

Editor's Summary

Deepening the Talent Pool

Whether you're talking about drafting for a professional sports team or hiring new lab staff, increasing the number of candidates improves your chances of the truly exceptional find. When it comes to vaccine vectors, the pool of human adenovirus candidates has been quite shallow. Although certain vectors are highly immunogenic in animal models, they can be neutralized by preexisting antibodies in humans. Yet, Colloca *et al.* show that viruses that are more rare in humans and are thus less likely to be neutralized are not as immunogenic.

Therefore, the authors deepened the vector pool by isolating more than 1000 adenovirus strains from chimpanzees. They identified vectors that grew in human cells and were not neutralized by human sera and prevented them from replicating. As with human adenoviral vectors, different simian vectors induced either more or less potent immune responses in mice. The more potent of these vectors were also immunogenic in humans. These chimp adenoviral vectors provide such embarrassment of riches that different vectors could be used for each vaccine target, lowering the chances of subsequent cross-reactive neutralization. Thus, these vectors serve as prime candidates for future vaccine development.

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Vaccine Vectors Derived from a Large Collection of Simian Adenoviruses Induce Potent Cellular Immunity Across Multiple Species

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Replication-defective adenovirus vectors based on human serotype 5 (Ad5) induce protective immune responses against diverse pathogens and cancer in animal models, as well as elicit robust and sustained cellular immunity in humans. However, most humans have neutralizing antibodies to Ad5, which can impair the immunological potency of such vaccines. Here, we show that rare serotypes of human adenoviruses, which should not be neutralized in most humans, are far less potent as vaccine vectors than Ad5 in mice and nonhuman primates, casting doubt on their potential efficacy in humans. To identify novel vaccine carriers suitable for vaccine delivery in humans, we isolated and sequenced more than 1000 adenovirus strains from chimpanzees (ChAd). Replication-defective vectors were generated from a subset of these ChAd serotypes and screened to determine whether they were neutralized by human sera and able to grow in human cell lines. We then ranked these ChAd vectors by immunological potency and found up to a thousandfold variation in potency for CD8⁺ T cell induction in mice. These ChAd vectors were safe and immunologically potent in phase 1 clinical trials, thereby validating our screening approach. These data suggest that the ChAd vectors developed here represent a large collection of non-cross-reactive, potent vectors that may be exploited for the development of new vaccines.

INTRODUCTION

Novel vaccines are needed for the prevention or treatment of diseases such as HIV, hepatitis C, malaria, tuberculosis, and cancers. Preclinical and clinical evidence supports the role of T cell immunity and, in particular, CD8⁺ T cells in the clearance of these diseases (1). One way to induce a CD8⁺ T cell response against a particular antigen is to express that antigen and suitable pathogen-derived innate activators intracellularly through gene delivery; genetic or gene-based vaccines co-opt physiological antigen processing and major histocompatibility complex (MHC) class I presentation to activate a CD8⁺ T cell response.

Replication-defective adenovirus 5 (Ad5) vectors have been extensively used for genetic vaccine delivery because Ad5 infects both replicating and nonreplicating cells, has a broad tissue tropism, propagates very efficiently in the available packaging cell lines, and has a scalable and affordable production process. Indeed, Ad5-based vectors elicit more potent antigen-specific CD8⁺ T cell than other genetic vaccine vectors based on poxviruses, lentiviruses, alphavirus, and naked DNA in animal models and human clinical trials (2–7). However, most humans

are exposed to Ad5 and develop high titers of anti-Ad5-neutralizing antibodies (nAbs). These preexisting Ad5 nAbs impair the immunogenicity of Ad5-based vaccines in animal models and in humans (3, 8–10) and may also potentially compromise their safety (11). Human adenovirus vectors based on rare serotypes such as Ad11, Ad24, Ad26, Ad34, Ad35, Ad48, Ad49, and Ad50, which are rarely neutralized in humans, have been proposed as alternatives to Ad5 and are currently being evaluated in a number of preclinical and clinical studies (12–16).

However, successful development of adenovirus vectors as genetic vaccine carriers will eventually depend not only on a low nAb frequency in the target population (seroprevalence) but also on their immunological potency as well as on the availability of cell substrates for scalable and reproducible production processes. We find here that adenovirus vectors from rare human serotypes induce lower levels of immune response than Ad5 in mice and nonhuman primates (NHPs), indicating that different adenovirus strains are not equivalent with respect to immunological potency. Instead, simian-derived adenoviral vectors had high immunological potency with low neutralization risk.

RESULTS

Adenovirus vectors from rare human serotypes are suboptimal immunogens in rodents and NHPs

There are 52 human adenovirus serotypes (17), but to date, very limited data compare the immunological potency of adenovirus vectors from different serotypes (18). Therefore, we performed a head-to-head comparison of the immunological potency of representative adenovirus

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serotypes from species B (Ad34 and Ad35), C (Ad5 and Ad6), and D (Ad24), which were chosen for their low seroprevalence in humans (Ad34, Ad35, and Ad24) or because they have been used in clinical trials (Ad5, Ad6, and Ad35).

Escalating doses [10^6 to 10^{10} viral particles (vp)] of replication-defective (E1-deleted) adenovirus vectors encoding HIV-1 gag (Ad-gag) were injected intramuscularly in BALB/c mice (five per group), and the relative immunological potency was defined as the minimal dose of vector capable of inducing a HIV-1 gag-specific T cell response in at least two of five animals within the same immunization group. Cellular immunity was measured 3 weeks after the immunization by ex vivo interferon- γ (IFN- γ) enzyme-linked immunospot (ELISpot) using a known gag CD8⁺ T cell epitope.

All vectors were immunogenic at the highest tested dose. However, although Ad5 and Ad6 were still immunogenic at lower doses (down to 10^5 vp), the other three vectors progressively lost the ability to induce a response with decreasing dose, with the minimal immunogenic dose being 10^{10} vp for Ad34, 10^9 vp for Ad35, and 10^8 vp for Ad24 (Fig. 1A). Thus, Ad5 and Ad6 showed a 10- to 100-fold higher immunological potency than Ad24 and at least 1000-fold higher immunological potency than the Ad34 and Ad35 vectors.

We performed similar dose-response experiments in NHPs by immunizing groups of three animals with 10^{10} and 10^8 vp of the Ad-gag vectors. Ad26 (another group D serotype that is currently in phase 1 clinical trials) was included in this study. Ad5 and Ad6 were the only vectors capable of inducing a T cell response at both tested doses, whereas Ad24, Ad34, and Ad35 showed a lower immunogenicity, even at 10^{10} vp (Fig. 1B). Ad26 had an intermediate phenotype; it induced cellular immunity comparable to that of Ad5 and Ad6 at the higher dose, but failed to elicit a response at the lower dose. Thus, the tested vectors displayed a similar ranking of immunological potency in mice and macaques.

Chimpanzee adenoviruses are potent genetic vaccine carriers

To identify candidate vaccine vectors with low seroprevalence and high immunological potency, we isolated several hundreds of adeno-

viruses from common chimpanzees (*Pan troglodytes*) and bonobos (*Pan paniscus* or pygmy chimpanzee) housed in animal facilities and zoos in Europe and in the United States.

By sequencing the capsid hexon gene, we classified chimpanzee adenovirus (ChAd) isolates according to the existing species of human adenoviruses (Fig. 2 and fig. S1). Most of the isolates belonged to species C and E (46 and 45%, respectively), whereas species B ChAd viruses were less frequently found (9%) and no isolate could be classified as species A, D, or F. A similar high frequency of isolation of B, C, and E adenoviruses was previously reported from NHPs (19).

ChAd-gag vectors from 26 different isolates were screened for immunological potency by dose-response in BALB/c mice. Similar to the human adenoviruses, ChAds displayed a wide range of immunogenicity, with the group C vectors being the most potent and the group B vectors inducing a T cell response only at very high doses (Fig. 3A and fig. S2). The group E vectors can be divided in two categories: those with high immunological potency (such as ChAd63, ChAd83, ChAd6, ChAd9, ChAd10, ChAd43, ChAd55, and ChAd147) and those characterized by a lower immunogenicity (ChAd4, ChAd5, ChAd7, ChAd16, ChAd38, ChAd146, ChAd149, and ChAd150). Some of the ChAd vectors were able to induce a T cell response at very low doses (1×10^6 to 3×10^6 vp), thus ranking in the same category of the clinically validated human Ad5 and Ad6 (4).

Consistent with the mouse data, ChAd3-gag and PanAd3-gag were immunogenic in macaques at both 10^{10} and 10^8 vp, indicating that the high level of immunological potency of these ChAd vectors is not a species-specific phenomenon (Fig. 3B).

ChAd vectors induced potent CD8⁺ T cell responses in mice and macaques, comparable to those elicited by the clinically validated Ad6 (Fig. 4, A and C). CD4⁺ T cells were also induced in mice and in NHPs, albeit at a lower level (Fig. 4, B and D).

ChAds have low seroprevalence in humans

Sera from 193 healthy individuals belonging to different geographical areas in Europe and the United States were tested for the ability to

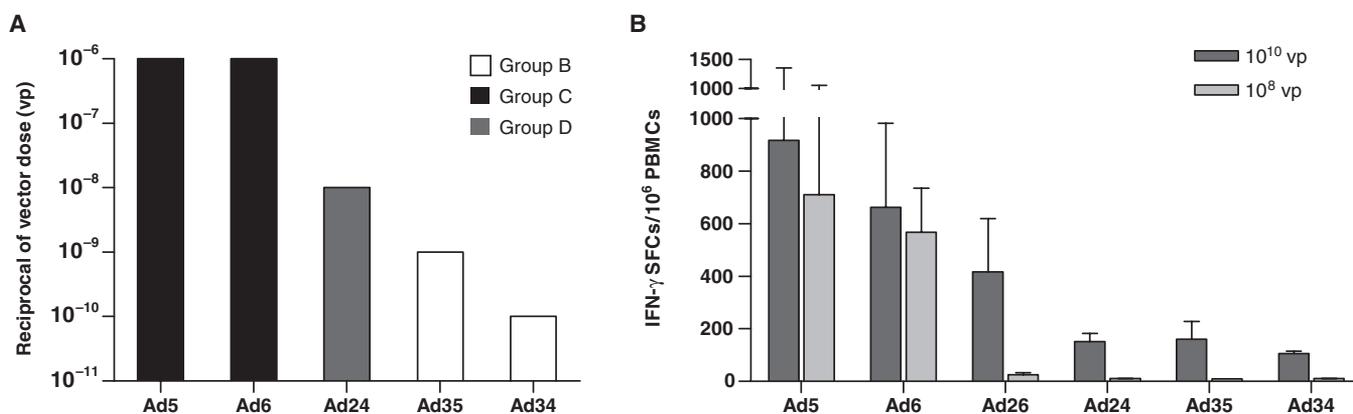


Fig. 1. Dose-response immunogenicity of human adenovirus in mice and macaques. **(A)** Immunological potency of human adenovirus vectors encoding for HIV-1 gag in BALB/c mice. Five animals per group were immunized intramuscularly with escalating doses of each adenovirus vector. IFN- γ ELISpot was performed on splenocytes collected 3 weeks later. Each bar represents the relative potency defined as the minimal adenovirus vector dose capable of inducing a HIV-1 gag-specific T cell response in at least two of five animals. Data are shown as the reciprocal of minimal dose. The

adenovirus serogroups are shown with different bar colors (white, group B; black, group C; dark gray, group D). **(B)** Immunological potency of human adenovirus vectors in macaques. Three animals per group were vaccinated intramuscularly with 10^{10} and 10^8 vp of each adenovirus vector encoding for HIV-1 gag. Four weeks after vaccination, T cell responses to a 15-mer peptide pool covering gag were measured by IFN- γ ELISpot on PBMCs. Data are expressed as IFN- γ SFCs per million PBMCs. The mean responses + SEM are shown for each immunization group.

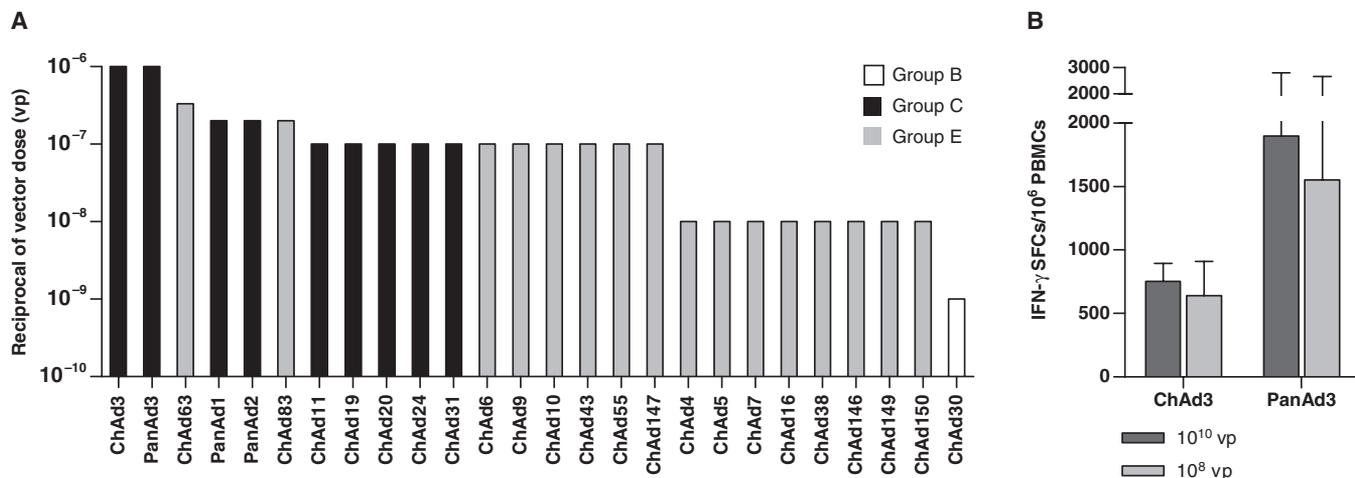


Fig. 3. Dose-response immunogenicity of ChAd in mice and macaques. **(A)** Immunological potency of chimpanzee adenovirus vectors encoding for HIV-1 gag in BALB/c mice was measured as described in Fig. 1. Data are shown as the reciprocal of minimal dose. The adenovirus serogroups are shown with different bar colors (white, group B; black, group C; light

gray, group E). **(B)** Immunological potency of chimpanzee adenovirus vectors in macaques. Three to six animals per group were vaccinated intramuscularly with 10¹⁰ and 10⁸ vp of each adenovirus vector encoding for HIV-1 gag. Data are expressed as IFN- γ SFCs per million PBMCs. The mean responses + SEM are shown for each immunization group.

ChAd3 was selected as a carrier for a candidate HCV vaccine. ChAd3-NSmut, shown here to induce high levels of T cell responses, was tested in a safety and immunogenicity phase 1 clinical trial in healthy volunteers. A similar vector based on the human Ad6 [Ad6-NSmut (21, 22)] was also tested in the same trial. The trial is described in detail in the ClinicalTrials.gov database (ID: NCT01070407), and all safety and immunogenicity results are described by Barnes *et al.* (23). Escalating doses (5 × 10⁸, 5 × 10⁹, and 2.5 × 10¹⁰ vp) of each vector were tested in groups of four to five healthy volunteers. ChAd3-NSmut was highly immunogenic and showed a clear dose-response effect with 100% frequency of responders at a dose of 2.5 × 10¹⁰ vp. Intracellular staining (ICS) analysis showed that ChAd3-NSmut primed a large number of IFN- γ -producing CD8⁺ T cells (range, 0.13 to 4% HCV-specific/total CD8 at 2.5 × 10¹⁰ vp; Fig. 7A). Antigen-specific CD4⁺ T cells producing IFN- γ were also detected, albeit at a lower frequency (fig. S4), which is consistent with the data obtained in mice and NHPs. Notably, the Ad6-NSmut showed comparable immunogenicity (range of IFN- γ -producing CD8⁺ T cells, 0.024 to 1.19% HCV-specific/total CD8; Fig. 7A).

The second vector, ChAd63, was used for the development of a genetic vaccine against *Plasmodium falciparum* malaria. For this candidate vaccine, we generated a ChAd63 encoding the liver-stage TRAP antigen fused to a string of *P. falciparum* CD4⁺ and CD8⁺ T cell epitopes mapped in

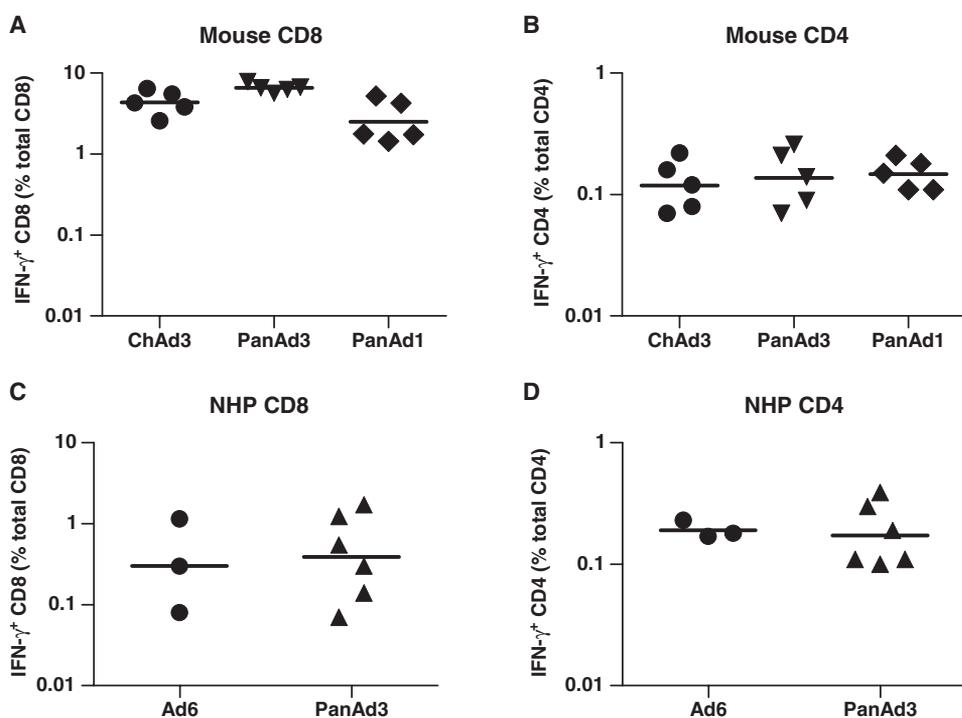


Fig. 4. Frequency of IFN- γ -secreting CD8⁺ and CD4⁺ T cells in mice and macaques. **(A to D)** Frequency of CD8⁺ and CD4⁺ T cells secreting IFN- γ in response to stimulation with a gag peptide pool was assessed using ICS and FACS (fluorescence-activated cell sorting) analysis in mice immunized with a dose of 10⁷ vp (A and B, CD8 and CD4, respectively) and macaques immunized with a dose of 10¹⁰ vp (C and D, CD8 and CD4, respectively). Each symbol corresponds to IFN- γ ⁺ CD8 or CD4 frequency in individual animals, expressed as the percentage of total CD8 or CD4. The horizontal line represents the geometric mean. Antigen-specific cells are undetectable in vaccination naive mice and macaques.

humans (ChAd63-METRAP). This vector was previously shown to induce high level of T cell responses in rodents and NHPs (24), low-level neutralization in African children living in a malaria endemic area

(25), and to protect against mouse malaria (26). ChAd63-METRAP induced potent T cell responses in human healthy volunteers in a phase 1 clinical trial (described in detail in the ClinicalTrials.gov database; ID: NCT01364883), as detected by IFN- γ ELISpot with a geometric mean response of 512 SFCs per million PBMCs at a dose of 5×10^{10} vp (Fig. 7B).

DISCUSSION

The human Ad5 vector induces a potent antigen-specific immune response but has had limited success as a vaccine vector because of a

high frequency of preexisting nAbs in the human population. Adenovirus vectors derived from strains that have not circulated widely in humans (“rare serotypes”) are under investigation as vaccine vectors based on the expectation that they would be as potent as Ad5 while being insensitive to neutralization by anti-Ad5 antibodies. However, our dose-response comparative studies in mice and NHPs show that adenovirus vectors from the rare human serotypes Ad24, Ad26, Ad34, and Ad35 are substantially less immunogenic (100- to 1000-fold) than the clinically validated Ad5 and Ad6. Indeed, Ad6 demonstrated comparable immunogenicity to Ad5 in humans (4), whereas preliminary data from a recent phase 1 clinical trial with an Ad35-based HIV candidate vaccine showed a rather low frequency of responders, with only 8 and 14% of subjects developing specific CD8⁺ and CD4⁺ T cells, respectively (27).

Chimpanzee-derived adenoviruses (CV68, CV33, CV23, CV32, and C1) belonging to three different serotypes were previously obtained from the American Type Culture Collection (ATCC) collection (28) and used as vaccine carriers for a number of different antigens from HIV, rabies, Ebola, malaria, and influenza. However, no comparative analysis of their immunological potency was carried out to aid in the prediction of their immunogenicity in humans. More recently, in two publications from the same group, a number of novel simian adenoviruses were described (19, 29), but also in this case only, very limited data on the relative immunological potency were generated (29).

To identify alternative adenovirus vectors with all the features necessary for vaccine development, we isolated several adenovirus strains from chimpanzees and generated a “library” of replication-defective adenovirus vectors. The choice of chimpanzee as a source of adenovirus isolates was based on the prediction that the substantial genetic diversity between individuals of the Pan species would extend to their pathogens (30–32), making it possible to isolate a large repertoire of different adenoviruses. Indeed, we isolated a large number of different adenoviruses from chimpanzees and bonobos, among which we could identify at least 26 different strains based on hexon homology. We confirmed that these strains represent individual serotypes by *in vitro* cross-neutralization assays and *in vivo* interference studies. Consistently, NHPs immunized with ChAd3-gag or ChAd3-NSmut followed by immunization with ChAd63 encoding the AMA-1 malaria antigen did not demonstrate any detectable interference between the different vaccine vectors (33). Thus, our collection of

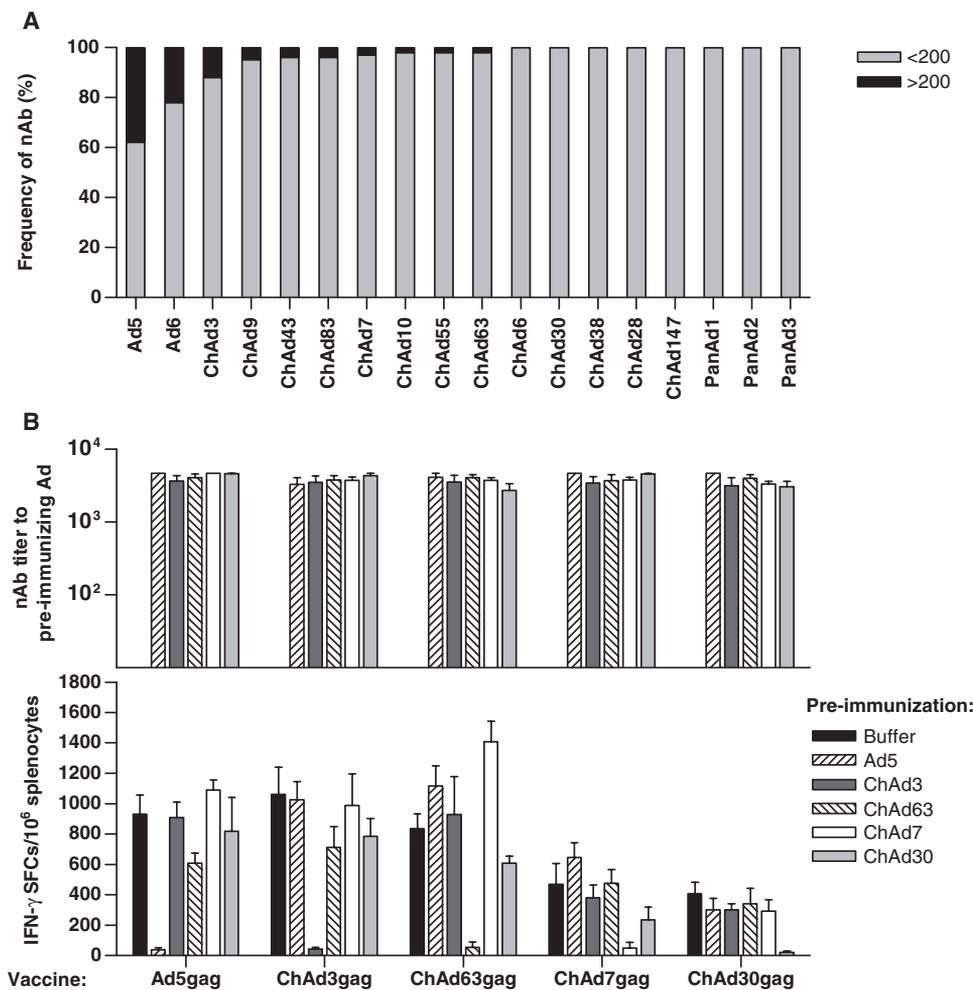


Fig. 5. nAb response and lack of cross-neutralization among different ChAd strains. **(A)** nAb titers to a panel of human and chimpanzee adenoviruses measured in sera collected from 193 Caucasian human healthy volunteers. The graph reports the percentage of individuals that show neutralizing titers above (black bar segment) or below (gray bar segment) 200 against each individual adenovirus tested. **(B)** Lack of cross-neutralization among different ChAd strains. ChAds belonging to different serological groups were selected according to the classification reported in Fig. 2. Mice were pre-immunized twice with 10^{10} vp of EGFP-expressing vectors (identified by different bar color and pattern), and neutralization titers to the preimmunizing vector were measured in sera (upper graph). Upper limit for the assay is >4608 , the highest dilution at which sera were tested. Mice were then vaccinated with 10^9 vp of HIV-1 gag-expressing vectors as reported in the lower graph x axis. Three weeks later, mice were tested for T cell response against gag by IFN- γ ELISpot. Bars represent the mean + SEM in each immunization group, expressed as neutralizing titer (upper graph) or IFN- γ SFCs per million splenocytes (lower graph).

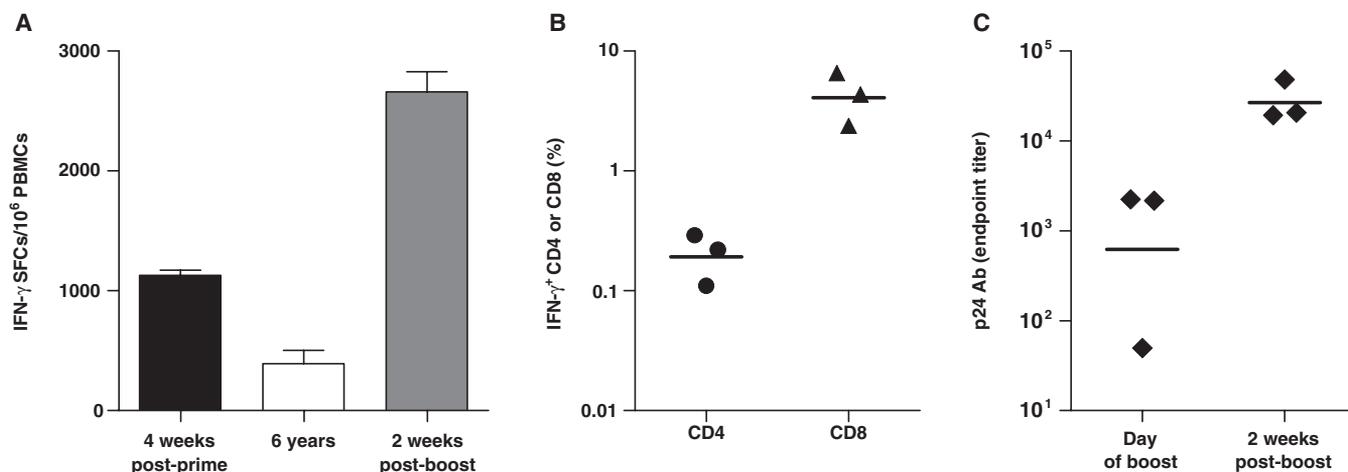


Fig. 6. Longevity of memory T and B cell response in macaques. Three macaques were vaccinated in early 2003 with 10^{10} vp of ChAd3-gag and boosted 6 years later with the same dosage of PanAd3-gag. **(A)** IFN- γ ELISpot response is shown at the peak after prime (4 weeks after vaccination), at the time of long-term boost (6 years), and 2 weeks after boost. Data are shown as the group mean + SEM response at each time point. **(B)** Identity and magnitude of the IFN- γ -secreting T cell subset,

non-cross-reacting ChAd vectors can be exploited for several vaccine applications after the strategy of “one vector–one disease” to avoid potential interference between different vaccines.

Replication-defective E1-deleted ChAd vectors efficiently propagated in PER.C6 and HEK293 cells, confirming the functional similarity of human Ad5 and chimpanzee adenovirus E1. In addition, these vectors did not form detectable replication-competent adenovirus during propagation in the HEK293 cells, suggesting that there is insufficient sequence homology between human and chimpanzee adenoviruses to allow for homologous recombination between the two genomes. Moreover, these data support the possible use of HEK293 cells for large-scale manufacturing of ChAds, a step necessary for adoption in the clinic.

All ChAds revealed significant diversity in the hypervariable regions of the hexon protein from the highly seroprevalent Ad5 and were not neutralized by anti-Ad5 antibodies *in vitro* and *in vivo*. Consequently, they were all found to be very rarely neutralized by antibodies present in humans.

In humans, many different approaches have failed to induce CD8⁺ T cell immunity even after positive results in preclinical animal studies. Our dose-response screening methodology allowed us to successfully predict immunological response in NHPs through mouse studies. Moreover, two of the ChAd vectors determined to be the most potent by our dose-response screen, ChAd3 and ChAd63, induced potent T cell immunity in 100% of immunized human volunteers. Indeed, the T cell responses were among the highest ever observed in humans with a single nonreplicating genetic vaccine vector. Notably, the ChAd-induced cellular response was characterized by extremely potent IFN- γ ⁺ CD8 T cells in all tested species, from rodents to humans, with high values of more than 1% antigen-specific IFN- γ ⁺ CD8 T cells in ChAd3 vaccinated healthy humans. Thus, a dose-response screen for immunological response may overcome some of the predictive limitations of preclinical models on human vaccination with adenoviral vectors.

Our dose-response screen in mice confirmed a high degree of immunostimulatory heterogeneity among ChAds similar to that observed

assessed by ICS and FACS analysis 2 weeks after PanAd3 long-term boost. Each symbol corresponds to IFN- γ ⁺ CD4 or CD8 frequency in individual animals, expressed as the percentage of total CD4 or CD8. The horizontal line represents the geometric mean. **(C)** Antibody (Ab) titers to p24 gag protein, assessed by ELISA and expressed as endpoint titers, for the three individual animals at the time of PanAd3-gag boost and 2 weeks later. The horizontal line represents the geometric mean.

for human adenoviruses. Nevertheless, by screening a large number of candidates, we were able to identify some ChAds with immunological potency equivalent to human Ad5 (ChAd3, ChAd63, ChAd83, PanAd1, PanAd2, and PanAd3), and several others with a slightly lower immunogenicity (within a factor of 5 to 10). The high level of immunogenicity of the top-ranking ChAd3 and PanAd3 was confirmed in NHPs, where they induced a T cell response comparable to that of Ad5, even at a low dose (10^8 vp). In addition, we have recently shown that another high-scoring ChAd vector (ChAd63) encoding the malaria TRAP antigen induced a very potent T cell response in rhesus macaques (24), indicating that the ability of ChAds to induce strong cellular immunity is not an antigen-dependent phenomenon.

The efficacy of vectors to stimulate the adaptive immune system correlated with phylogenetic classification into the different species according to the homology of their hexon protein sequence. One possible connection between species differentiation and immunostimulatory ability is differences in cell tropism (34): Adenovirus receptor use strongly influences cell tropism and likely also contributes to recognition by immune cells (35). Group B adenoviruses (such as the human Ad11, Ad34, and Ad35 and the chimpanzee ChAd30) recognize the CD46 surface protein and infect dendritic cells more efficiently than group C isolates *in vitro* (36). Therefore, group B adenoviruses were predicted to be a more effective class of genetic vaccine carrier; however, they were the least potent among all human- and chimpanzee-derived adenovirus vectors we tested. Instead, the most potent adenoviruses (group C and E) use the coxsackievirus and adenovirus receptor (CAR) to infect a variety of different cell types. It is possible that efficient infection of professional antigen-presenting cells by the group B adenoviruses leads to a more rapid elimination of the vector, thereby reducing the level and longevity of expression of the encoded antigen. Indeed, group B adenoviruses have low prevalence in both humans and chimpanzees, and a recent report shows that engagement of CD46 by Ad35 inhibits activation and proliferation of CD4 T cells and interleukin-2 production (37). Although further work needs to be done to characterize

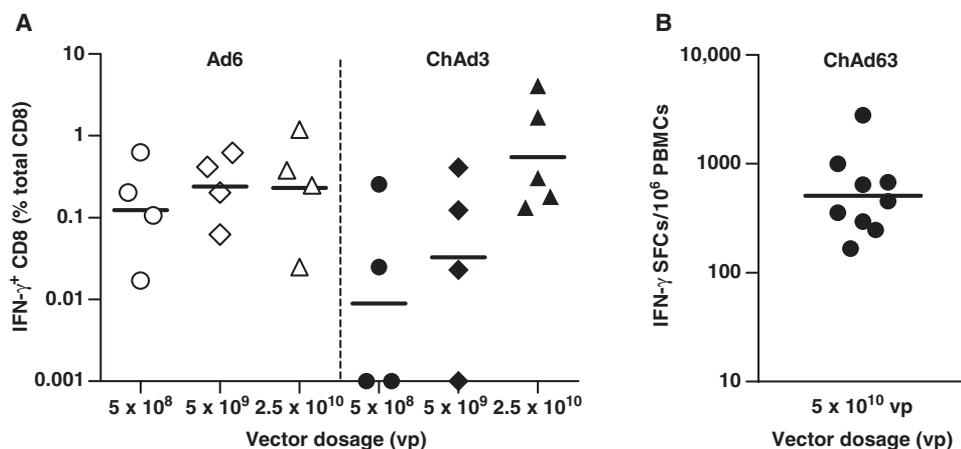


Fig. 7. Immunogenicity of human and chimpanzee adenovirus in humans. **(A)** Magnitude of the IFN- γ -secreting CD8 T cell subset by dose level assessed by ICS and FACS analysis after ChAd3-NSmut and Ad6-NSmut. Each symbol corresponds to IFN- γ ⁺ CD8 frequency in individual subjects, expressed as the percentage of total CD8. Horizontal line represents geometric mean. PBMCs were stimulated with two mixtures of three HCV NS pools covering either NS3-NS4 or NS5A-NS5B regions of the HCV vaccine insert or with DMSO, the peptide pool diluent, as the negative control. Data are expressed as the total NS response calculated by adding up reactivity to the two peptide mixtures corrected of background. **(B)** IFN- γ ELISpot data from human healthy volunteers vaccinated once with the indicated dosage of malaria adenovirus vaccine ChAd63-METRAP ($n = 9$) as individual responses to the vaccine insert. Peak response was measured 2 or 4 weeks after vaccination. Horizontal line represents geometric mean.

the mechanism governing different immunogenicity between vector species, this classification scheme, in conjunction with a dose-response immunological screen, should improve vector selection for future vaccine development.

The success of vaccine-induced T cell immunity in clearing infected cells before the onset of an acute disease or the establishment of a chronic infection likely depends on two factors: (i) the number of CD8⁺ T cell effectors that are present at the time of the infection and that can rapidly recognize and eliminate infected cells and (ii) the presence of a pool of memory T cells that are capable of rapid re-expansion upon encountering pathogen-derived antigen. Our finding that NHPs immunized with ChAd3 developed HIV gag-specific IFN- γ ⁺ T cells that persisted for more than 5 years suggests that ChAds have the potential to induce high frequencies of long-lasting antigen-specific T cells with effector function. Furthermore, these long-lived T cell pools also displayed the typical features of a memory population: They underwent rapid expansion in vivo upon boosting with a second non-cross-reacting ChAd vector encoding the same HIV gag antigen. If these observations hold true in humans, these vectors may be able to generate the memory T cell responses to targeted antigens needed for successful vaccination.

These studies are currently being extended into humans (23) and early clinical trials highlight the feasibility of this approach. ChAd vectors should serve as a new resource for human vaccine development.

MATERIALS AND METHODS

ChAd isolation and amplification

Stool specimens were collected in viral transport medium (VTM; Microtest M4-R Multi-Microbe Transport Medium, Remel Inc.) and then frozen or frozen directly at -70°C . The specimens were kept frozen

at $\leq 70^{\circ}\text{C}$ until they were processed for inoculation into cell cultures. At that time, the specimens were thawed and then vortexed in excess of chilled VTM. After the specimens had dissociated into suspensions, they were centrifuged for 10 min at 1500 to 1800 rpm. The supernatants were filtered through 0.8- and 0.2-mm syringe filters in series, and then the filtered material was inoculated into cell cultures. Each processed specimen was inoculated into tube cultures and shell vial cultures seeded with HEK293 cells or A549 cells cultivated in Dulbecco's modified Eagle's medium, 10% fetal bovine serum, and 1% penicillin-streptomycin. Cultures were visually monitored for cytopathic effect (CPE) for at least 21 days after inoculation. Cell monolayers showing clear sign of CPE were detached, suspended in the culture supernatant, and then stored at -70°C . Adenoviruses were cloned by infecting HEK293 cells seeded in 96-well plates. The virus cloning was performed by limiting dilution of the cell lysate obtained at the first passage of the virus amplification.

Five isolated clones were picked up and serially propagated. After two to three serial passages on HEK293 cells, a large-scale preparation of adenovirus was performed on cells planted on one to two two-layer cell factories (Nunc) (2×10^6 cells per cell factory). Purified viral particles were obtained from cell lysate by two ultracentrifugation steps on cesium chloride density gradients following a standard procedure.

Classification of the isolates

An initial classification of the new isolates was obtained by sequence analysis of the hypervariable region 7 (HVR7) of the hexon gene. To this end, two sets of primers were designed on the highly conserved regions flanking HVR7: TGTCCTACCARTCTTGCTTGA and GTGGAARGGCACGTAGCG; TGTCCTACCAGCTCTTGCTTGA and GTTCATGTAATCGTAGGIGTTG. These sets of primer pairs annealed on all isolates we have obtained so far. In addition, a pair of primers was designed to amplify hexon HVR1 to HVR6 (CAY-GATGTGACCACCGACCG and GTGTTYCTGTCYTGAAGTC).

The HVR7 and/or HVR1 to HVR6 were amplified by polymerase chain reaction (PCR) with purified viral DNA or crude HEK293 lysate as template. The PCR was performed following this protocol: 4 μl of crude lysate or 100 ng of purified viral DNA in 45 μl of reaction mixture containing 2 \times Master Mix (GoTaq Colorless Master Mix, Promega Corp.) and 10 pmol of each primers. Then, 5 μl of the reaction mixture was analyzed on 1% agarose gel containing ethidium bromide to detect the expected amplicon.

The PCR product was then purified to remove excess nucleotides and primers (with Wizard SV Gel and PCR Clean-Up System, Promega Corp.) and subjected to sequencing reaction with the same primers of the PCR. Based on HVR7 and HVR1 to HVR6 sequence alignment, we classified the new isolated viruses into the subgroups (B, C, and E) proposed for human adenoviruses (17).

The nomenclature adopted for our isolates reflects the species from which they were isolated [Ch from chimpanzees (species *P. troglodytes*)

and Pan from paniscus (species *P. paniscus*) and the progressive number that was assigned to each virus during the isolation history.

Human and chimpanzee adenovirus vectors

Human Ad5, Ad6, Ad24, Ad26, Ad34, and Ad35 were obtained from ATCC. All different expression cassettes inserted in adenovirus, ChAd, and PanAd vectors were based on human cytomegalovirus (HCMV) promoter and BGH pA. HIV-1 gag, HCV NS, *P. falciparum* ME-TRAP, and SEAP expression vectors were first cloned under HCMV and BGH pA control and then transferred in the different ChAd vectors containing HCMV/BGH pA cassette by homologous recombination in BJ5183 cells.

Vector construction, rescue, and characterization are described in detail in Supplementary Materials and Methods.

Animals and immunization

Six-week-old female BALB/c and C57BL/6 mice were purchased by Charles River. Male rhesus macaques (*Macaca mulatta*) of Chinese origin were housed at the Italian National Research Council primate facility (Rome, Italy) or at Istituto di Ricerche di Biologia Molecolare (Rome, Italy). The animal care routine and experimental procedures were in compliance with national and international laws and policies (EEC Council Directive 86/609; Italian Legislative Decree 116/92; Gazzetta Ufficiale della Repubblica Italiana n. 40, Feb. 18, 1992). The ethical committee of the Italian Ministry of Health approved this research. During handling, the animals were anesthetized.

To determine immunological potency, we immunized a series of five mice per group with escalating doses of each adenoviral vector suspended in 100 μ l of adenovirus stabilization buffers A195 or A438 (see Supplementary Materials and Methods) and injected them bilaterally in the quadriceps muscles. Three weeks after vaccination, mice were euthanized to measure T cell responses in splenocytes. In a second set of experiments, mice were preimmunized twice every 2 weeks with 10^{10} vp of Ad5, ChAd3, ChAd63, ChAd7, or ChAd30 expressing enhanced green fluorescent protein (EGFP) or mock-vaccinated with buffer. Pre-immunized mice were then immunized once with 10^9 vp of the above vectors encoding HIV gag in all possible vector combinations. Immune response was tested on splenocytes 2 weeks after immunization.

Macaques were immunized intramuscularly in the deltoid with adenovirus diluted in 0.5 ml of adenovirus stabilization buffer. At serial time points, EDTA-treated blood was drawn, PBMCs were prepared by standard technique with Accuspin tubes containing Histopaque-1077 (Sigma) and used for immunological assays.

Human clinical trials

All volunteers gave written informed consent before participation, and the studies were conducted according to the principles of the Declaration of Helsinki and in accordance with Good Clinical Practice. The HCV vaccine phase 1 trial (HCV001) was registered with the European Clinical Trial database (EudraCT number: 2007-004259-12) and with the ClinicalTrials.gov database (ID: NCT01070407). The malaria vaccine clinical trial (VAC043) was registered with the European Clinical Trial database (EudraCT number: 2010-023824-26) and with the ClinicalTrials.gov database (ID: NCT01364883).

Antigens for immunological assays

For BALB/c mouse experiments, a 9-mer peptide encoding the HIV gag major H-2 Kd CD8 epitope (AMQMLKETI) was used as antigen in ELISpot assay at a final concentration of 0.5 μ g/ml. For mouse ICS

and macaque ELISpot and ICS assays, a pool of 122 15-mer peptides overlapping by 11 amino acids dissolved in dimethyl sulfoxide (DMSO), covering the entire HIV gag protein, was used as antigen at a final concentration of 4 μ g/ml of each peptide. DMSO and concanavalin A were used as negative and positive controls, respectively. For HCV vaccine clinical trial, a set of 494 15-mer peptides overlapping by 11 amino acids encompassing NS3 to NS5B proteins of the NS region from HCV genotype 1b, BK isolate, were arranged in six pools covering NS3 protease (NS3p), NS3 helicase (NS3h), NS4, NS5A, and NS5B (split in two pools, NS5B-I and NS5B-II). Pools were used at a final concentration of 3 or 1 μ g/ml of each single peptide in the ELISpot and ICS assays, respectively. For the malaria vaccine clinical trial, 20-mer peptides overlapping by 10 amino acids, spanning ME and TRAP protein, were used for ex vivo ELISpot as described (38).

Immunological assays

IFN- γ ex vivo ELISpot and ICS with mouse splenocytes and monkey or human PBMCs, adenovirus neutralization assays, and antibody titration enzyme-linked immunosorbent assay (ELISA) with monkey sera were performed according to well-established standard techniques, described in detail in Supplementary Materials and Methods.

SUPPLEMENTARY MATERIAL

www.sciencetranslationalmedicine.org/cgi/content/full/4/115/115ra2/DC1
Materials and Methods

Fig. S1. Comparison of amino acid sequence of hexon proteins.

Fig. S2. Representative dose-response experiments with ChAd and PanAd vectors.

Fig. S3. Long-term mouse immunogenicity.

Fig. S4. CD4⁺ and CD8⁺ T cell responses in representative human volunteers.

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