza viruses is a global public health concern. Conventional influenza vaccines targeting the highly variable surface glycoproteins hemagglutinin and neuraminidase must antigenically match the emerging strain to be effective. In contrast, “universal” vaccines targeting conserved viral components could be used regardless of viral strain or subtype. Previous approaches to universal vaccination have required protracted multi-dose immunizations. Here we evaluate a single dose universal vaccine strategy using recombinant adenoviruses (rAd) expressing the conserved influenza virus antigens matrix 2 and nucleoprotein.

**Methodology/Principal Findings:** In BALB/c mice, administration of rAd via the intranasal route was superior to intramuscular immunization for induction of mucosal responses and for protection against highly virulent H1N1, H3N2, or H5N1 influenza virus challenge. Mucosally vaccinated mice not only survived, but had little morbidity and reduced lung virus titers. Protection was observed as early as 2 weeks post-immunization, and lasted at least 10 months, as did antibodies and lung T cells with activated phenotypes. Virus-specific IgA correlated with but was not essential for protection, as demonstrated in studies with IgA-deficient animals.

**Conclusion/Significance:** Mucosal administration of NP and M2-expressing rAd vectors provided rapid and lasting protection from influenza viruses in a subtype-independent manner. Such vaccines could be used in the interval between emergence of a new virus strain and availability of strain-matched vaccines against it. This strikingly effective single-dose vaccination thus represents a candidate off-the-shelf vaccine for emergency use during an influenza pandemic.

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Introduction

Pandemic influenza represents a major threat to global public health, with the potential for sudden emergence and explosive transmission of virus strains to which humans have little or no serologic immunity. Conventional influenza vaccines function by inducing antibodies against the highly variable surface glycoproteins hemagglutinin (HA) and neuraminidase (NA), and currently take at least 6 months to prepare and distribute once a potential pandemic strain has been identified [1,2]. This was highlighted by the 2009 swine origin H1N1 pandemic virus: the newly emergent virus was identified in April but sufficient vaccine for mass immunization was not available until October. Meanwhile the virus was spreading in the community [3,4].

So-called “universal” influenza vaccines providing cross-protective immunity in a strain- and subtype-independent manner could mitigate the impact of newly emergent virus strains when strain-matched vaccines are not yet available [5–7]. Universal vaccines would provide heterosubtypic immunity directed against viral components conserved among all influenza A viruses, and could be stockpiled for use in controlling outbreaks. While heterosubtypic immunity does not prevent infection, it can reduce morbidity and mortality and promote accelerated viral clearance [5,6], resulting in a shortened period of viral shedding and reduced transmission of virus.

In recent years considerable attention has been paid to the challenge of generating effective immune responses at mucosal surfaces. Mucosal surfaces are sites of pathogen entry, replication and pathology, making immune responses at these locations critical for effective immunity [8]. Candidate influenza vaccines delivered intranasally and inducing mucosal immunity include live-attenuated viruses [9], adjuvanted killed vaccines [10,11], and recombinant viral vectors. Recombinant adenovirus (rAd) vectors are potently immunogenic [12,13] and induce effective pathogen-specific mucosal immunity when delivered intranasally [14–17]. rAd vectors are under investigation in more than 300 human
Intranasal rAd immunization triggers strong mucosal immune responses

Anatomically compartmentalized immune responses are observed in DNA prime-rAd boost immunization [21], so we explored this for single-dose rAd immunization. Mice were immunized i.m. or i.n. with A/NP+M2-rAd or rAd expressing influenza B virus nucleoprotein (B/ NP-rAd) which serves as a specificity control to rule out innate immune protection due to the rAd vector. Antibody, T-cell, and cytokine responses were analyzed at one and ten months post-immunization.

At one month post-A/NP+M2-rAd immunization, serum IgG levels against M2e were high and equivalent between mice vaccinated i.n. and i.m. [Figure 2A], but IgG levels in bronchoalveolar lavage (BAL) were higher after i.n. than i.m. immunization. IgA against M2e was detectable only following i.n. immunization, with levels in BAL considerably higher than in serum. These differences in the antibody response were maintained at 10 months post-immunization [Figure 2B]. Similar results were seen for antibodies against rNP [Figure S1].

Elevated pro-inflammatory cytokine levels in BAL (IFN-γ, mKC, IL-12) were seen after i.n. but not i.m. rAd immunization, regardless of the transgene, at both one and 10 months post-immunization [Figure 2C and D]. Levels of other cytokines in BAL (IL-1β, IL-2, IL-4, IL-5, IL-10 and TNF-α) were low at both one and 10 months and equivalent between groups (data not shown). Serum cytokine levels were low and similar between groups at both one and 10 months (data not shown).

T-cell responses against conserved viral epitopes were assessed by IFN-γ ELISPOT analysis of spleen [Figure 2E and F] and lung cells [Figure 2G and H]. At one month post-immunization, strong responses to the immunodominant H2-Kd restricted NP147–155 epitope were seen in spleen after i.m. but not i.n. immunization with A/NP+M2-rAd. The converse was observed in lungs, with
Figure 2. Immune responses after single-dose rAd immunization. BALB/cAnNCr mice were immunized with $5 \times 10^9$ particles each of A/NP–rAd and M2–rAd, or $1 \times 10^{10}$ particles of B/NP–rAd i.n. or i.m., or were unimmunized (naive). Analyses were performed at one month (A, C, E, G) or 10 months (B, D, F, H) post-immunization. (A, B) M2e-specific IgG (left panels) and IgA (right panels) responses in serum and BAL were measured by ELISA. (C, D) Cytokine concentrations in serum were measured by ELISA. (E, F) IFN-γ–secreting cells in the spleen were measured by ELISPOT. (G, H) IFN-γ–secreting cells in the lung were measured by ELISPOT.
approximately 3-fold more NP147–155-specific lung cells per mouse following i.n. compared to i.m. immunization. Responses to MHC-II restricted epitopes (M2e2–24 and NP55–69) followed a similar pattern but were of lower magnitude. Responses to rAd epitopes Hex486–494 and Dbp413–421 were independent of the transgene and much higher in spleen after i.m. than after i.n. immunization, but were similar in magnitude in lungs regardless of immunization route. Similar compartmentalization of the T-cell response was observed at 10 months post-immunization. Responses to the dominant NP147–155 epitope in both spleen and lungs were similar in magnitude to those at 1 month. In contrast, the M2e2–24-specific response in the spleen was lower than at 1 month but was considerably greater in the lungs of A/NP+M2-rAd i.n. mice. The response to rAd epitopes was much lower at 10 months than at one month regardless of the route of administration or transgene.

Tetramer staining confirmed that animals immunized with A/NP+M2-rAd i.n. had more NP147–155-specific CD8+ T cells recoverable from lungs than i.m. immunized animals (Figure 3A). Compared to i.m. rAd immunized mice, a greater proportion of tetramer positive cells from lungs of i.n. rAd immunized mice showed an effector memory (CD62Llo) phenotype, and the majority were also CD127hi (Figure 3B). A smaller proportion of cells with this activated phenotype were seen in the NP147–155-tetramer negative population, which contains T cells specific to other epitopes including the rAd vector. More CD8+ T cells (both

Figure 3. Lung T cell phenotyping. BALB/cAnNCr mice were immunized with 5×10⁹ particles each of A/NP-rAd and M2-rAd, or 1×10¹⁰ particles of B/NP-rAd i.n. or i.m., or were unimmunized (naive). Lung T cells were isolated and analyzed by flow cytometry. (A) Total number of Kd-NP147–155 tetramer+ T-cells recovered from the lungs of mice, as determined by multicolor flow cytometry. Bars show mean ± SEM of 3 animals per group. (B) Phenotypic analysis of lung CD8+ T cells. Pie charts show relative proportions of central memory (CD62Lhi), effector memory (CD62Llo, CD127hi) and activated effector memory (CD62Llo, CD127lo) among tetramer positive (upper pies) and tetramer negative (lower pies) CD8+ T-cells. (C) Activation status of lung CD8+ T cells, as determined by staining for tetramer vs. CD69. Each plot shows one representative mouse per group of 3 mice assessed. Numbers in plots indicate % of CD8+ T cells per quadrant. * Indicates a statistically significant difference (P<0.05) compared to all other groups.

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tertamer positive and negative) expressed the early activation marker CD69 after i.n. than i.m. immunization (Figure 3C), indicating that not only did i.n. rAd immunization result in greater virus-specific CD69+ T-cell responses in the lung, but these cells also possessed a more activated phenotype than cells induced by i.m. immunization.

Intranasal rAd immunization provides long-lived protection from virulent influenza virus challenge

Immunized animals were tested for protection against challenge with a highly pathogenic H5N1 strain with pandemic potential (A/VN1203) at both one and 10 months post-immunization. At one month after immunization, all animals in the A/ΔNP+ΔM2-rAd i.n. group were protected and had little weight loss (Figure 4). In contrast, the A/ΔNP+ΔM2-rAd i.m. group suffered significant weight loss and 30% died by day 11. Control animals died by day 8. Lung virus titers 3 days post-challenge were significantly lower in mice immunized with A/ΔNP+ΔM2-rAd by either route than in controls, with titers significantly lower after A/ΔNP+ΔM2-rAd i.n. than i.m. immunization. At day 5, only A/ΔNP+ΔM2-rAd i.n. immunized mice had significantly lower titers than controls. When challenged 10 months after immunization, control animals died with comparable kinetics to those challenged at 1 month. All A/ΔNP+ΔM2-rAd i.n. immunized animals were protected with little weight loss, but A/ΔNP+ΔM2-rAd i.m. immunized animals suffered more severe weight loss than at 1 month and 37.5% succumbed to infection (Figure 4D and E). Lung virus titers at both days 3 and 5 post-challenge were significantly lower than controls in mice immunized with A/ΔNP+ΔM2-rAd via either route. Interestingly, virus titers in A/ΔNP+ΔM2-rAd immunized mice were lower at 10 months than at one month (Figure 4G and F). Despite these reduced lung virus titers, the A/ΔNP+ΔM2-rAd i.m. group had worse outcomes than at one month. The reasons for this are unclear.

Protection was also assessed in immunized animals challenged with a virulent mouse-adapted H3N2 strain [X-79] (Figure S2). Complete protection was seen in mice immunized with A/ΔNP+ΔM2-rAd via either route when challenged with X-79 one month after vaccination, although significantly greater weight loss (P<0.05) was observed for i.m. than i.n. immunized animals. Lung virus titers at day 3 were lower in the A/ΔNP+ΔM2-rAd groups than in control groups, with titers significantly lower (P<0.05) after i.n. compared to i.m. immunization. At day 5, lung virus titers were further reduced and similar between A/ΔNP+ΔM2-rAd i.m. and i.n. groups. At 10 months post-A/ΔNP+ΔM2-rAd immunization, complete protection with minimal weight loss was maintained in mice immunized i.n., but was waning in animals immunized i.m. (75% protection with severe weight loss). This is reflected in day 3 lung virus titers, which were significantly lower in A/ΔNP+ΔM2-rAd i.n. mice than in all other groups.

IgA is not required for protection

As virus-specific IgA was detectable after i.n. but not i.m. rAd immunization, the role of this response in enhanced protection was examined. BALB/c-IgA−/− mice were immunized with A/ΔNP+ΔM2-rAd i.n. or i.m., or with B/ΔNP-rAd i.n. as a control. M2e-specific IgG was detectable in serum 2 weeks post-immunization (Figure 5A) and the IFN-γ ELISPOT response in PBMCs (Figure 5B) was similar to wild-type mice (see Figure S3B). When challenged with A/FM one month post-immunization all control animals died, while i.m. immunized animals lost considerable weight and 50% died (Figure 5C and D). The i.n. immunized animals lost significantly less weight than i.m. immunized mice and survived challenge, indicating that while IgA may play a role following i.n. rAd immunization, it is not required for protection and is likely not the sole reason for superiority of mucosal immunization.

Intranasal immunization rapidly induces protection

Universal vaccines could be deployed when strain-matched vaccines are unavailable, providing interim protection early in a pandemic. To evaluate this potential, we examined how rapidly protection develops. Mice were immunized i.n. or i.m., then challenged with A/FM at various times post-immunization and monitored for survival and weight loss (Figure 6). Control animals immunized with B/ΔNP-rAd lost significant weight and died. Partial protection was observed in A/ΔNP+ΔM2-rAd i.m. mice challenged one week post-immunization although animals lost significant weight. This partial protection was lost at 2 and 3 weeks but began to reappear at 4 weeks and good protection was observed at 6 months despite significant weight loss. Animals immunized with A/ΔNP+ΔM2-rAd i.n. showed little protection at one week but had 90% protection at 2 weeks and complete protection with little weight loss from 3 weeks out to at least 6 months post-immunization.

Examination of antibody levels in the serum revealed that IgG specific to M2e was detectable within one week of i.m. immunization and within 2 weeks for i.n. immunization (Figure S3A). IgG reached maximal levels by 5 weeks and remained high for at least 6 months. Serum IgA responses became detectable in i.n. immunized mice by 2 weeks after immunization for M2e. The IgA response was absent in the case of i.m. immunization. Again, development of a virus-specific serum IgA response correlated with protection for the i.n. rAd immunized groups. However there was no serum antibody correlate for the partial protection observed in i.m. immunized mice at one week; although IgG responses against M2e were observed early after i.m. immunization, they increased further at weeks 2–5 as protection decreased.

As assessed by IFN-γ ELISPOT, virus specific T cell levels in peripheral blood were modest one week after i.m. rAd immunization, higher at 2–4 weeks, and then declined to approximately one third of peak levels at 6 months post-immunization (Figure S3B). As expected, strong responses were seen against the immunodominant NP147–155 peptide, with lower responses directed to NP55–65 and M2e2–24 helper T cell epitopes. Peripheral blood T-cell responses to NP147–155 following i.n. rAd immunization were lower and rose later than after i.m. immunization but did not decline by 6 months (Figure S3B).

Intranasal rAd immunization confers accelerated challenge virus clearance

Kinetic studies of lung virus titers were performed to assess rate of virus clearance after A/FM challenge. Maximal titers were seen 2 days post-challenge in all groups (Figure 7) with B/ΔNP-rAd immunized mice retaining high titers through day 7. A/ΔNP+ΔM2-rAd i.m. immunized mice had peak virus titers similar to those of B/ΔNP-rAd mice but exhibited a steady decrease in titer from day 3 onward, with virus clearance complete by day 10. Mice immunized with A/ΔNP+ΔM2-rAd i.n. showed significantly lower titers (P<0.001) as early as day 2 post-challenge and cleared virus more rapidly than the other groups, with clearance complete by day 7.

Discussion

The recent emergence of an unanticipated pandemic virus has highlighted the need for influenza vaccines providing broad coverage across multiple strains and subtypes. This study demonstrates that a single dose of rAd vaccines expressing two
highly conserved influenza virus antigens (NP and M2) protects from virulent influenza virus challenges with multiple widely divergent subtypes. Protection is greatly enhanced by i.n. administration, develops by 2 weeks post-immunization, and persists for at least 10 months. Intranasal rAd vaccination decreased lung virus titers and accelerated challenge virus clearance relative to i.m. vaccination. This reduction in virus titers corresponded to greatly reduced morbidity, as shown by weight loss, compared to controls and i.m. immunized animals.

Antibody and T-cell responses against both NP and M2 were seen. Previous work suggests that dominant protective mechanisms are likely M2e-specific antibodies that limit viral spread [24] and

Figure 4. Morbidity and mortality after H5N1 challenge following single-dose rAd immunization. Groups of 10 (at one month) or 8 (at 10 months) BALB/cAnNCr mice were challenged with $10^{2.84}$ EID$_{50}$ (~10 LD$_{50}$) of A/VN1203 one month (A, B, C) or 10 months (D, E, F) after immunization. (A, D) Survival after challenge. (B, E) Weight loss after challenge. When challenged one month post-boosting, statistically significant differences in weight loss ($P<0.05$) were observed between A/NP+M2-rAd i.n. and all other groups at days 4–12. When challenged at 10 months, weight loss in A/NP+M2-rAd i.n. immunized mice was significantly ($P<0.05$) different from all other groups from days 4–16. (C, F) Virus titers in the lungs at days 3 and 5 after challenge as determined by plaque assay. Bars show log$_{10}$ geometric mean titer ± SEM of 4 mice per group. The dashed line shows limit of detection. * indicates a statistically significant difference ($P<0.05$) compared to all other groups at the same time point; ** indicates a significant difference from B/NP-rAd and naïve groups.

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Figure 5. IgA is not required for protection after i.n. rAd immunization. Groups of 6 IgA$^{-/-}$-BALB/c mice were immunized with $5 \times 10^9$ particles each of A/NP-rAd and M2-rAd i.n. or i.m., or $1 \times 10^{10}$ particles of B/NP-rAd i.n. (A) IgG responses against M2e were measured by ELISA using serum obtained 2 weeks after immunization. The dashed line indicates limit of detection. (B) Antigen-specific T-cell responses at 3 weeks post-immunization were determined in peripheral blood pooled from these animals by IFN-γ ELISPOT using NP$_{42-55}$, NP$_{55-69}$ or M2e$_{2-24}$ peptides as stimulus. Unstimulated cells (no peptide) were used as a control. Bars show mean ± SEM of triplicate wells for each group per stimulus. At one month post-immunization, animals were challenged with $10^3$ TCID$_{50}$ (100 LD$_{50}$) of A/FM and monitored for survival (C) and weight loss (D). Error bars in weight loss graph indicate mean ± SEM. Statistically significant differences in weight loss ($P<0.05$) were observed between A/NP+M2-rAd i.n. and i.m. at days 2–15, between A/NP+M2-rAd i.n. and B/NP-rAd at days 3–13, and between A/NP+M2-rAd i.n. and B/NP-rAd at days 2, 3, 9 and 13.

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Figure 6. Kinetics of protection after single-dose rAd immunization. BALB/cAnNCr mice were immunized with 5×10^9 particles each of A/NP-rAd and M2-rAd, or 1×10^10 particles of B/NP-rAd i.n. or i.m. and challenged with 10^3 TCID_{50} (≈100 LD_{50}) of A/FM at 1, 2, 3, or 4 weeks or 6 months later. Left panels show survival and right panels show weight loss after challenge. Groups consisted of 10 mice per immunization per challenge time.

Error bars in weight loss graphs indicate mean ± SEM. Statistically significant (P<0.05) differences in survival were as follows: A/NP+M2-rAd i.n. was significantly different from A/NP+M2-rAd i.m. at weeks 2, 3, and 4, and from B/NP-rAd i.n. or i.m. at all times; A/NP+M2-rAd i.m. was significantly different from B/NP-rAd i.m. at 2 weeks and 6 months, but not different from B/NP-rAd i.n. at any time. In terms of weight loss, no statistically
significant differences were seen between groups at week 1. At week 2 A/NP+M2-rAd i.n. differed (P<0.05) from all other groups on days 2–7, and also from B/NP-rAd i.n. on day 1; A/NP+M2-rAd i.m. was significantly different from B/NP-rAd i.n. on days 2–3 and from B/NP-rAd i.m. on day 1. At week 3 A/NP+M2-rAd differed from all other groups on days 2–8; B/NP-rAd i.n. differed from all other groups on day 3. At week 4 A/NP+M2-rAd i.n. was significantly different from all other groups on days 2–15; A/NP+M2-rAd i.m. differed from B/NP-rAd i.m. on day 11. At 6 months A/NP+M2-rAd i.n. was significantly different from all other groups on days 2–10, and from A/NP+M2-rAd i.m. on days 11–15.

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mediated antibody-dependent cellular cytotoxicity [25], and NP-specific CD8+ T cells eliminating infected cells by cytolysis [26]. M2e and NP-specific helper T cells probably make a lesser contribution as direct anti-viral effectors. A role for NP-specific antibody has also been proposed [27], but the significance of this in protection is debated. As with prime-boost studies [20,21], the A/NP+M2 rAd vector combination was superior to either component alone in providing protection, which may be due to induction of complementary effector mechanisms including virus-specific IgA and T cells in the respiratory tract.

Targeting multiple viral antigens, in this case NP and M2, clearly has advantages over using a single antigen. Immunization with either M2-rAd or A/NP-rAd alone confers less protection than using a mixture of these two rAd vectors (Figure 1). The A/NP+M2-rAd combination also has potential advantages in preventing emergence of viral escape mutants, which have been reported for NP [28,29] and M2 [30]. However, even under selective pressure from monoclonal antibodies, very few escape mutant sequences were observed for M2e [30] suggesting that this region is biologically constrained. Combination vaccines also reduce the possibility that individuals with certain HLA types may be overall non-responders to the vaccine. The latter is a potential problem with M2 due to the small size of this protein (97 amino acids) and the limited number of potential epitopes it may contain, but less of a concern with the larger NP (498 amino acids) within which multiple epitopes for numerous MHC-I and –II alleles have been identified [31,32]. It is possible that incorporation of additional antigens (such as M1 or PB1) into heterosubtypic vaccines may further broaden and enhance the response.

HA-expressing rAd influenza vaccines intended to induce strain-matched neutralizing antibodies have been studied in mice [33,34], chickens [35], and humans [36], but would require regular reformulation to accommodate antigenic variation in HA. Recent studies show that monoclonal antibodies against conserved epitopes in the HA stem region can neutralize viruses of several subtypes [37–39]. However, such antibodies do not react with all HA subtypes, so multiple such immunogens would be needed to cover all subtypes. NP-expressing rAd5 and chimp rAd vectors given i.m. provided partial protection from both H1N1 and H5N1 challenges in mice [40], but required a higher vaccine dose than reported here. Our findings demonstrate that a single dose of the A/NP+M2-rAd combination given i.m. provided complete protection against highly virulent H1N1, H3N2, and H5N1 influenza virus challenges.

Compared to the DNA prime-rAd boost approach previously reported [21], the single-dose rAd strategy reported here has the advantage of a streamlined vaccination protocol. Significantly, it obviates the requirement for multiple vaccine doses used in prime-boost regimens (3 doses of DNA given at 2 week intervals by i.m. injection, followed by rAd given i.m. or i.n. one month later is typical). We have previously demonstrated that for NP-based immunization, DNA priming enhances protection over that of i.m. rAd alone [41]. However, results reported here suggest that priming is less critical when rAd is given intranasally.

Concern has been raised about the possibility of adenovirus vectors accessing the central nervous system. Although transgene expression in the olfactory bulb occurs after i.n. administration of rAd vectors to mice, this is transient, low level, and not associated with inflammation [42,43]. Another potential limitation of rAd vaccines is host immunity to the vector which may interfere with vaccination [12]. This is a particular concern for Ad5-based
vectors, to which much of the human population has neutralizing antibody [44]. While experimental animals mount strong immune responses against rAd vectors, this is not always the case in humans. In gene therapy trials some individuals do not make neutralizing antibody responses against rAd given i.n., even after repeated administrations [45]. Other reports suggest that rAd immunization may induce de novo T-cell responses against the transgene despite pre-existing immunity, albeit lower levels than in seronegative individuals [46]. To circumvent immunity, vectors based on rare adenovirus serotypes [47], non-human primate adenoviruses [48], or chimeric viruses [49] have been suggested in place of current Ad5 vectors.

The rAd doses used here are broadly similar to those used in other vaccine studies. It may be possible to use lower rAd doses. Preliminary results using A/NP:rAd given i.n. suggest that similar levels of protection are achieved with a 10-fold lower rAd dose (Figure S4). Studies to assess minimum protective doses for the A/NP-M2-rAd combination are ongoing. An effective rAd vaccine dose for humans would have to be addressed in future clinical trials, and may not require a dose proportionate to body weight. Further vector optimization (for example by encoding both NP and M2 on a bicistronic vector) may be possible, and could allow vaccine dose to be reduced still further. It should be noted that since rAd-based vaccines targeting conserved antigens would not need to be changed frequently, vaccine manufacture could occur on an ongoing basis to produce a stockpile, rather than on a seasonal basis as is the case for current influenza vaccines.

Immune correlates of protection are needed for new vaccine types. IgA is not required for protection, but may play a role in protection when present, and could provide a useful correlate. Serum IgG responses do not correlate with protection. They are similar between i.n. and i.m. rAd immunizations which differ in protection (Figure 2A and B; Figure S1), and develop earlier after i.m. than i.n. rAd, strengthening at 2 weeks as protection decreases (Figure S3A). Mucosal IgG appears more promising, as i.n. rAd immunization induced higher BAL IgG responses than i.m. rAd. Interestingly, this indicates that i.n. rAd immunization likely induces IgG-secreting cells resident within the respiratory tract; if antibody reached the BAL by transudation from serum (where IgG levels are similar between i.n. and i.m. immunized mice), then BAL IgG levels would be equivalent regardless of immunization route.

Ideally, correlates of protection should be feasible to assess in humans with non-invasive sampling methods. Anatomical compartmentalization of cellular immune populations after i.n. immunization (ref. 21 and this study) complicates the matter. Cellular correlates can be identified, for example IFN-γ secreting virus-specific T cells in the lungs, but cannot be directly measured in humans. However, the frequency of IFN-γ secreting cells in blood increases between 1 and 6 months after i.n. immunization (Figure S3B), suggesting equilibration between lung and blood T cell pools over time. If the relevant lung T cells possess a distinctive phenotype of memory, homing or activation markers, perhaps low numbers of comparable cells could be detected in the circulation.

Our studies using a non-replicating viral vector rather than productive infection are in agreement with reports that virus-specific T cells resident in the lungs after clearance of viral infection exhibit an activated phenotype in both mice [50,51] and humans [52]. While the classic paradigm is that during recall responses memory T cells activated in draining lymph nodes recirculate back to the site of infection to clear pathogen, T cells already present in tissue and re-activated locally may be able to mediate immediate effector function to control virus [5]. This agrees with our observation that virus titers were significantly reduced from 2 days post-challenge, with a trend for lower titers at day 1, in i.n. but not i.m. immunized animals.

Detection of elevated cytokine levels (IFN-γ, mKC, IL-12) in BAL at both one and 10 months after i.n. rAd vaccination (Figure 2C and D) is surprising. This was transgene-independent, and thus due to the rAd vector. mKC (CXCL1) is a powerful neutrophil chemotactant and functional homolog of human IL-8/CXCL8 [53,54]. IFN-γ is immunostimulatory and secreted by various cells including activated T cells and macrophages [55]. The elevated IFN-γ levels in BAL after i.n. rAd immunization could result from continued IL-12 secretion. IL-12 promotes differentiation of CD4+ T cells towards a Th1 phenotype [56] and maintains CD4+ IFN-γ+ T cell effector function [57]. Continued IL-12 expression in BAL after i.n. rAd immunization may maintain CD4+ T cell activation, which could sustain the strong virus-specific CD8+ T-cell responses observed in the lung.

We have not yet identified the cellular source of the cytokines seen in BALs, but rAd can infect immature dendritic cells (DC) from both mice and humans, causing them to mature and secrete IL-12 [58,59]. This occurs independently of transgene expression [58] via a TLR9/MyD88 dependent pathway in vitro [60]. Earlier studies demonstrated that exposure to an aerosolized antigen induced an activated CD11c+CD11b+ DC subset in BAL that retained antigen presenting function for several weeks after antigen exposure [61].

Other studies have been interpreted as showing persistence of influenza virus antigen after infection, and have suggested that this maintains the activation of virus-specific T cells generated in response to infection [51,62–64]. In contrast to the situation with natural influenza virus infection, previous studies have demonstrated persistence of both vector genome and antigen expression for at least a year following i.m. rAd immunization of mice [65]. This sustained antigen expression appears important for supporting the activated phenotype of transgene-specific T cells following rAd immunization [65,66], although maintenance of memory T cell populations eventually becomes antigen-independent [66]. Differences in expression pattern likely explain the dichotomy between the T-cell responses against the transgenes (NP and M2 which are driven by a constitutively active CMV promoter) which were sustained over the duration of the study and responses against the rAd vector (the E1, E3 deleted vector backbone driving a constitutively active CMV promoter) which declined substantially over this time. Ultimately, the combination of prolonged low-level antigen presentation and Th1 cytokine production may be optimal for maintaining protective mucosal immune responses.

With their ability to induce potent innate and adaptive immune responses and to deliver antigen to intracellular processing and presentation pathways, rAd vectors may be particularly well suited for vaccination against viruses and other intracellular pathogens. Here we demonstrate their potential as an emergency, fast-acting vaccine inducing long-lasting protective immunity in the respiratory tract. Heterosubtypic rAd vaccines could be stockpiled in advance since regular reformulation would be unnecessary, and could be delivered by nasal spray, facilitating widespread administration by limited healthcare personnel. During a large scale virus outbreak or pandemic, reducing disease severity by vaccination, even while allowing mild infection, could greatly reduce the burden on healthcare facilities. Conserved antigen vaccines do not provide the type of sterilizing immunity mediated by HA-specific neutralizing antibody, but would permit only a mild, transient natural influenza virus infection. This transient infection would further boost heterosubtypic immunity and induce
neutralizing antibodies against the exact virus strain circulating in the community, preventing re-infection.

Materials and Methods

Ethics statement
All animal protocols and procedures were approved by Institutional Animal Care and Use Committees at the Center for Biologics Evaluation and Research (CBER; Protocol #1991-06) and/or the Centers for Disease Control and Prevention (CDC; Protocol #1619) in animal facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. All experiments were performed according to institutional guidelines.

rAd vaccines
Recombinant adeno-viruses vectors (Ad5-ΔE1ΔE3) expressing A/NP, B/NP or consensus M2 have been described previously [22,41]. Briefly, Pac I-linearized A/NP- or B/NP-containing shuttle vectors were recombined with a cosmid containing Ad5 genomic DNA using Cre-recombinase. M2-rAd was constructed using the ViraPower Adenoviral Expression System (Invitrogen, Carlsbad, CA) by subcloning the M2-consensus sequence into the pAd/CMV/V5-DEST Gateway vector using LR Clonase. In each case, recombinants were transfected into 293 cells for recovery of rAd vectors. High titer rAd stocks were prepared by ViraQuest Inc. (North Liberty, IA) and stored at -80°C in PBS with 3% sucrose. Viral particle concentration was determined by absorbance at 260 nm. All rAd stocks were confirmed negative for replication-competent adeno-virus by passage on non-permissive cells.

Influenza viruses
The highly virulent, mouse-adapted virus A/FM/1/47-ma (H1N1) [A/FM] (67) originally provided by Earl Brown (University of Ottawa, Canada), the mouse-adapted A/Philippines/2/82 X A/PR/8/34 (H3N2) reassortant [X-79] (68), and the systemically replicating avian strain A/Vietnam/1203/2004 (H5N1) [A/VN1203] (69) have been described previously. Virus stocks were prepared as previously described [20]. Experiments involving H5N1 virus were conducted under enhanced BSL-3 conditions at CBER.

Experimental animals, immunization, and challenge infection
Female BALB/cAnNCr mice aged 8–12 weeks purchased from the National Cancer Institute, Frederick, MD were used for all experiments, except where indicated. IgA−/− mice were originally obtained by MTA from Baylor University [70] and backcrossed onto the BALB/c background in our colony using marker-assisted accelerated backcrossing (MAX-BAX) technology from Charles River Laboratories (Wilmington, MA). BALB/c-IgA−/− mice were bred in the CBER colony. Intramuscular injection of rAd vaccine used a 100 μl volume split evenly between each rear quadriceps muscle; intranasal immunization used a 50 μl volume delivered under isoflurane anesthesia. H1N1 and H3N2 challenge infections were performed under isoflurane anesthesia; 2,2,2-trichloroethanol in tert-amyl alcohol (Avertin; Aldrich Chemical Co., Milwaukee, WI) given i.p. was used as anesthetic for H5N1 challenge. Following challenge infection, animals were monitored for weight loss and survival. In this paper the term “protection” is defined as survival following challenge infection, and does not imply complete absence of virus replication.

Mucosal sampling
Mice were euthanized with ketamine/xylazine, and broncho-alveolar lavage (BAL) and lung cells were obtained as described previously [21].

Peptides and proteins
The following peptides were synthesized by the CBER core facility: NP147-155 (TYQYRTALVD), NP55-69 (RLIQNSL-TIERMVS), and M2e2-24 consensus sequence (SLLTEVET-PIRNEWGCRCNDSSD) corresponding to the surface exposed M2 ectodomain region [M2e]. The MHC-I restricted adenovirus 5 hexon (Hex419–454: KYPSPSNVK) and DNA-binding protein (Dbp419–427: FALSNAEDL) peptides [71] were synthesized by GenScript (Piscataway, NJ). Recombinant nucleoprotein (rNP) from strain A/PR/8/34 (H1N1) was purchased fromImagenex (San Diego, CA).

T cell ELISPOT
Interferon (IFN)-γ ELISPOT was performed as previously described [22] on lung and spleen cells by stimulation with indicated peptides.

Flow cytometry
Lung and spleen T cell phenotypes were assessed by surface staining with CD3-εFluor450, CD8-εFluor780, CD62L-PE-Cy7, CD69-PE, CD127-PerCP-Cy5.5 (all from eBiosciences, San Diego, CA), NP147-155-H2-K1 Tetramer-APC (NIH Tetramer core facility, Atlanta, GA), and Live/Dead fixable viability stain for 488 nm excitation (Invitrogen, Carlsbad, CA). 50,000 events were acquired on a BD-FACS Canto II and data analyzed with FlowJo (TreeStar, Ashland, OR) software. Thresholds of positivity were identified using fluorescence minus one controls for each color on cells from each sample group.

Cytokine Analysis
Cytokine levels in BAL were assessed using the Meso Scale Discovery mouse Th1/Th2 9-plex ultra-sensitive kit (MSD, Gaithersburg, MD), according to manufacturer’s instructions. This kit allows simultaneous quantitation of murine IL-1β, IL-2, IL-4, IL-5, IL-10, total IL-12, IFN-γ, mKC, and TNF-α.

Antibody analysis
Antibody levels in serum and BAL were assessed by ELISA, as described previously [20,21]. Data are expressed as endpoint titers, defined as the highest dilution of sample giving an OD 405 nm reading greater than 3 SD above the mean of the naive samples.

Virus titration
Virus titers in tissues were determined by plaque assay (for A/VN1203) or TCID50 (for A/FM) as described previously [21,72].

Statistical analysis
SigmaStat v 3.5 (Systat Software, Point Richmond, CA) was used for all statistical analyses. Body weight and virus titers analyses used one way ANOVA, followed by pairwise multiple comparisons via the Holm-Sidak method. Survival analysis was performed by the Log-Rank method.

Supporting Information
Figure S1 Serum and BAL antibody responses to nucleoprotein. Mice were immunized with 5×10⁸ particles each of A/NP-rAd
and M2-rAd, or 1 x 10^10 particles of B/NP-rAd i.n. or i.m., or were unimmunized (naive). rNP-specific IgG (left panels) and IgA (right panels) responses in serum and BAL were measured by ELISA as described one month (A) and 10 months (B) after immunization. Bars show mean ± SEM of 3 mice per group. The dashed line indicates limit of detection. Statistically significant differences are indicated as follows: * P < 0.05 compared to all other groups; ** P < 0.05 compared to B/NP-rAd and naive groups. 

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**Figure S2** Morbidity and mortality after H3N2 challenge following single dose rAd immunization. Groups of 10 (at one month) or 8 (at 10 months) mice were challenged with 5 x 10^3 TCID_{50} (=100 LD_{50}) of X-79 one month (A, B and C) or 10 months (D, E and F) after immunization. (A, D) Survival after challenge. (B, E) Weight loss after challenge. When challenged one month post boosting statistically significant differences in weight loss (*P < 0.05) were observed between A/NP+M2-rAd i.n. and i.m. groups at days 1–7, between A/NP+M2-rAd i.n. and B/NP-rAd or naive groups at days 1–15, and between A/NP+M2-rAd and B/NP or naive groups at days 5–15. When challenged at 10 months weight loss in A/NP+M2-rAd i.n. immunized mice was significantly (P < 0.05) different from all other groups from days 3–11 and 13–15. (C, F) Virus titers in the lungs at days 3 and 5 after challenge, as determined by TCID_{50}. Bars show log_{10} geometric mean ± SEM of 4 mice per group, or 5 mice per group at 10 months. The dashed line shows limit of detection. * indicates a statistically significant difference (P < 0.05) compared to all other groups at the same time point; ** indicates a significant difference from B/NP-rAd and naive groups; {dagger} indicates a significant difference from B/NP-rAd groups. Note that day 5 titers were not assessed at 10 months. 

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**Figure S3** Kinetics of the immune response after single-dose rAd immunization. Mice were immunized with 5 x 10^9 particles each of A/NP-rAd and M2-rAd, or 1 x 10^10 particles of B/NP-rAd i.n. or i.m. (A) M2e-specific IgG (left panels) and IgA (right panels) responses in serum collected 1, 2, 3, or 5 weeks or 6 months post-immunization were measured by ELISA. Bars show endpoint titer, as determined by IFN-γ ELISPOT. (B) peripheral blood from 10 mice per group was collected and pooled at 1, 2, 3, or 4 weeks or 6 months post-immunization, as indicated. T-cell responses were determined using NP_{147-155}, NP_{55-69} or M2e_{2-24} peptides as stimuli. Unstimulated cells (no peptide) were used as a control. Bars show mean ± SEM of triplicate wells for each group per stimulus. For the A/NP+M2-rAd i.m. group at 2 weeks, specific T-cell responses could not be determined due to high background observed in all test wells. This is indicated by HB in (B). This high background was not seen in a repeat experiment. 

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**Figure S4** Dose titration of i.n. A/NP-rAd immunization. Groups of 12-week old BALB/cAnNGr mice were immunized i.n. with 1 x 10^{10} (10 mice), 1 x 10^{9} (5 mice), or 1 x 10^{8} (5 mice) particles of A/NP-rAd. 4 weeks after immunization, animals were challenged i.n. with 10^{3} TCID_{50} (100 LD_{50}) A/FM/1/47-ma (H1N1) and monitored for survival (A) and weight loss (B). 

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**Author Contributions**

Conceived and designed the experiments: GEP SLE. Performed the experiments: GEP MRS CYL JAM MRQ KVH MBP CP TMT. Analyzed the data: GEP MRS CYL JAM MRQ KVB CP TMT SLE. Wrote the paper: GEP SLE.

**References**

1. Carrat F, Flahault A (2007) Influenza vaccine: the challenge of antigenic drift. Vaccine 25: 6852–6862.
2. Leroux-Roels I, Leroux-Roels G (2009) Current status and progress of prepandemic and pandemic influenza vaccine development. Expert Rev Vaccines 8: 401–425. 10.1586/ere.09.15.
3. ‘Interim results: influenza A (H1N1) 2009 monovalent vaccination coverage — United States, October-December 2009.’ MMWR Morb Mortal Wkly Rep 59: 44–48.
4. Dawood FS, Jain S, Finelli L, Shaw MW, Lindstrom S, et al. (2009) Emergence of a novel swine-origin influenza A (H1N1) virus in humans. N Engl J Med 360: 2605–2615.
5. Brown LE, Kelso A (2009) Prospects for an influenza vaccine that induces cross-protective cytotoxic T lymphocytes. Immunol Cell Biol 87: 300–308.
6. Epstein SL (2003) Control of influenza virus infection by immunity to conserved viral features. Expert Rev Antivir Infect Ther 1: 627–638.
7. Schotsaert M, De FM, Fiers W, Saelens X (2009) Universal M2 ectodomain-based influenza A vaccines: preclinical and clinical developments. Expert Rev Vaccines 8: 499–508.
8. Relyakov IM, Ahlers JD (2009) What role does the route of immunization play in protective immunity against mucosal pathogens? J Immunol 183: 6823–6829.
9. Murphy BR, Cuollo KG (2002) Principles underlying the development and use of live attenuated cold-adapted influenza A and B virus vaccines. Vital Immunol Med Microbiol 8: 62–73.
10. Takada TM, Renuh M, Clements JD, Katz JM (2001) Mucosal delivery of inactivated influenza vaccine induces B-cell-dependent heterosubtypic cross-protection against lethal influenza A H5N1 virus infection. J Virol 75: 5141–5150.
11. Tompkins SM, Zhao ZS, Lo CY, Misplon JA, Liu T, et al. (2007) Matrix protein 2 vaccination and protection against influenza viruses, including subtype H1N1. Emerg Infect Dis 13: 426–435.
23. Rao SS, Kong WP, Wei CJ, Van HN, Gorres JP, et al. (2010) Comparative efficacy of hemagglutinin, nucleoprotein, and matrix 2 protein gene-based vaccination against H5N1 influenza in mouse and ferret. PLoS One 5: e9812.

24. Zebedee SL, Lamb RA (1988) Influenza A virus M2 protein: monoclonal antibody resistance of virus growth and detection of M2 in virions. J Virol 62: 2762–2772.

25. Jegerlehner A, Tissot A, Lechner F, Sebbel P, Erdmann I, et al. (2002) A molecular assembly system that renders antigens of choice highly repetitive for presentation to human T cell responses. Vaccine 20: 3101–3112.

26. Karzon DT (1996) Cytoxic T cells in influenza immunity. Semin Virol 7: 265–271.

27. Carragher DM, Kaminski DA, Mosquita MK, Hartson L, Randall TD (2008) A novel role for non-neutralizing antibodies against nucleoprotein in facilitating resistance to influenza virus. J Immunol 181: 4168–4176.

28. Roos AC, de Mutsert G, Graus YM, Foucher RA, Sinnicolaas K, et al. (2002) Sequence variation in a newly identified H1A-B13 restricted epitope in the influenza A virus nucleoprotein gene and escape from recognition by cytoxic T lymphocytes. J Virol 76: 2567–2572.

29. Voeten JT, Bestebroer TM, Nierszwoop NJ, Foucher RA, Oosterhuis AD, et al. (2006) Antigenic alterations in the influenza A virus (H3N2) nucleoprotein and escape from recognition by cytoxic T lymphocytes. J Virol 74: 6080–6097.

30. Chakraborty D, Mozdzanowska K, Feng J, Zhang M, Gerhard W (2005) Influenza type A virus escape mutants emerge in vivo in the presence of antibodies to the cytoxidm of nucleoprotein 2. J Virol 79: 6644–6654.

31. Bui HH, Peters B, Assarsson E, Mbawuike I, Sette A (2007) Ab and T cell responses conserved between the hemagglutinin of influenza A virus H1 and H2 strains. J Virol 67: 2532–2535.

32. Sui J, Hwang WC, Perez S, Wei G, Aird D, et al. (2009) Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. Nat Struct Mol Biol 16: 255–263.

33. Roy S, Kohlinger GP, Lin J, Figueredo J, Calcedo R, et al. (2007) Partial replication-defective adenovirus gene transfer vector. Genet Vaccines Ther 6: 5.

34. Ekiert DC, Bhabha G, Elsliger MA, Friesen RH, Jongeneelen M, et al. (2009) Vaccination against H5N1 influenza in mouse and ferret. PLoS One 5: e9812.

35. Epstein SL, Kong WP, Misplon JA, Lo CY, Tumpey TM, et al. (2005) Protection of non-human primates from lethal challenges with H5N1 influenza A virus by replication-defective adenovirus vector expressing nucleoprotein. Vaccine 23: 5404–5410.

36. Holman DH, Wang D, Raja NU, Luo M, Moore KM, et al. (2008) Multi-antigen vaccines based on complex adenovirus vectors induce protective immune responses against H5N1 avian influenza viruses. Vaccine 26: 2627–2639.

37. Zhang Y, Chirmule N, Gao GP, Qian R, Croyle M, et al. (2001) Acute cytokine response following intranasal murine influenza virus infection. J Infect Dis 194: 1638–1649.

38. Tatsis N, Tsema L, Robinson ER, Giles-Davis W, McCoy K, et al. (2006) Chimpanzee-origin adenovirus vectors as vaccine carriers. Gene Ther 13: 421–429.

39. Roberts DM, Nanda A, Havenga MJ, Albinik P, Lynch DM, et al. (2006) Hexon/chimeraic adenovirus type 5 vectors circumvent pre-existing anti-vector immunity. Nature 441: 239–243.

40. Hogan RJ, Usherwood EJ, Zhong W, Roberts AD, Dutton RW, et al. (2001) Activated antigen-specific CD8+ T cells persist in the lungs following recovery from respiratory virus infection. J Immunol 166: 1013–1023.

41. Lammli TT, Turner DL, Klonowski KD, Lefrancois L, Cadle LS (2006) Residual antigen presentation after influenza virus infection affects CD8 T cell activation and migration. Immunity 24: 439–449.

42. de Bree GJ, van Leeuwen EM, Oot TA, Jansen HM, Jonkers RE, et al. (2005) Selective accumulation of antigen-specific CD8+ T cells specific for respiratory viruses in the human lung. J Exp Med 202: 1433–1442.

43. Boizic CR, Kolakowski LF Jr., Gerard NP, Garcia-Rodriguez C, von Zeckull-Guldenbach C, et al. (1995) Expression and biologic characterization of the murine chemokine KC. J Immunol 154: 6948–6957.

44. Zlotnik A, Yoshie O (2000) Chemokines: a new classification system and their role in immunity. Immunity 12: 121–127.

45. Young HA, Hardy KJ (1995) Role of interferon-gamma in immune cell regulation. J Leukoc Biol 58: 373–381.

46. Szabo SJ, Sullivan BM, Peng SL, Glimcher LH (2003) Molecular mechanisms regulating Th1 immune responses. Annu Rev Immunol 21: 713–758.

47. Stohle I, Gurunathan S, Pruscin C, Sacks DL, Glagovich N, et al. (2000) The role of antigen and IL-12 in sustaining Th1 memory cells in vivo. IL-12 is required to maintain memory/effector Th1 cells sufficient to mediate protection to an infectious parasitic challenge. Proc Natl Acad Sci U S A 97: 8427–8432.

48. Zhang Y, Chirmule N, Gao GP, Qian R, Croyle M, et al. (2004) Acute cytokine response to systemic adenoviral vectors in mice is mediated by dendritic cells and macrophages. Mol Ther 5: 697–707.

49. Zhong L, Granelli-Piperno A, Choi Y, Steinman RM (1999) Recombinant adenovirus is an efficient and non-perturbing genetic vector for human dendritic cells. Eur J Immunol 29: 964–972.

50. Yamaguchi T, Kawabata K, Koizumi N, Sakurai F, Nakashima K, et al. (2007) Role of MyD88 and TLR9 in the innate immune response elicited by serotype 5 adenoviral vectors. Hum Gene Ther 18: 753–762.

51. Julia V, Hessell E, Malherbe L, Glagovich N, O’Gara A, et al. (2002) A restricted subset of dendritic cells captures airway antigens and remains able to activate specific T cells long after antigen exposure. Immunity 16: 271–283.

52. Jelley-Gibbs DM, Brown DM, Dibble JP, Haynes L, Eaton SM, et al. (2005) Unexpected prolonged presentation of influenza antigens promotes CD4 T cell memory generation. J Exp Med 202: 697–708.

53. Jelley-Gibbs DM, Dibble JP, Brown DM, Strutt TM, McKinstry KK, et al. (2007) Persistent depots of influenza antigen fail to induce a cytotoxic CD8 T cell response. J Immunol 178: 7565–7570.

54. Kim TS, Hufford MM, Sun J, Fu YX, Braciale TJ (2010) Antigen persistence and the control of local T cell memory by migrant respiratory dendritic cells: implications for their use as vaccines. Blood 110: 1916–1923.

55. Finn JD, Bassett J, Millar JB, Grinsnlstein N, Yang TC, et al. (2009) Persistence of transgene expression influences CD107+ T-cell expansion and maintenance following immunization with recombinant adenovirus. J Virol 83: 12027–12036.

56. Smeets CA, Brown EG (1994) The influenza virus variant A/FM/1/47-MA possesses single amino acid replacements in the hemagglutinin, controlling virulence in, and the matrix protein, controlling virulence as well. J Virol 68: 590–594.

57. Chen KS, Quinnan GV, Jr. (1988) Induction, persistence and strain specificity of haemagglutinin-specific secretory antibodies in lungs of mice after intragastric administration of inactivated influenza virus vaccines. J Gen Virol 69: 2779–2784.

58. Maines TR, La XH, Erb SM, Edwar L, Guerner J, et al. (2005) Avian influenza (H5N1) viruses isolated from humans in Asia in 2004 exhibit increased virulence in mammals. J Virol 79: 11788–11800.

59. Harriman GR, Bogue M, Rogers P, Finegold M, Pacheco S, et al. (1999) Targeted deletion of the IgA constant region in mice leads to IgA deficiency with alterations in expression of other Ig isotypes. J Immunol 162: 2521–2529.

60. McKeever T, Fang A, Bett AJ, Casimiro DR, Chastain M (2004) T-cell response to adenovirus hexon and DNA-binding protein in mice. Gene Ther 11: 791–796.

61. Zeng H, Goldsmith C, Thawatsupha P, Chittaganpitch M, Waicharoen S, et al. (2007) Highly pathogenic avian influenza H5N1 viruses elicit an attenuated type 1 interferon response in polarized human bronchial epithelial cells. J Virol 81: 12439–12449.