Genetic immunization in the lung induces potent local and systemic immune responses

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Successful vaccination against respiratory infections requires elicitation of high levels of potent and durable humoral and cellular responses in the lower airways. To accomplish this goal, we used a fine aerosol that targets the entire lung surface through normal respiration to deliver replication-incompetent recombinant adenoviral vectors expressing gene products from several infectious pathogens. We show that this regimen induced remarkably high and stable lung T-cell responses in nonhuman primates and that it also generated systemic and respiratory tract humoral responses of both IgA and IgG isotypes. Moreover, strong immunogenicity was achieved even in animals with preexisting antiadenoviral immunity, overcoming a critical hurdle to the use of these vectors in humans, who commonly are immune to adenoviruses. The immunogenicity profile elicited with this regimen, which is distinct from either intramuscular or intranasal delivery, has highly desirable properties for protection against respiratory pathogens. We show that it can be used repeatedly to generate mucosal humoral, CD4, and CD8 T-cell responses and as such may be applicable to other mucosally transmitted pathogens such as HIV. Indeed, in a lethal challenge model, we show that aerosolized recombinant adenoviral immunization completely protects ferrets against H5N1 highly pathogenic avian influenza virus. Thus, genetic immunization in the lung offers a powerful platform approach to generating protective immune responses against respiratory pathogens.

mucosal responses | tuberculosis | platform immunization

The generation of potent cellular and humoral responses at mucosal surfaces has been a goal of immune intervention against tuberculosis (TB), influenza, respiratory syncytial virus (RSV), and HIV, among others. We hypothesized that genetic immunization—i.e., using recombinant adenoviral (rAd) vectors expressing immunogens of choice—would be ideal for this purpose. First, the immunogen would be continuously produced for an extended period (days) by transduced cells, rather than eliminated quickly from mucosa by the proteolytic environment. Second, as a genetic immunization, there is no need to use an adjuvant. Finally, previous systemic vaccination (e.g., i.m.) studies suggests that eliciting an immune response with recombinant vectors is more likely to result in effector responses (1) that provide protection rather than in the negative regulatory responses typically induced by protein immunization in the mucosa.

Respiratory delivery of recombinant vectors offers a platform for targeting vaccines encoding antigens from pathogens such as TB, RSV, and influenza. By expressing the immunogen in a vector at the local mucosal site, protective humoral and cellular pathogen-specific immune responses can, in principle, be generated and remain at high levels at the portal of entry for these pathogens. There is reason to believe that the T-helper 1 (Th1) or cytotoxic T-lymphocyte cellular responses necessary to protect against TB (2, 3) and RSV (4) are stimulated through genetic immunization (reviewed in ref. 1).

Several mucosally delivered vaccines that induce protective humoral immunity have been licensed for use in humans by the US Food and Drug Administration. These vaccines include oral typhoid and rotavirus vaccines, the nasal influenza vaccine, and oral live attenuated polio vaccine (not used in the United States since 2000). Aerosol vaccination also has been used successfully. In animals, for example, aerosol vaccines are used to immunize poultry against Newcastle disease (5). Similarly, aerosol measles vaccination of 4 million Mexican schoolchildren in 1989–1990 demonstrated a seroconversion rate of 52–64% (similar to s.c. administration) and an overall efficacy of 96%, with excellent public acceptance and fewer side effects than s.c. vaccination (6). Thus, there is an excellent safety profile for lung vaccination, at least in the setting of standard (nonrecombinant) vaccines designed to elicit humoral immunity.

Here we test whether aerosol delivery of recombinant adenoviruses can be used safely to induce broad-based humoral and cellular immunity to organisms that are transmitted by mucosal routes and, importantly, whether the induced response is qualitatively more suited to providing protective immunity. We show in nonhuman primates that this vaccine regimen induces very high, stable T-cell responses localized to the lung with functions suited to provide broad protection against pathogens, as well as humoral responses in the peripheral blood, distal mucosal sites, and respiratory tract. In addition, this regimen induces protective immunity against a lethal challenge with H5N1 (pandemic) influenza in ferrets.

Finally, we demonstrate that, because of the low anti-vector immune responses elicited and the insensitivity to preexisting antivector immunity, this regimen can be used multiple times with different inserts to generate immune responses against a variety of pathogens. Thus, aerosolized adenoviral vectors can be used repeatedly to generate humoral, Th1, and cytotoxic responses and hence may be highly useful as a general platform for vaccination against respiratory infections such as TB, influenza, and RSV.

Results

Immunogenicity of Aerosolized Recombinant Adenoviral Serotype 5: Cellular Responses. We studied the qualitative and quantitative aspects of immune responses elicited by immunization with replication-deficient recombinant adenoviral serotypes 5 (rAd5) and 35 (rAd35) delivered to the lung in defined particle sizes using an Investigational eFlow Nebulizer System. To determine if this approach could generate local, systemic, and mucosal adaptive

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Conflict of interest statement: A patent application for the use of aerosolized adenoviruses as a vaccine has been filed with S.S.R., C.A.A., G.J.N., and M.R. as inventors.

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responses, we immunized six rhesus macaques in a pilot experiment. For each animal, a mixture of 10^{11} particles of rAd5 encoding simian immunodeficiency virus Envelope (SIV Env) and 10^{11} particles encoding an SIV group antigen (Gag)-polymerase (Pol) fusion protein (7) were administered by aerosol. Two different drop sizes were used to test whether immunogenicity is affected by delivery to the upper airways (11 μm aerosol) vs. the entire lung (4 μm) (8). Animals were immunized twice with a 3-wk interval, and T-cell and antibody responses were measured in peripheral blood mononuclear cells (PBMC) and in cells obtained by bronchoalveolar lavage (BAL).

Fig. 1 shows the quantification of the CD4 and CD8 T-cell responses elicited by the vaccine, i.e., responses specific to the SIV Env and SIV Gag-Pol inserts. PBMC T-cell responses measured by IFN-γ ELISpot peaked at week four, 1 wk after the second immunization (Fig. 1*A*), and declined to background levels by 3–6 mo. The 4-μm aerosol induced roughly threefold greater responses than the 11-μm aerosol. Intracellular cytokine staining (ICS) measurements showed similar kinetics, with the peak reaching 0.5–1% of CD4 or CD8 memory cells producing cytokines in response to in vitro SIV peptide stimulation and falling to an undetectable level over the next few months. No responses were detectable at any time in jejunal tissue or inguinal or mesenteric lymph node biopsies. The hierarchy of responses produced to the rAd5-encoded inserts is the same as reported for i.m. immunization (9): Env generates the largest responses, followed by Pol and Gag.

In contrast to the transient nature of the PBMC T-cell responses, T-cell responses measured in the BAL fluid were substantially higher and much more durable (Fig. 1*B* and Fig. S1). Furthermore, unlike the systemic T-cell responses, there is no difference in the magnitude of the responses in the BAL cells induced by 4-μm vs. 11-μm aerosols.

An important facet of a vaccine-induced T-cell response is its quality, that is, the repertoire of functions that are elicited (10). Aerosol immunization induced CD4 and CD8 T-cell responses in the BAL fluid that are highly polyfunctional (simultaneously making IFN-γ, TNF, and IL-2) (Fig. 1 *C* and *D*). This response differs substantially from the peripheral T-cell response induced by i.m. rAd5, in which IFN-γ–only T cells predominate. The production of IL-2 and the polyfunctional nature of the cells are highly desirable from the standpoint of generating persistent, highly effective T cells (10). The polyfunctional T cells induced by the aerosolized vaccination also secrete much larger amounts of cytokine on a per-cell basis (as estimated by the median fluorescence intensity), as has been described previously for optimized polyfunctional effector T cells (11).

To determine the dose–response to immunization with rAd5 by this route, we immunized nine macaques with rAd5 encoding HIV Env clade A with doses ranging from 10^9–10^{11} particles. Over this range, we found that the total magnitude of the BAL T-cell response is not demonstrably affected (Fig. 1*C*). Nevertheless, we detected clear differences in the quality of the T-cell response (Fig. 1*D*). As has been demonstrated previously for rAd5 immunization in mice (11), higher doses of rAd5 are associated with responses that are less polyfunctional. In particular, high-dose immunization shifts the balance of CD4 and CD8 T cells from a polyfunctional response toward one comprised primarily of only IFN-γ.

Potent Immunogenicity of the rAd5 Vector-Encoded SIV Env in Seropositive Animals. Although rAd5 vectors elicit potent T-cell responses in humans, they have been shown to be substantially less immunogenic when administered i.m. to individuals who have preexisting immunity to Ad5 (Ad5 seropositive) because of prior Ad5 infection (12). Nevertheless, as we show here, preexisting Ad5 immunity does not impair the immunogenicity of aerosol-administered rAd5.

For these studies, three animals previously immunized with 10^{11} rAd5 SIV Env aerosol only and three that were immunized simultaneously by aerosol and i.m. were reimmunized 6 mo later with rAd5 HIV Env. Note that both T- and B-cell responses to SIV and HIV Env proteins are distinct and do not cross-react. The BAL T-cell response to SIV Env following the initial immunization was robust (Fig. 1*E*, *Left*). In addition, neutralizing antibodies reactive with Ad5 were detectable at varied levels in sera from the immunized animals (Table 1).

Six months or more after the initial immunization, the previously immunized animals were immunized with rAd5 HIV EnvA administered by aerosol at a dose (10^{10} particles) that induces strong T-cell responses in the BAL fluid when given by aerosol to Ad5-naïve animals (Fig. 1*E*, *Right*). All six of the immunized animals, including three with high levels of neutralizing Ad5 antibodies, generated very strong CD4 T-cell responses to the HIV Env proteins encoded by the second rAd (Fig. 1*E*, *Center*). The CD8 T-cell responses, although trending lower in animals previously exposed to Ad5, were still strong (1–5% of CD8 T cells)

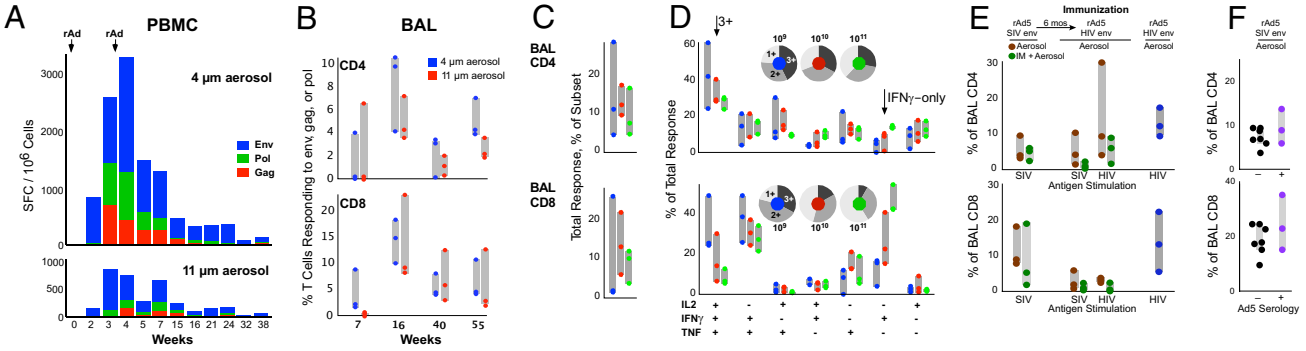

Fig. 1. Cellular immune responses induced by aerosolized rAd5 immunization. (*A*) After rAd5 immunization at week 0 and week 3, T-cell responses were measured by IFN-γ ELISpot to SIV Gag-Pol and Env peptides in PBMC. SFC, spot-forming cells. (*B*) Robust and persistent BAL CD4 and CD8 T-cell responses were measured by ICS. The responses to SIV Gag-Pol and Env peptides are summed. (*C*) A dose titration of rAd5 showed little effect on the total BAL T-cell magnitude when measured 8 wk after a single immunization. (*D*) The quality of the T-cell response induced by various doses of rAd5. The fraction of the response that is polyfunctional (3+) or solely IFN-γ is indicated by arrows. The quality also is summarized in pie charts depicting the polyfunctionality of the response: Antigen-specific cells secreting a single cytokine (1+) are depicted in light gray, cells secreting two cytokines (2+) are shown in dark gray, and cells secreting all three cytokines (3+) are shown in black. (*E*) Three animals were immunized with 10^{11} particles of rAd5 encoding SIV Env and Gag-Pol by aerosol (brown) or were immunized by aerosol and i.m. simultaneously (green); de novo responses to the SIV proteins are shown (*Left*). (Neutralizing titers in the serum following this first immunization are shown in Table 1.) These six animals then were immunized with 10^{10} particles of aerosolized rAd5 encoding HIV EnvA (*Center*); shown are the memory responses to SIV proteins (*Center Left*) and the de novo responses to HIV proteins (*Center Right*). For comparison, the de novo HIV responses in three different animals that received only aerosolized HIV Env rAd5 are shown (*Right*). (*F*) Ten animals were immunized with 5×10^9 particles of rAd5 encoding SIV Env. Seven of these animals were seronegative for Ad5 at the time of immunization (black), and three were seropositive from naturally acquired infection (purple).

and were higher than achieved with other vaccination routes. Notably, the second rAd5 immunization did not eliminate the T-cell responses to the original immunization (SIV Env), showing that the responses generated by the initial immunization persist through additional heterologous immunizations.

In a separate experiment, we immunized 10 naïve animals with aerosolized rAd5 encoding SIV antigens. Three of these animals were Ad5 seropositive from previously acquired (natural) infection. The BAL CD4 and CD8 T-cell responses following immunization of the seropositive animals were as high or higher than those in the seronegative animals (Fig. 1*F*), indicating that natural infection with Ad5 does not limit the immunogenicity of aerosolized recombinant Ad5 vectors.

Data from these studies show that aerosolized rAd5 immunization elicits strong cellular responses that peak in the periphery within 4–6 wk and subsequently decrease to background levels. BAL cellular responses, in contrast, are very strong and durable. The quality of the T-cell response is best at lower doses of rAd5. Importantly, the ability to generate de novo T-cell responses in the BAL cells is not strongly affected by previous exposure to Ad5 administered either systemically or by aerosol.

Immunogenicity of Aerosolized rAd5: Humoral Responses. In addition to T-cell responses, antibody responses at mucosal surfaces are critical for mediating protection against many viral and bacterial infections. Thus, we measured local and systemic antibody responses to gene inserts as well as to the rAd5 vector used in these immunizations.

The anti-vector (anti-Ad5) antibody responses induced by aerosol immunization with the vector (Ad5) itself at doses of 10^9–10^{11} particles is very poor (Table 1). Neutralizing titers reach detectable levels only after repeated immunization with the highest dose (10^{11} particles) and at best are still dramatically lower than responses observed following i.m. alone or i.m. plus aerosol immunization.

In contrast, aerosolized rAd5 elicits robust humoral responses to the antigens encoded by the inserts. Serum IgG levels peak ~6 wk after immunization and persist for at least 6 mo (Fig. 2*A*). IgA levels in the serum also are detectable, albeit at much lower levels (Fig. 2*B*). As with the anti-vector humoral responses (Table 1) and the transient systemic cellular responses (Fig. 1*A*), the 4-μm aerosol drop size generated stronger humoral responses to the gene inserts than did the 11-μm aerosol.

Significantly, from a protective standpoint, in addition to systemic humoral responses, aerosol immunization induces antibody responses at both local and distal mucosal sites. In the dose–response study, IgG and IgA responses were readily detectable at mucosal sites at the highest dose. However, at even the lowest immunogenic dose (Fig. 2 *C* and *D*), aerosol immunization results in the presence of both IgG and IgA at mucosal sites. Levels of specific antibody at distal mucosa are consistent with transudation from serum. However, the specific IgA levels at the local mucosal site are much higher than in serum, indicative of locally produced secretory IgA and not solely transudated serum IgA.

As with cellular responses to aerosolized rAd5, humoral responses to the antigens encoded by the gene inserts are not affected by the presence of neutralizing anti-vector antibodies (Fig. S2). In essence, aerosolized rAd5 induces equivalent humoral responses irrespective of whether the rAd5-immunized animal has detectable anti-Ad5 levels in serum or preexisting levels of anti-Ad5 antibodies, if present.

Thus we conclude that aerosolized rAd5 immunization is effective at generating humoral responses when delivered by 4-μm drop size. Moreover, this mode of immunization may result in long-term production of secretory IgA at local mucosal sites.

Aerosolized Immunization Is More Effective than Intranasal Immunization. Because intranasal (i.n.) delivery of vaccines has been studied for many years and is clinically approved for influenza vaccination, we compared the efficacy of aerosol versus intranasal (i.n.) rAd5 delivery. Thus, we immunized rhesus macaques with 10^{10} particles of rAd5 encoding HIV Envelope (HIV Env) delivered by 4 μm aerosol and four similar animals with the same dose rAd5 delivered i.n. Animals were immunized twice, 4 wk apart. Results demonstrate that with the rAd vector used here, which encodes HIV Env proteins, aerosolized immunization in the lung is superior to i.n. delivery of the same vector.

Cellular responses to rAd administered i.n. were weak but detectable in BAL cells and PBMC (Fig. S3*A*). Both CD4 and CD8 T cells specific for HIV Env were present. However, cellular and humoral responses in the aerosol-delivery group, which were consistent with those described above (Figs. 1 and 2), were substantially higher. Aerosol delivery elicited almost 10-fold greater CD4 and CD8 responses than i.n. delivery. The quality of the T-cell response was similar for CD8 cells; for CD4 cells, the aerosol elicited a greater proportion of the more-differentiated IFN-γ–producing cells, whereas i.n. elicited a greater proportion of TNF-producing cells. Thus, aerosol delivery induces a much stronger cellular responses than does i.n. delivery.

Induction of antigen-specific IgG (Fig. S3*B*) and IgA (Fig. S3*C*) was similar between the two routes, although there was a trend toward greater induction by the aerosol route than by the i.n. route. Of note, aerosol immunization induced fourfold higher vaginal IgG responses than i.n. delivery; however, these responses were concordant with the higher serum levels and simply may reflect transudation of serum Ig. Importantly, both i.n. and aerosol delivery elicited higher levels of IgA in the respiratory mucosa than in serum, indicative of the production of secretory IgA at this site.

Immunogenicity of Aerosolized rAd35 Encoding TB Antigens. The generation of potent and durable T-cell responses in the lung may be highly desirable for containing respiratory pathogens such as TB.

Table 1. Induction of anti-vector antibodies

First regimen				Second regimen*	
Route (dose)	Drop size (μm)†	No. of times‡	Ad5 IC90§	Route (dose)	Ad5 IC90¶
AE (10^{11})	4	2	432*, 1,249, 1,144	AE (10^{10})	322
AE (10^{11})	11	2	nd*, nd*, nd	AE (10^{10})	nd, nd
AE (10^{9})	4	1	nd, nd, nd		
AE (10^{10})	4	1	nd, nd, nd		
AE (10^{11})	4	1	44, nd, nd		
i.m. + AE (10^{10})	4	1	>8,800*, >8,800*, >8,800*	AE (10^{10})	>8,800, >8,800, >8,800

AE, aerosol; nd, below the limit of detection, <1:12. For each group, three animals were immunized.

*These animals were immunized with another regimen 6–14 mo after the first immunization. All immunizations in the second regimen were single administrations using a 4-μm aerosol drop size. Immunogenicity for these six animals (and three Ad-naïve animals) is reported in Fig. 1*E* and. Fig. S2.

†*Materials and Methods*.

‡Number of administrations (spaced at 3-wk intervals).

§Serum dilution that inhibits 90% of Ad5 infectivity, measured 8–40 wk after the primary immunizations.

¶IC90 was measured 8 wk after the second regimen.

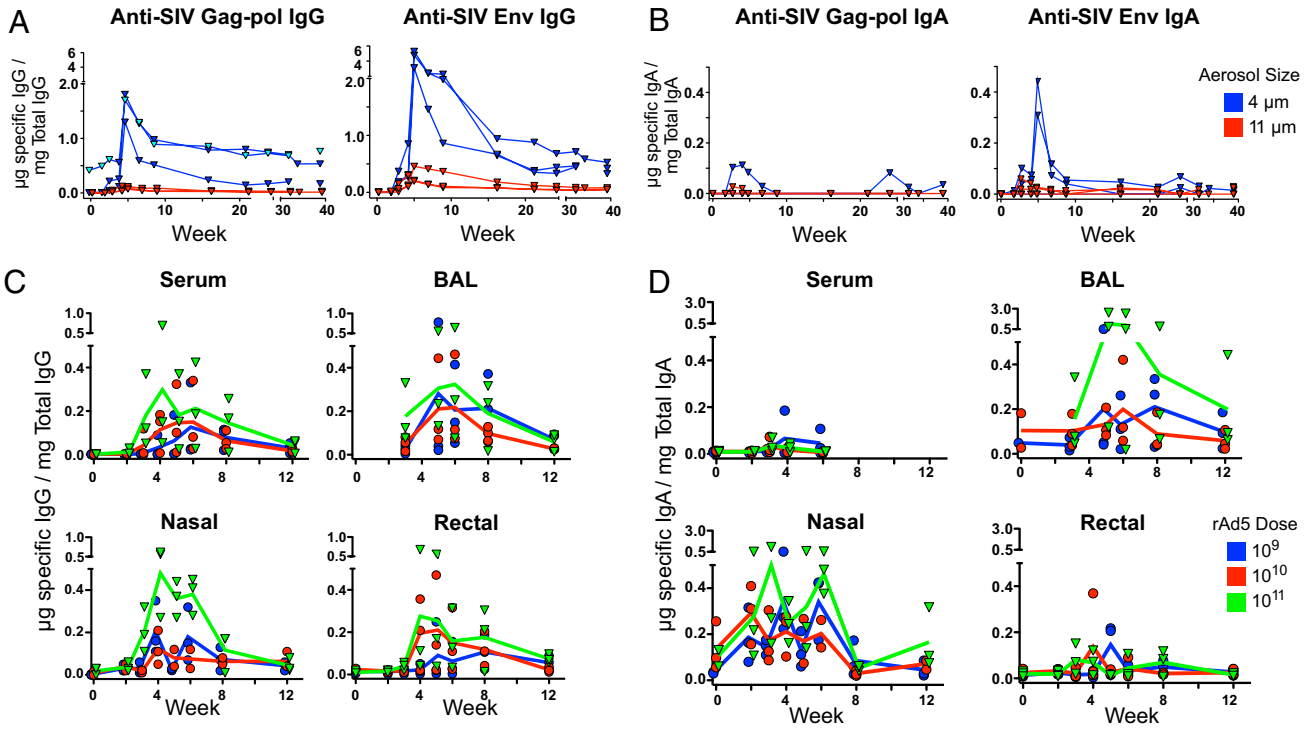

Fig. 2. Humoral responses induced by aerosolized rAd5 immunization. (*A* and *B*) Induction of IgG (*A*) and IgA (*B*) to the vector inserts, SIV Gag-Pol (*Left*) and SIV Env (*Right*), were measured in serum. Data are shown as specific activity, i.e., micrograms of antigen-specific antibody of a given isotype per milligrams of total antibody of that isotype for each animal ($n = 3$ per group). The symbols filled with light blue represent one animal that had high preimmunization anti-gag-pol cross-reactivity. (*C* and *D*) rAd5 dose–response induction of HIV EnvA-specific IgG (*C*) and IgA (*D*) is shown in serum and three mucosal sites.

To test this concept, we performed a pilot immunization of rhesus macaques using an rAd35 vector encoding the TB antigens 85A/B and mTB10.4, currently in clinical trials with i.m. delivery (13).

We found that rAd35 is highly immunogenic in the lungs when given by aerosol to rhesus macaques (Fig. 3*A*). A single dose of even 10^9 particles of rAd35 generated measurable T-cell responses; a second dose boosted the response in most animals. Higher doses gave very strong T-cell responses ranging from 25–50% of CD8 T cells and from 3–12% of CD4 T cells. Notably, in BAL fluid the responses induced even by two i.m. immunizations with 10^{11} particles were 10- to 50-fold lower than the response induced by a single aerosol immunization.

In this experiment, three of the animals across the dose groups were Ad35 seropositive from previous (naturally acquired) infection. However, consistent with the data in Fig. 1 *E* and *F*, there was no decrease in the BAL T-cell responses in these animals compared with the seronegative animals. Finally, as with rAd5, the quality of the T-cell response varied with dose, with the lower doses of rAd35 giving more polyfunctional T-cell responses (Fig. 3*B*).

Aerosolized rAd5 Protects Against Influenza. To determine whether aerosolized rAd5 vaccines could protect against challenge with a respiratory pathogen, we immunized ferrets with rAd5 encoding influenza HA (Indonesia strain) by 4-μm aerosol or by i.m. injection. rAd5 has been demonstrated to afford protection against influenza after i.m. immunization (14). Eighteen days after immunization, the animals were challenged i.n. with a lethal dose of H5N1 avian influenza (Vietnam 1203/04). Both vaccine groups were protected completely, with 100% survival (Fig. 4*A*) and nearly complete protection against weight loss (Fig. 4*B*). Vaccinated animals also showed significant reductions in nasal viral loads by day 4 (Fig. 4*C*).

To address the mechanism of protection, we quantified the cellular and humoral responses elicited by the vaccine at the time of challenge. Cellular immunity in ferrets is difficult to quantify, because few monoclonal antibodies are available for intracellular cytokine or ELISpot assays. Thus, we measured BAL-associated T-cell responses in five animals from each group by quantitative RT-PCR (15) and thereby confirmed that robust cellular immunity is induced by the aerosolized (but not by i.m.) administration of the vaccine (Fig. 4*D*).

Importantly, vaccination by either route also elicits neutralizing titers to the vaccine strain (Fig. 4*E*). Although heterologous neutralization of the challenge strain was undetectable in sera from both vaccine groups at the time of challenge, the mechanism of protection is most likely the humoral response. Among vaccinees, one animal with neutralizing titers below detection showed the highest nasal viral loads and significant weight loss (but still controlled the virus and survived, indicating that the protection might have been mediated by something other than a traditional neutralizing antibody response).

Discussion

Optimally protective vaccines against HIV, TB, and influenza will require generation of humoral and cellular responses at mucosal sites to prevent or substantially limit infection. Protection from TB will require a strong local cellular response to circumvent the several-week delay in the trafficking of T cells to the lung after infection. Similarly, protection against RSV will likely require a local, high-level Th1-biased cellular response.

Here we show that rAd vectors delivered directly to the lung with normal respiration are ideally suited for this purpose. Genetic immunization by aerosol is safe, immunogenic, and elicits effector rather than regulatory immune responses. In addition, as we show here, the systemic and mucosal response profile throughout the body is distinct from that of i.m. or i.n. administration and has a number of properties that make it potentially useful as a protective vaccine against TB, RSV, or other pathogens.

Protection against TB pathogenesis requires T-cell responses. However, after TB infection, T-cell responses in the lung are not detectable for several weeks. Even in animals immunized with bacillus Calmette–Guérin (BCG), lung T-cell responses are not

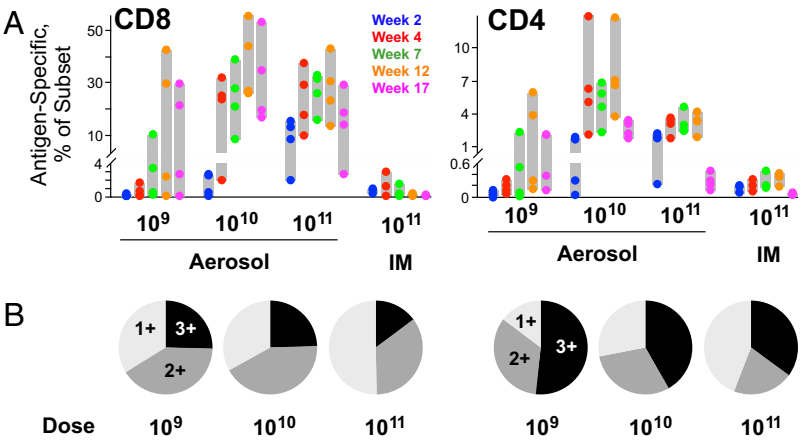

Fig. 3. Cellular immunity induced by aerosolized rAd35 immunization. (*A*) rAd35 encoding the TB antigens 85A, 85B, and 10.4 was given by 4-μm aerosol administration to four animals at three different doses and to three animals by i.m. injection (10^{11} particles). Animals were immunized at week 0 and week 8. Shown are the 85A/B-specific responses for BAL CD4 and CD8 T measured by ICS as in Fig. 1*B* at the indicated time points postimmunization. (*B*) The quality of the BAL CD4 and CD8 T-cell responses to 85A/B peptides (week 17) as shown for rAd5 in Fig 1*E*.

observed until 14 d after TB infection. Furthermore, cellular responses to BCG immunization consist of mainly CD4 T cells, although CD8 T cells probably play a critical role in protection against TB (2, 3). Consequently, BCG vaccination is only mildly protective against pulmonary infection.

Similarly, T-cell responses may be central to protection against RSV disease, where induction of Th1-biased or CD8 T-cell responses in the respiratory tract has been shown to be potentially important at the time of infection (4). Furthermore, although protection against pathogens such as influenza requires humoral responses, effective cellular responses at affected mucosal sites may well participate in and augment protection.

Induction of good mucosal immunity may benefit from vaccination at the target site of the infection. Nevertheless, vaccine delivery to mucosal sites is typically achieved via i.n. or oral routes, which thus far have proved inadequate. To date, oral administration of rAd vectors has not elicited potent immune responses; as we show here, i.n. delivery is far less potent than aerosol delivery.

A number of features of the aerosolized rAd immunization argue for its use to deliver immunogens in mucosal vaccines. First, the immunization is highly effective, in part because of the relatively long-term expression of rAd-encoded immunogens produced by cells infected with the nonreproducing rAd vector. Secondly, the immunization is largely silent with regard to the generation of anti-vector immune responses. As we have shown, anti-adenoviral responses are nearly absent despite induction of strong humoral immunity (equivalent to i.m. immunization) to the vaccine insert.

The failure to respond to the vector is explained in part because rAd does not encode vector proteins that could serve as immunogens and in part because the rAd particles that do not infect cells are rapidly eliminated from lung mucosa and hence are cleared before anti-vector humoral responses can be induced. In contrast, rAd introduced via i.m. delivery can act as a protein depot for immunization. This anti-rAd response potentially compromises subsequent i.m. vaccination with rAd encoding other immunogens (16).

Remarkably, immunity to Ad5 does not compromise the ability to respond to aerosol vaccination with rAd-encoded immunogens. As we showed, the induction of local cellular responses and systemic humoral responses is largely unaffected by preexisting anti-vector antibodies. This ability to overcome preexisting immunity is important, because populations targeted for vaccination often

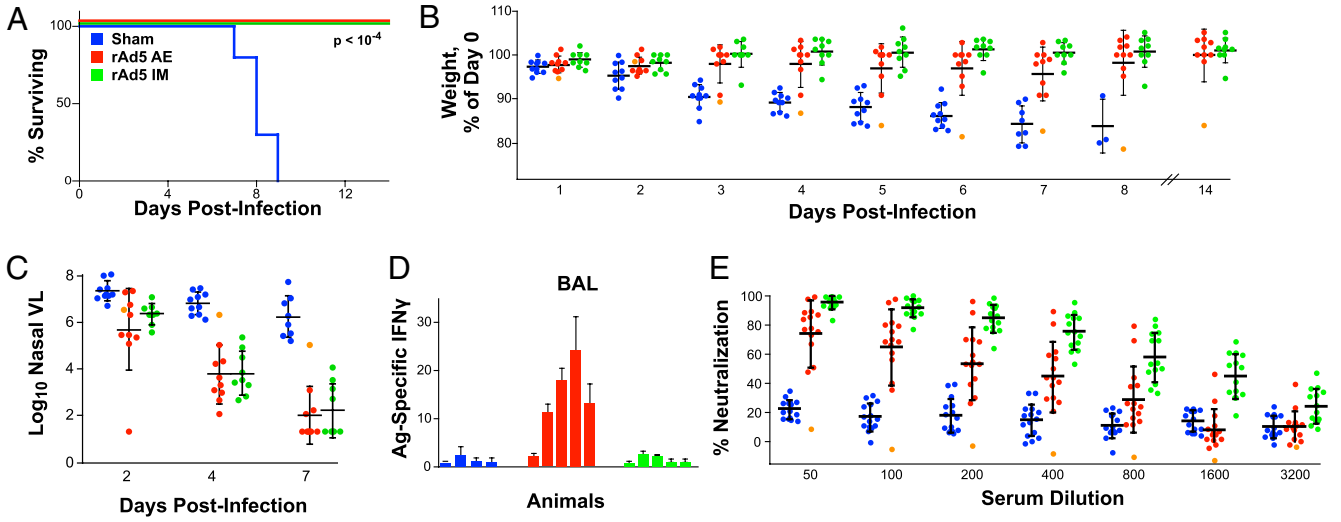

Fig. 4. Aerosolized rAd5 provides protective immunity to live viral challenge. Three groups of ferrets (n = 15 each) were immunized using a sham rAd5 (by aerosol) or an rAd5-encoding influenza HA (Indonesia) by i.m. or aerosol. Eighteen days later, five animals in each group were euthanized to measure cellular responses; the remaining 10 animals were challenged with a lethal dose of H5N1 (Vietnam). (*A*) Protection in vaccinated groups was 100%. (*B*) Relative weight compared with day 0. (*C*) Viral loads measured from nasal swabs. (*D*) Induction of IFN-γ mRNA in BAL cells following restimulation with HA peptides ex vivo; each bar represents one animal measured in triplicate. (Error bar: +1 SD.) (*E*) Neutralization of the Indonesia H5N1 vaccine strain by sera from all 15 animals at the time of challenge. Note: a single animal in the aerosol group, indicated by the orange symbol, showed no neutralization, higher nasal viral loads, and significant weight loss but met no other criteria for euthanasia before the termination of the experiment.

have high rates of adenoviral seroprevalence, particularly in the developing world (17). Ad5 seroprevalence has raised concerns that the utility of adenoviral vectors in humans may be limited (12). Aerosol immunization may remove a key barrier to rAd immunization for selected pathogens.

Immunization with aerosolized rAd generates durable, high-magnitude, polyfunctional effector CD4 and CD8 T-cell responses in the lung, ideal for regimens aimed at controlling TB, RSV, or influenza. It elicits humoral responses both at mucosal and systemic sites, including IgA at local mucosal sites. Because such delivery may elicit responses at distal sites such as the rectum and vagina (18), it may serve as an adjunct to other immunization modalities that generate systemic T-cell and B-cell responses against sexually transmitted pathogens such as HIV.

In summary, aerosolized adenoviral delivery to the lung can be used repeatedly with different immunogens to generate potent cellular and humoral immune responses. Thus, it introduces a platform vaccine approach potentially capable of enabling induction of the humoral, Th1, and CD8 responses necessary to protect against lethal influenza viral challenge and other mucosal infections. Indeed, the rapid, durable, large cellular responses induced in the lung via aerosol immunization may facilitate induction of protection against TB and other diseases by shortening the delay in trafficking of antigen-specific T cells to the lung. Thus, we expect that this approach will prove applicable in a range of infectious diseases that thus far have defeated current immunization strategies.

Methods

Aerosolization and Immunization of Adenoviral Particles. We used the Investigational e-Flow Nebulizer System (PARI Pharma) (Fig. S4) in compliance with the recommendations by the company and the Institutional Biosafety Office at the National Institutes of Health (NIH), outfitted with aerosol heads generating droplets with a mass median diameter (MMD) of 4.4 μm (±1.6 μm, geometric SD) to deliver immunogen deep into the lung or with heads generating droplets with an MMD of 11.4 μm (±1.8 μm) to target primarily the upper airways (8). Information about delivery to and evaluation of animals can be found in *SI Methods*.

rAd immunogens. All rAd5 vectors are E1, E3, E4-deleted, replication-incompetent vectors expressing HIV, SIV, or influenza antigens under control of a CMV promoter. The rAd35 vector expresses the mTB85 A/B + 10.4 fusion protein and also is replication incompetent. Expression of adenoviral genes is minimized by deletion of the early proteins (7, 13, 14).

Animals. All animal studies were approved by the Animal Care and Use Committees of the Vaccine Research Center, National Institute of Allergy and Infectious Diseases, NIH, and of Bioqual, Inc. Fifty-five colony-bred Indian-origin rhesus macaques and 65 ferrets, housed and cared for in accordance with local, state, federal, and institute policies in an American Association for Accreditation of Laboratory Animal Care-accredited facility, were used in this study. Blood and tissue specimens were collected before immunization and at weekly or monthly time points following immunization. Ferrets were procured from Triple F Farms and tested seronegative for H5 and H1 influenza antibodies before study initiation. All animals were monitored carefully by veterinary staff (Figs. S5 and S6); evaluations can be found in *SI Methods*. To date, no adverse events have been associated with aerosol immunization.

Influenza Challenge. Ferrets were challenged with 10^5 50% egg infective dose (EID_{50}) delivered in 0.25 mL medium into each nostril. Body weights and temperature were collected before challenge and daily after influenza challenge. Nasal washes for viral load were collected on days 1, 2, 4, 6, and 7 postinfection. Remaining animals were euthanized 14 d after challenge. Lungs and trachea were collected and fixed from animals euthanized because they were moribund or euthanized per schedule.

Immune Assays. Assays on PBMC were performed on cyropreserved cells at a single assay time. Assays on BAL cells were performed fresh. All antibodies were purchased from Becton Dickinson, Inc., either conjugated or unconjugated, and were derivatized in our laboratory. ICS (19) and ELISpot analysis was performed by standard methods (*SI Methods*). For T-cell responses in ferrets, we used a modified RT-PCR assay to quantify cytokine mRNAs (20, 21). Humoral immune assays were performed by ELISA as previously described (22).

Histology. Lung tissue fixed with 4% formaldehyde was sectioned at 5 μm, H&E stained using standard methods, and evaluated by a board-certified pathologist (S.R.).

Viral Neutralization Assays. Adenoviral neutralizing titers were performed by the NVITAL Laboratory based on a published assay (23). Influenza virus neutralization was performed as described (24).

Statistics. Comparisons of distributions were by the Student's *t* test, using either JMP (SAS Institute) or SPICE (Vaccine Resarch Center, NIH).

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