Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity

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Recent studies of human immunodeficiency virus type 1 (HIV-1) infection in humans and of simian immunodeficiency virus (SIV) in rhesus monkeys have shown that resolution of the acute viral infection and control of the subsequent persistent infection are mediated by the antiviral cellular immune response[1–11]. We comparatively assessed several vaccine vector delivery systems—three formulations of a plasmid DNA vector, the modified vaccinia Ankara (MVA) virus, and a replication incompetent adenovirus type 5 (Ad5) vector—expressing the SIV gag protein for their ability to elicit such immune responses in monkeys. The vaccines were tested either as a single modality or in combined modality regimens. Here we show that the most effective responses were elicited by a replication-incompetent Ad5 vector, used either alone or as a booster inoculation after priming with a DNA vector. After challenge with a pathogenic HIV–SIV hybrid virus (SHIV), the animals immunized with Ad5 vector exhibited the most pronounced attenuation of the virus infection. The replication-defective adenovirus is a promising vaccine vector for development of an HIV-1 vaccine.

Two independently performed immunization and viral challenge studies (studies A and B) were conducted in rhesus macaque monkeys (*Macaca mulatta*). The immunization groups and experimental design for each study are summarized in Table 1. In study A, 21 rhesus monkeys were divided into six groups, including an unimmunized control cohort of six animals and five immunized groups of three monkeys each. Study B used 14 monkeys, three each in the immunized groups and eight in the unimmunized control group. Each of the test vectors expressed the identical SIVmac239 *gag* gene that had been codon optimized for expression in mammalian cells. The plasmid DNA vector was formulated in either phosphate-buffered saline (PBS) solution, a solution containing a nonionic blocked copolymer adjuvant (CRL1005)[12], or a solution containing monophosphoryl lipid A adsorbed onto aluminium phosphate (MPL/alum). All immunized animals were genotyped for the MHC class I *Mamu-A*01* allele and, with the exception of one monkey in the DNA/PBS group, all were positive for this allele. Two of the six and four of the eight control animals in studies A and B, respectively, were also positive for *Mamu-A*01*. The presence of this MHC allele allowed us to analyse the responses of T cells positive for the CD8 antigen ($CD8^+$) to the test vaccines; we used a tetramer reagent designed to detect T-cell receptors capable of binding an immunodominant SIV gag epitope, p11CM (residues 181–189), presented by the *Mamu-A*01* MHC protein[13,14].

All the vaccines were delivered by intramuscular inoculation. In the first study, the two viral vector vaccines were administered at weeks 0 and 6 followed by a booster dose at week 32; the preparations of DNA plasmid vector vaccine were delivered at weeks 0, 4 and 8 followed by a booster at week 25. In the second study, the DNA vector priming inoculations were also administered at weeks 0, 4 and 8. The viral vector boost inoculations were then given at week 32. As noted in Table 1, four of the control animals in study B (both positive and negative for *Mamu A*01*) were immunized with

Table 1 Immunization and challenge schedule

Group no.	Monkey no.	Priming immunization and schedule	Boost immunization and schedule	Challenge*
Study A				
1	115Q, V388, 127T†, 068T†, V446†, V391†	None	NA	NA
2	97X011, T271, T282†	SIV *gag* DNA, 5 mg, weeks 0, 4 and 8	SIV *gag* DNA, 5 mg, week 25	12
3	S202, 047R, 079R	SIV *gag* DNA with CRL1005, 5 mg, weeks 0, 4 and 8	SIV *gag* DNA with CRL1005, 5 mg, week 25	12
4	058R, 088R, 108R	SIV *gag* DNA with MPL/Alum, 5 mg, weeks 0, 4 and 8	SIV *gag* DNA with MPL/Alum, 5 mg, week 25	12
5	122G, 126G, 15G	MVA–SIV *gag*, 10^9 p.f.u., weeks 0 and 6	MVA–SIV *gag*, 10^9 p.f.u., week 32	12
6	54G, 56G, 82F	Ad5–SIV *gag*, 10^{11} particles, weeks 0 and 6	Ad5–SIV *gag*, 10^{11} particles, week 32	12
Study B				
7	040F‡, 97N114‡, 89Q, AW35, 99D142†‡, 99D158†‡, 99D221†, 99D149†	None	NA	NA
8	98D273, 98D318, 98D359	SIV *gag* DNA with CRL1005, 5 mg, weeks 0, 4 and 8	MVA–SIV *gag*, 10^9 p.f.u., week 32	6
9	98C040, 98C081, 98C084	SIV *gag* DNA with CRL1005, 5 mg, weeks 0, 4 and 8	Ad5–SIV *gag*, 10^{11} particles, week 32	6

See text for vaccine descriptions. The boost immunization in study A was conducted simultaneously for all groups in the study. NA, not applicable; p.f.u., plaque-forming units.
* Time of challenge is indicated as weeks after boost immunization. The animals in study A and study B were challenged separately. However, all animals within each study were challenged simultaneously.
† These monkeys did not express the *Mamu-A*01* MHC allele.
‡ These monkeys were mock immunized with 10^{11} particles of empty Ad5 vector at week 32.

an 'empty' Ad5 vector (10^{11} particles) that did not contain the SIV *gag* gene as a control for possible nonspecific immune effects on viral challenge.

The levels of circulating p11CM-specific $CD8^+$ T cells for the *Mamu-A*01*-expressing monkeys were monitored by the tetramer assay. In general, all of the monkeys developed p11CM-specific cellular immune responses after the initial immunization series (Fig. 1). These responses in study A were highest in the Ad5-vaccinated monkeys, followed in order by the DNA/CRL1005, MVA, DNA/MPL/alum and DNA/PBS cohorts. In the Ad5-vaccinated monkeys, the numbers of p11CM-specific cells reached levels as high as 4.0% of the circulating $CD8^+$ T-cell population before declining to 0.3–1.0% at the time of the booster inoculation. For all three animals, administration of the third dose of the Ad5 vector resulted in an additional increase of p11CM-specific cells, with levels maintained at about 1.0–2.0% of circulating $CD8^+$ T cells at the time of virus challenge. Staining with p11CM tetramers was notably lower at challenge in the monkeys from the remaining immunized groups within this study. The greatest p11CM-specific responses were seen in study B, in those monkeys that received priming immunizations with DNA/CRL1005 followed by the Ad5-vector boost (Fig. 1). After the booster inoculation, these animals exhibited peak levels of p11CM-specific T cells of 20.0–30.0% of circulating $CD8^+$ T cells, with levels ranging from 5.0% to 25.0% at challenge. The group that received the prime-boost combination of DNA/CRL1005 and MVA exhibited post-boost peaks in p11CM tetramer staining of 3.0–5.0%, with levels of less than 1.0% at virus challenge. As expected, staining was not detected in any monkey after immunization using tetramer reagents for *Mamu-A*01*-restricted determinants in the viral env (p41, residues 431–439) and pol (p68, residues 621–629) proteins (data not shown)[15].

The functional nature of the tetramer-positive $CD8^+$ T cells was confirmed in all of the immunized monkeys by an ELISPOT assay designed to determine the number of antigen-specific T cells that secreted interferon-γ (IFN-γ) when stimulated with either the p11CM peptide or a pool of peptides derived from SIV gag (see Supplementary Information Table 1). The relative levels of IFN-γ-secreting cells in the animals correlated well with the tetramer-staining results. Intracellular IFN-γ-staining assays confirmed that these responses were primarily mediated by $CD8^+$ T cells (data not shown). The functional nature of the responding T cells in the immunized monkeys was further confirmed by a bulk culture killing assay for cytotoxic T lymphocytes (CTLs) (data not shown).

At 12 weeks (study A) or 6 weeks (study B) after the final immunization, all monkeys were challenged by intravenous injection with 50 MID_{50} (50% monkey infectious doses) of the pathogenic SHIV 89.6P[16]. The challenge of the control and immunized animals within the context of each of the two independent studies occurred concurrently. Most of the immunized *Mamu-A*01*-positive monkeys responded to the challenge with a marked increase in staining of p11CM-specific $CD8^+$ tetramers, reaching 10.0–40.0% of total $CD3^+$ $CD8^+$ circulating T cells at about 3 weeks after challenge (see Supplementary Information Fig. 1). Although the post-challenge expansion seen in the cohort of DNA/CRL1005 prime followed by Ad5 boost was relatively modest, the animals in this group maintained substantial levels of tetramer staining-specific cells, of 10.0–30.0%. By contrast, the *Mamu-A*01*-positive monkeys in both unimmunized control groups developed much weaker p11CM-tetramer responses after challenge.

Counts of $CD4^+$ T cells and levels of plasma virus were followed over time for all monkeys after challenge (Fig. 2). Each of the animals in both control groups exhibited acute $CD4^+$ T-cell lymphopaenia and peak viral loads of 10^8–10^9 viral RNA copies per ml plasma at about 3 weeks after challenge, consistent with reported

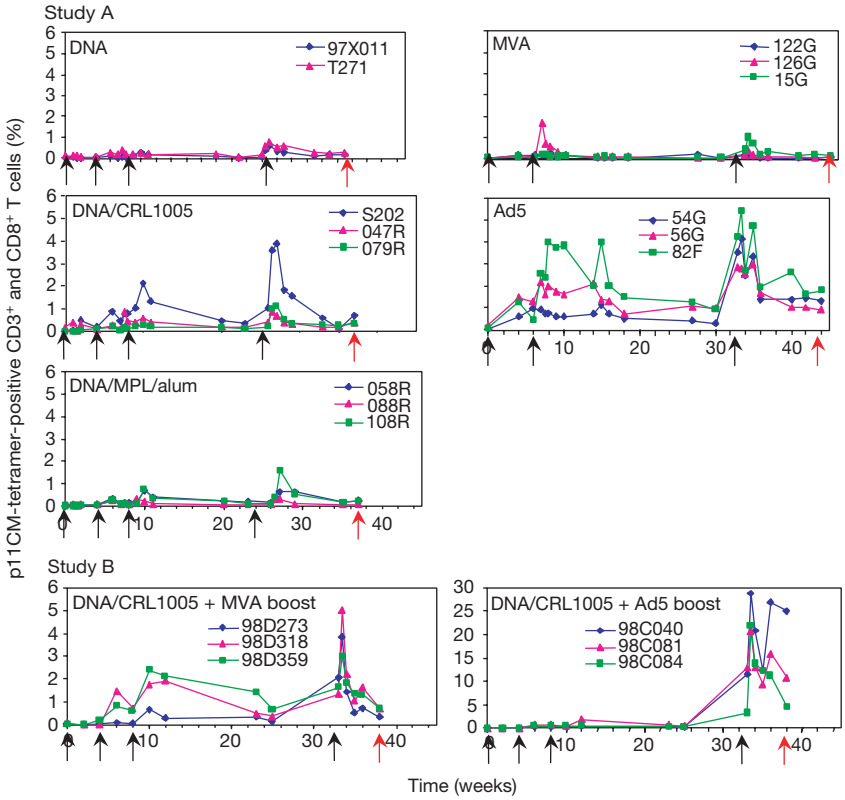

Figure 1 Quantification of $CD8^+$ T-cell responses by tetramer analysis during immunization. The percentage of $CD3^+$ $CD8^+$ cells that bind tetramers are shown for each vaccine group. The black arrows indicate the time of each immunization; the red arrow represents the time of SHIV challenge. Note that the *y*-axis scale in the right panel of study B is different from the remaining panels.

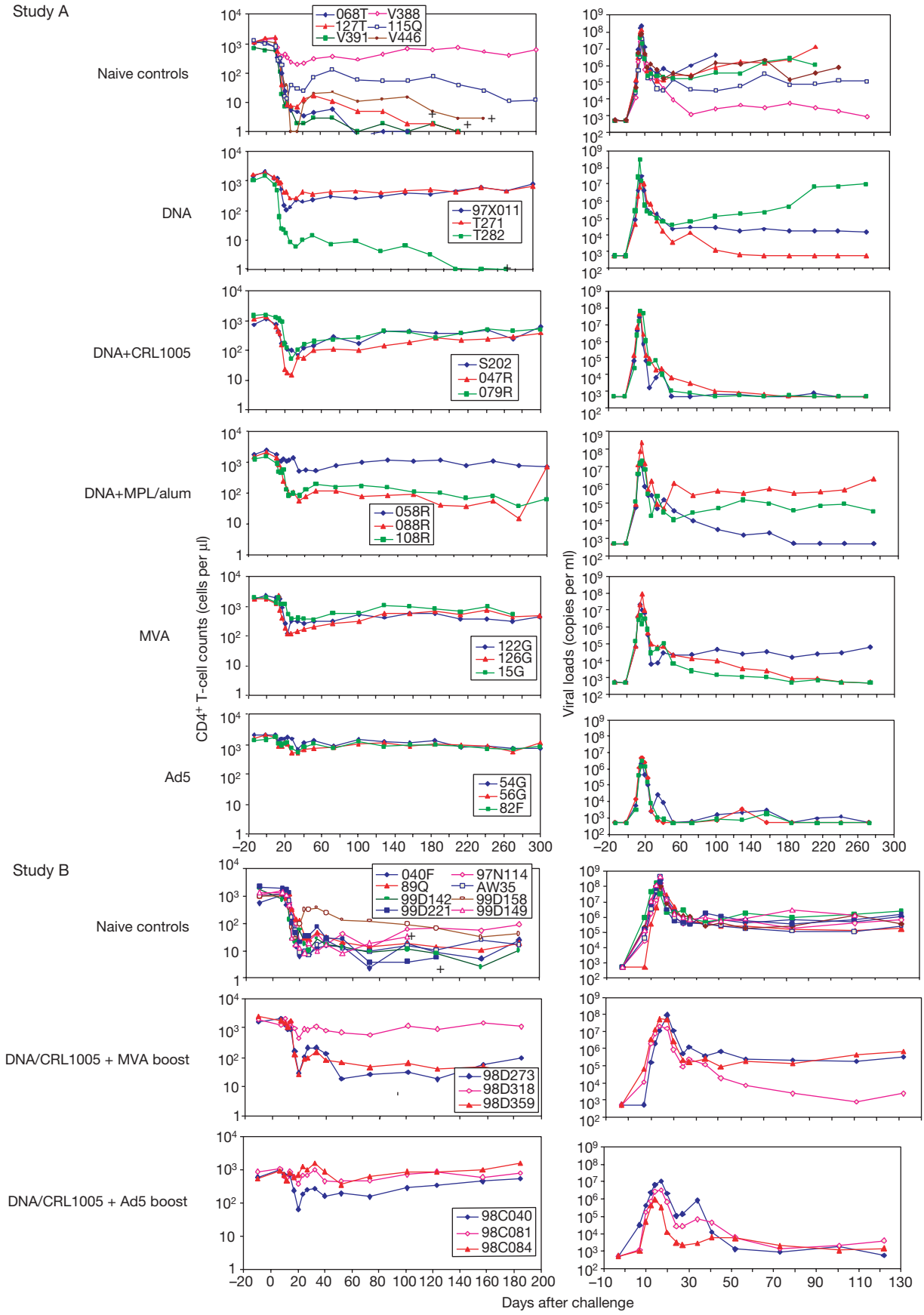

Figure 2 Post-challenge viraemia and CD4$^+$ T-cell counts. For CD4$^+$ cell counts, total lymphocyte numbers were multiplied by the per cent CD3$^+$ CD4$^+$ lymphocyte staining based on flow cytometry and reported as the number of cells per microlitre of whole blood. Plasma viral levels were determined by a branched DNA-amplification assay with a detection limit of 500 viral RNA copies per ml (Bayer Diagnostics).

characteristics of infection with this virus in rhesus monkeys[16]. With the exception of one animal in study A, all of the control monkeys experienced dramatic loss of $CD4^+$ T cells, with counts remaining below 100 cells μl^{-1} during the chronic phase of the infection. In these animals, plasma viral loads did not drop below 10^5 vRNA copies per ml. These observations were noted in both *Mamu-A*01*-positive and -negative animals, suggesting that the expression of this specific *Mamu-A*01* allele does not influence the course of infection after challenge with SHIV 89.6P. Control animals in study B that were immunized with an 'empty' Ad5 vector did not exhibit any differences in plasma viral load or $CD4^+$ T-cell loss compared with the remaining controls.

During the acute phase of the infection, most monkeys immunized with either the DNA or MVA vectors in study A or with the DNA/CRL1005–MVA vector prime–boost combination in study B exhibited an acute $CD4^+$ T-cell lymphopaenia similar to that in the control animals. The peak loads of plasma virus in these immunized animals were also indistinguishable from those in the controls. However, by about 70 days after challenge, many of the immunized monkeys exhibited some evidence of a positive immunization benefit, as manifested by control of viraemia and recovery of $CD4^+$ T-cell counts. This was particularly noticeable in the monkeys that had been immunized with four doses of the DNA/CRL1005 vaccine.

Nonetheless, the greatest degree of attenuation of the SHIV infection was provided in both studies by immunization with the Ad5-vector vaccine, either alone or as a boost after priming with the DNA vector formulated in CRL1005 (Fig. 2). With a single exception, the $CD4^+$ T-cell counts in these vaccinated monkeys remained above 500 cells μl^{-1} at all times and recovered to around 1,000 cells μl^{-1} by 70 days after challenge. In contrast to the other vaccine groups, the peak viraemia levels in the Ad5-vaccinated monkeys were 10- to 50-fold lower than those in the unimmunized monkeys, and all controlled the chronic loads of plasma virus to levels close to the assay detection limit (500 viral RNA copies per ml). One Ad5-immunized animal in study B, 98C040, did exhibit a somewhat prolonged peak viraemia with an associated acute loss of $CD4^+$ T cells. Yet, by 120 days after challenge, this monkey also manifested both a noted control of viraemia and recovery of $CD4^+$ T cells.

The observations of viraemia and $CD4^+$ T cells that were made with peripheral blood samples were confirmed by *in situ* staining analyses of lymph node biopsies (data not shown).

Serum samples collected after the challenge were assayed for neutralizing antibody against SHIV 89.6P. For most of the immunized animals, and for all of the Ad5-immunized monkeys, measurable neutralizing antibody responses were not detected until 42 days after challenge (data not shown). This is much later than the relatively rapid post-challenge neutralizing antibody responses reported in animals from previous studies that had been immunized with vectors expressing the viral *env* gene[9,10]. Also, the elicitation of neutralizing antibodies after challenge in our studies was not dependent on the type of vaccine used. These observations support the conclusion that the early mitigation of viral infection reported here was due solely to the vaccine-elicited gag-specific cellular immune responses. Any potentially confounding effects of the initial post-challenge neutralizing antibody response were controlled by avoiding the use of the *env* gene for immunization.

Immunodeficiency-related symptoms, such as prolonged weight loss, opportunistic infections and chronic diarrhoea, have been observed in five of the six unimmunized control monkeys from study A to date (~1 year after challenge). These five monkeys have been euthanized owing to their deteriorating physical condition (according to the recommendation of the attending veterinarians and under institutional guidelines). In contrast, the immunized animals, with two exceptions, generally have remained healthy during this time. The two monkeys that did not remain healthy (T282 and 088R from the DNA and the DNA/MPL/Alum vaccine groups, respectively) exhibited immunodeficiency-related symptoms beginning about six months after challenge, and we euthanized T282 at day 290. These two animals had developed relatively low immune responses after immunization and had poorly controlled viraemia after challenge. The monkeys immunized with the Ad5 vector have remained completely healthy throughout the post-challenge interval.

The unimmunized control monkeys from study B have all shown various degrees of immunodeficiency-related disease during the six months after challenge. Four of the eight controls have been euthanized owing to their deteriorating health. Two of the euthanized controls were *Mamu-A*01* positive while two were not, again suggesting that the expression of this specific MHC class I allele does not influence the course of infection or disease after challenge with SHIV 89.6P. None of the vaccinated monkeys from study B have yet exhibited any signs of immunodeficiency or suffered any consistent weight loss.

The results of the two studies reported here demonstrate that vaccine-elicited cellular immune responses against SIV gag can successfully attenuate infection and mitigate disease progression in a stringent model of monkey challenge using a highly pathogenic SHIV strain. The most notable vaccine-mediated effects were seen in those animals that had been immunized with the replication-incompetent Ad5 vector, either alone or as a booster inoculation after priming with a DNA vector formulated in CRL1005. The best pre-challenge immune responses in both studies were exhibited by the Ad5-immunized monkeys. After virus challenge, these Ad5-immunization-mediated responses were effective in reducing the level of peak viraemia, minimizing loss of $CD4^+$ T cells during the infection's acute phase and maintaining low levels of viraemia during the chronic phase, thereby permitting almost complete recovery of $CD4^+$ T cells. It is of interest that these beneficial post-challenge effects were seen in both immunization groups that involved the use of the Ad5 vector, although the vaccine-elicited cellular immune response in the heterologous prime–boost cohort (study B) was almost fivefold greater than that seen in the cohort immunized with the Ad5 vector alone (study A). Boosting with the MVA vector after DNA/CRL1005 priming was not nearly as effective in mitigating the effects of virus challenge; yet, the pre-challenge immune responses seemed similar to those elicited by immunization with only the Ad5 vector. Care must be used in making such quantitative comparisons across two independent studies. Nonetheless, these observations suggest that the Ad5 vector was superior with regard to both the quantity and, possibly, the quality of the elicited immune response. Further immunization and challenge studies are in progress to evaluate the nature of the Ad5-vector-mediated systemic and local (lymph node) virus-specific cellular immune response immediately before and after challenge in an attempt to establish an unequivocal immunological correlate of the antiviral effect. The relevance of the SHIV 89.6P monkey challenge model system used in these studies to human HIV-1 infection is not firmly established. The potential of the vaccines studied in this model cannot presently be extrapolated to predict human immunogenicity or efficacy. This assessment awaits the outcome of ongoing safety and immunogenicity clinical trials. □

Methods

Animals

Rhesus monkeys (*Macaca mulatta*) were maintained in accordance with the institutional animal care protocols of Merck Research Laboratories, New Iberia Research Center, and Primedica. Monkeys were typed for expression of the *Mamu-A*01* allele according to PCR sequence-specific primers methods as previously described[17]. Animals that were positive for the PCR reaction were subsequently confirmed by DNA sequencing of the amplified region. Twelve weeks after the final immunizations, monkeys were challenged intravenously with 50 MID_{50} of cell free SHIV-89.6P[16]. Monkeys were monitored for clinical signs of disease progression and cared for under the guidelines established by the National Institutes of Health (NIH) 'Guide for the Care and Use of Laboratory Animals'.

Vaccines

The codon-optimized SIVmac239 *gag* gene was constructed by annealing a series of overlapping oligonucleotides. The authenticity of the synthetic gene was confirmed by DNA sequencing. The gene was cloned into V1R vector, which has been described previously[18,19]. Plasmid DNA vaccines were formulated either in PBS alone, or in PBS containing the selected adjuvants: 7.5 mg of a nonionic block copolymer (CRL1005, CytRx); or 0.5 mg of monophosphoryl lipid A (MPL, Corixa) absorbed onto 0.7 mg of aluminium, as aluminium hydroxyphosphate (Adju-Phos, HCI Biosector). For study B, a modification of the DNA/CRL1005 was employed on the basis of ongoing studies that suggested this formulation provided improved immune responses compared with the initial formulation. For this formulation, 5 mg of DNA was added to 7.5 mg of CRL1005 mixed with 0.75 mM benzalkonium chloride (Ruger). Before immunization, the formulation was warmed slowly to room temperature from a frozen stock.

The synthetic SIV *gag* gene was also used for construction of the MVA and adenoviral vectors. The method for generation of recombinant MVA has been described elsewhere[20]. Briefly, the SIV *gag* gene was cloned into the pSC59 shuttle vector. This plasmid was designed to insert the transgene fragment into a viral thymidine kinase region, and to drive the transgene from a synthetic early/late promoter. Recombinant virus was identified and subsequently cloned again from single plaques six times with confirmation of maintenance of transgene by detecting expression by immunostaining gag.

The adenoviral vector was based on a serotype 5 adenovirus that had been rendered incompetent to replicate by deletion of the E1 and E3 viral genes, and was propagated subsequently in E1-expressing 293 cells. Recombinant adenovirus expressing the codon-optimized SIV *gag* gene was prepared as follows. The adenoviral shuttle vector, pHCMVIBGHpA1, contains Ad5 sequences of nucleotides 1–341 and 3,534–5,798 and an expression cassette containing the human cytomegalovirus (HCMV) promoter with intron A and the bovine growth hormone polyadenylation signal. The codon-optimized full-length SIV *gag* was digested from a parental plasmid with *Bgl*II and then cloned into a *Bgl*II-digested shuttle vector, pHCMVIBGHpA1. The shuttle vector was recombined with an adenoviral backbone plasmid, pAd-ΔE1E3, which contains the complete Ad5 genome except the E1 and E3 regions, to generate a pre-adenoviral plasmid, pAd5-SIVgag. This plasmid was linearized by digestion with *Pac*I and transfected into 293 cells to generate Ad5-SIVgag. The recombinant adenovirus was grown in large quantities by multiple rounds of amplification in 293 cells. The virus was purified by caesium chloride gradient centrifugation. Viral DNA was extracted by proteinase K digestion and confirmed for genomic integrity by restriction enzyme digestion of the viral genome, and PCR and DNA sequencing of the transgene cassette. SIV gag expression was verified by western blot analysis of COS and 293 cells infected with the virus. Virus quantities were expressed in terms of viral particles, based on determination of the nucleic acid content from purified virus.

Tetramer staining

The methods for preparing p11CM tetramer reagent and staining lymphocytes within whole blood have been described elsewhere[9]. Samples were analysed by flow cytometry on a FACScalibur (Becton Dickinson). Gated $CD3^+$ $CD8^+$ T cells were examined for staining with tetrameric Mamu-A*01–p11CM complex. The CTL epitope p11CM has been reported as a dominant epitope in SIV gag restricted to the *Mamu-A*01* MHC class I allele. For each sample, 30,000 gated $CD3^+$ $CD8^+$ T-cell events were collected and analysed on CellQuest program (Becton Dickinson), and the results presented as per cent tetramer-positive cells within $CD3^+$-and-$CD8^+$-positive events.

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Eventual AIDS vaccine failure in a rhesus monkey by viral escape from cytotoxic T lymphocytes

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Potent virus-specific cytotoxic T lymphocyte (CTL) responses elicited by candidate AIDS vaccines have recently been shown to control viral replication and prevent clinical disease progression after pathogenic viral challenges in rhesus monkeys[1–4]. Here we show that viral escape from CTL recognition can result in the eventual failure of this partial immune protection. Viral mutations that escape from CTL recognition have been previously described in humans infected with human immunodeficiency virus (HIV)[5–10] and monkeys infected with simian immunodeficiency virus (SIV)[11–13]. In a cohort of rhesus monkeys that were vaccinated and subsequently infected with a pathogenic hybrid simian–human immunodeficiency virus (SHIV), the frequency of viral sequence mutations within CTL epitopes correlated with the level of viral replication. A single nucleotide mutation within an immunodominant Gag CTL epitope in an animal with undetect-