Immunoprophylaxis against AIDS in macaques with a lentiviral DNA vaccine

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Abstract

We earlier reported that immunization of macaques with a reverse transcriptase-deleted $SHIV_{KU2}$ ($\Delta rtSHIV_{KU2}$) plasmid that contained HIV-1 (HXB2) *env* and SIV *gag–nef* induced protection against AIDS caused by challenge virus SHIV89.6P with a heterologous *env*. We further deleted *vif* and *integrase* from $\Delta rtSHIV_{KU2}$ and substituted the 3′LTR with SV40 poly A sequences, creating $\Delta 4SHIV_{KU2}$ (M) and a parallel construct containing *gag–nef* of $HIV-1_{SF2}$, $\Delta 4SHIV_{KU2}$ (H). Six macaques received two intramuscular injections of the (M) DNA, and another six received three injections of the (H) DNA. Three of the latter group received two post-challenge boosts with (M) DNA vaccine. Seven virus control macaques were inoculated with SHIV89.6P. All twelve immunized macaques were challenged with SHIV89.6P virus, and CMI responses were measured by ELISPOT assays.

Virus control animals all developed progressive infection, whereas vaccinated macaques from both groups controlled virus replication, with plasma viral loads dropping to undetectable levels between weeks 6 and 126 p.i. This DNA vaccine was efficacious even though it encoded Env, Gag, and Nef that were genetically distinct from the proteins in the challenge virus. The DNA vaccine induced broad-based protection without using viral proteins to boost the immunity.
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Introduction

The availability of a safe and effective vaccine to prevent AIDS remains an elusive goal and thus necessitates various approaches to the problem. The use of live-attenuated simian immunodeficiency virus (SIV) and chimeric SIV/human immunodeficiency virus (SHIV) viruses in the macaque model of HIV-1 infection had provided proof of concept that

virus replication can be controlled for long periods of time in infected individuals to halt disease progression. The concept of using live-attenuated vaccines for HIV-1 however was abandoned for safety reasons and also because the immunity induced by the live SHIV vaccines was contingent on persistent infection by the vaccine virus. Challenged immunized macaques that subsequently eliminated their vaccine virus succumbed to AIDS caused by resurgent challenge virus that had been maintained in a latent state in the presence of persistent vaccine virus (Mackay et al., 2004). Thus, the live SHIV vaccines likely did not induce memory immune responses.

The use of plasmid DNA encoding HIV-1 genes as a vaccine has proven an excellent alternative to live vaccines because they induce memory CMI responses, the arm of the immune response that is closely associated with control of the virus (Letvin et al., 1997, 1998; MacGregor et al., 2002;

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McKay et al., 2004; Schmitz et al., 1999; Singh et al., 2005; Yasutomi et al., 1996). Most of the current DNA vaccines utilize either CMV (Tang et al., 1992), β-actin, or muscle-specific desmin promoter (Cazeaux et al., 2002) to drive expression of one or two fused genes of HIV-1 including the *env*, *gag*, *pol* (Amara et al., 2001; Amara et al., 2002a; 2002b), and *tat* (Ensoli and Cafaro, 2000). DNA vaccines comprised of multiple plasmids encoding different HIV-1 proteins have been used to obtain a broader spectrum of immunity than individual plasmids expressing single proteins (Kong et al., 2003). The use of these plasmid DNA vaccines proved to be safe and immunogenic in macaques, but nevertheless required boosts with viral proteins (Letvin et al., 1997), or viral proteins expressed by various vector systems including recombinant pox virus (Robinson et al., 2000), modified vaccinia virus Ankara (Amara et al., 2002a; Barouch et al., 2001; Earl et al., 2002), and adenovirus (Casimiro et al., 2003; Kostense et al., 2004) to be efficacious in preventing AIDS.

Because of potential logistical problems involved with prime/boost strategy in carrying out vaccination in underdeveloped countries, we undertook studies to explore whether a new type of DNA vaccine could be efficacious without the need for viral protein boosts. In an earlier report, we had shown that the genes of SHIV$_{KU2}$, a virus known for its high replication efficiency in macaques, could be used as a vaccine after the genome was made non-infectious by deletion of the RT portion of the *pol* gene (Singh et al., 2005). We also showed that inoculation of mice in the gastrocnemius muscle with a noninfectious ΔRT Δint Δvif SHIV construct resulted in the production of viral p24 in myocytes and infiltrating mononuclear cells (Hegde et al., 2005). In the current study, we report that after additional deletions of *integrase* and *vif* and substitution of SV40 poly A sequences for the 3′*LTR*, the genome of SHIV$_{KU2}$, still remained immunogenic and efficacious in macaques. The Δ4SHIV$_{KU2}$ DNA utilized promoter/enhancer sequences in the 5′LTR of SIV$_{mac}$239 to drive expression of Gag, Protease, Vpr/Vpx, Tat, Rev, Vpu, Env, and Nef. The *gag*, *env*, and *nef* could be exchanged by genetic engineering techniques from those of SIV to those of HIV-1. The DNA was incorporated into a plasmid and tested for expression in transfected cell cultures prior to immunization of cynomolgus macaques by intramuscular injections of the DNA. In this study, we used two constructs, Δ4SHIV$_{KU2}$ (M) (M DNA) and Δ4SHIV$_{KU2}$ (H) (H DNA), respectively, to immunize macaques to explore the breadth of the protective antiviral responses by using SHIV89.6P that has the SIV *gag–nef* as challenge. The first vaccine construct (M) utilized the macaque adapted *env* of HIV-1 HXB2, thereby providing a heterologous *env* gene. The *gag–nef* genes of the (M) DNA were similar to those of the challenge virus. The second construct (H) contained the *gag–nef* of HIV-1$_{SF2}$, thereby obtaining a DNA molecule that encoded distinctly different *env*, *gag*, and *nef* genes from corresponding proteins of the challenge virus. Macaques prophylactically immunized with either construct were protected from persistent productive replication of the challenge virus. These results indicated that

this type of vaccine that facilitated synthesis of several HIV-1 proteins in a part of a single cycle of replication of the virus has the potential for eliciting broad-based protection and, importantly, that protection could be obtained without need for using viral proteins to boost immunity.

Results

The construction of the gene-deleted DNA vaccines Δ4SHIV$_{KU2}$ (M) [(M) DNA] and Δ4SHIV$_{KU2}$ (H) [(H) DNA] is shown diagrammatically in Fig. 1. These constructs are based on the backbone of SIVmac239 and lack the *RT*, *integrase*, *vif*, and 3′ LTR. The *env* genes are derived from HIV-1 HXB2, and the *gag*, *pro*, and *nef* are from SIVmac$_{239}$ and HIV-1$_{SF2}$, respectively. The SV40 polyadenylation signal was cloned in 3′ to the *nef* gene.

Transfection of (M) and (H) DNAs resulted in transient expression of viral proteins in HEK293

Human HEK293 cells were transfected with the constructs using a standard protocol reported earlier Hegde et al., 2005. Transfected cells were monitored through day 14 for the production of p27 or p24. Protein was prepared from harvested cells in a parallel culture incubated with [^{35}S] methionine and cysteine, and proteins were immunoprecipitated and separated by SDS polyacrylamide gel electrophoresis. The immunoprecipitation study showed that the plasmid DNAs expressed gp160, gp120 and p55, p27/p24, Gag-pol, and Gag of both constructs (Fig. 2A), with peak production of the Gag protein at concentrations of 30–40 ng/ml between day 2 and 4 (Fig. 2B). Supernatant fluids and lysates of transfected cells were examined for infectivity in Jurkat cell cultures that are highly susceptible to replication and development of CPE caused by SHIV$_{KU2}$. These inoculated cultures did not develop CPE, and PCR for *gag* in cell lysates was negative (data not shown).

Macaque infection with SHIV89.6P$_{–CY}$

All seven control macaques inoculated with the challenge virus, SHIV89.6P$_{–CY}$, developed highly productive infections within 2 weeks after inoculation; plasma viral RNA concentrations peaked at levels varying from 4×10^7 to 3×10^9, with set points varying from approximately 10^4 to 10^6 copies/ml plasma (Figs. 3A, B). Four of the seven animals developed a precipitous loss of CD4$^+$ T cells by 3 weeks after inoculation, and this was maintained in all four to the end of the study at week 135. Three animals retained CD4 counts above 200 until weeks 59–93 p.i. One animal, 13-1030, maintained a count of 586 CD4$^+$ T cells/ml blood (Fig. 3C). Except for this animal, all of others developed minimal ELISPOT titers (Fig. 3D). Four of the seven animals died with AIDS at various points during the observation period as indicated in Fig. 3B. Note that the groups of animals were not infected simultaneously, thus the numbers of weeks post-infection vary in the figures. Of the three
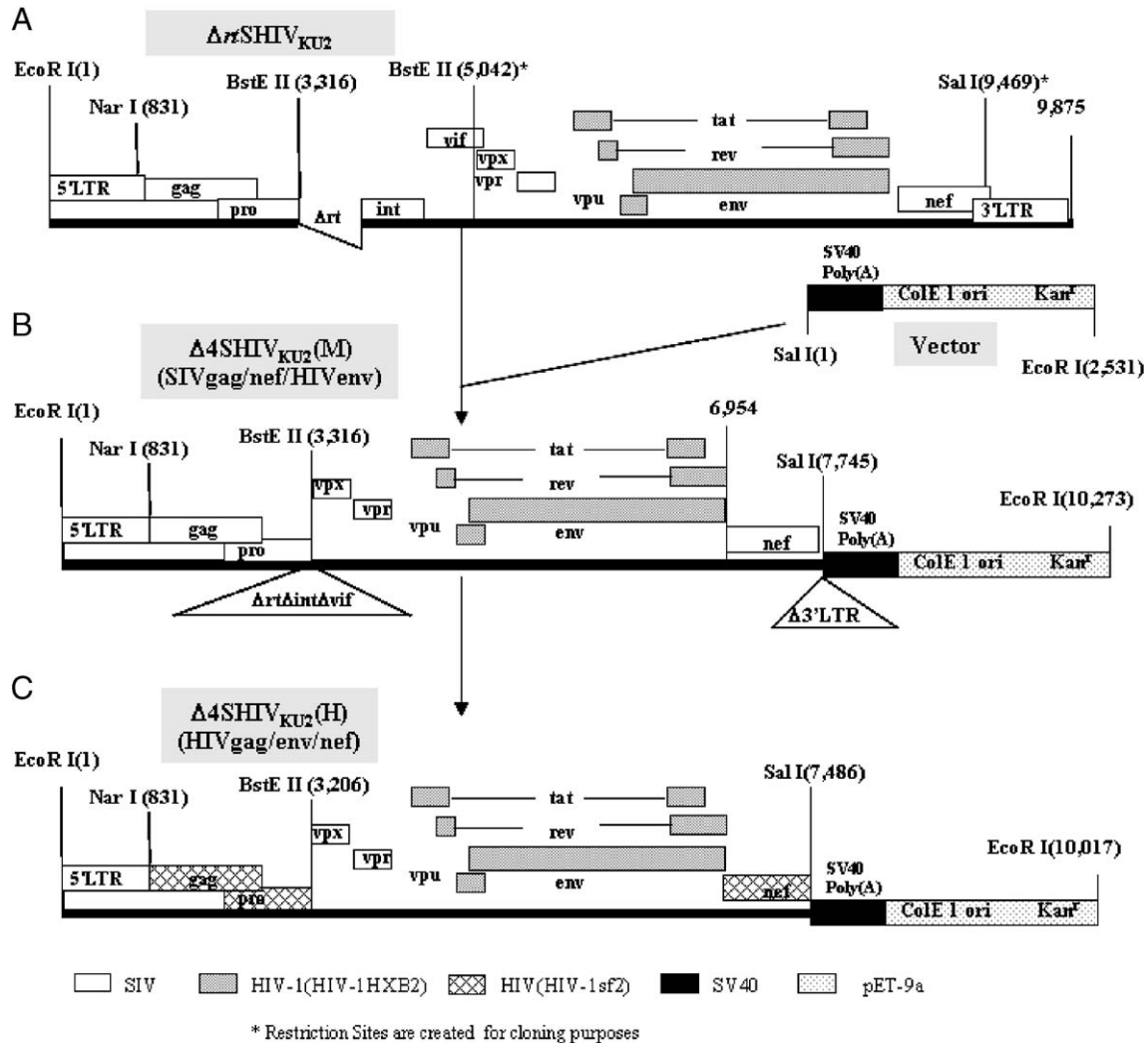
Fig. 1. Schematic diagram illustrating sequential steps in the construction of $\Delta4SHIV_{KU2}$ DNA vaccines, (M) and (H), with (M) containing the *gag* and *nef* of SIV origin and *env* from HIV-1 and (H) containing the *gag*, *env*, and *nef* of HIV-1$_{SF2}$. Detailed descriptions of the cloning steps are given in Materials and methods.

survivors, 13-1030, 13-1031, and 4226, the latter two have maintained viral RNA concentration in excess of 10^4 copies/ml plasma, CD4$^+$ T cell counts less than 100 cells/ml blood, and negligible ELISPOT responses. Thus, these two animals are at risk for developing AIDS. Macaque 13-1030 is the only member of the group that has maintained relative control over the virus. Nevertheless, this animal has a viral RNA concentration of almost 10,000 copies/ml plasma.

Immunization of six macaques with $\Delta4SHIV_{KU2}$ (M)

The six animals immunized with DNA expressing SIV Gag–Nef developed ELISPOT responses to SIV Gag peptides, and were challenged with SHIV89.6P$_{CY}$ between 5 and 12 weeks after the last boost (Fig. 4A). Following challenge, all animals became infected, with viral RNA titers peaking a week later at concentrations varying from 10^3 to 10^7 copies/ml (Fig. 4B). However, these titers declined rapidly, and viral RNA became undetectable in individual animals between week 6 and 126. All of the animals

controlled plasma virus to less than 3000 viral RNA copies/ml by weeks 2–40 (Fig. 4B).

ELISPOT responses surged 1 week following challenge and levels fluctuated in individual animals as shown in Fig. 4A. The ELISPOT responses in general declined during the following 64 weeks. Three of the animals became negative at this point in contrast to the other three that had responses of 170–400 at week 98 (Fig. 4A). None of the animals developed any significant loss of CD4$^+$ T cells (Fig. 4C). Thus, the vaccine protected all of the animals from AIDS, with viral RNA in plasma becoming undetectable in five of the six animals within 1 year after challenge (Fig. 4B).

Immunization of six macaques with (H) DNA

In the preceding experiment, we showed that the (M) DNA induced protection against a virus with a different Env, but homologous Gag–Nef. In the experiment reported below, we sought to determine whether the vaccine with HIV-1 *gag–nef* genes would elicit protection against
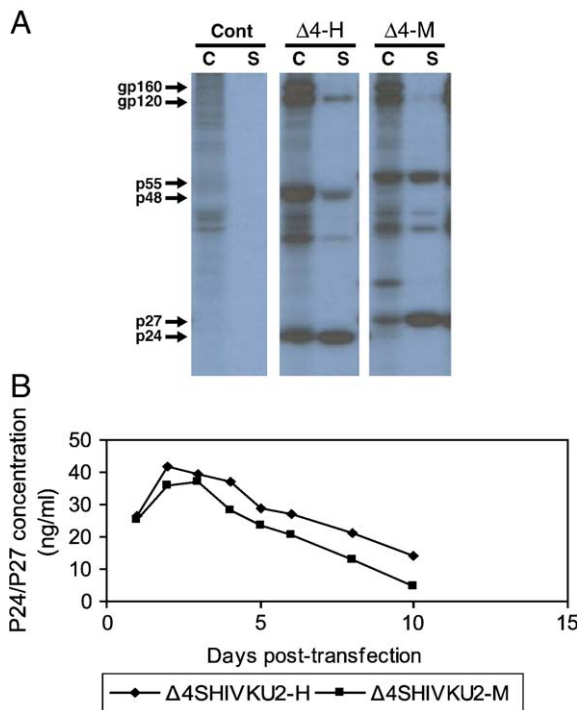
Fig. 2. Production of viral proteins from DNA vaccine constructs. (A) Immunoprecipitation data showing viral proteins encoded by vaccines (H) and (M) that were secreted into the supernatant fluid (S) and present in cell extracts (C) of transfected HEK293 cells. HEK293 cells were transfected with 4.75 μg of vaccine construct and 15.5 μl of ExGen 500. At day 2 p.i., proteins were extracted from cell lysates and supernatant fluids using protein A Sepharose (catalog number 17-0780-01, Amersham Bioscience AB, Sweden) and normal and immune human or macaque sera used to precipitate various proteins. The immunoprecipitates were separated by SDS-polyacrylamide electrophoresis in 10% gels, dried, and exposed to X-ray film. Lanes 1, 2, uninfected cells; lanes 3, 4, cells infected with Δ4SHIV$_{KU2}$(H); lanes 5, 6, cells infected with Δ4SHIV$_{KU2}$(M). C, cell-associated proteins; S, supernatant proteins. (B) Quantification of Gag p27 of the (M) construct and p24 of the (H) construct secreted into the supernatant fluids of transfected HEK293 cells. Samples of supernatant medium were harvested at the indicated times. ELISAs were performed on the fluids and standards, and the p24 or p27 concentrations were determined by regression from the standard curves. The transfected cells were rinsed three times following harvest of fluids at each sampling period. Thus, the reported values represent the amount of viral proteins secreted into the supernatant fluids during the previous 24 h.

SHIV89.6P$_{-CY}$ that has the SIV *gag–nef*. The animals were immunized three times within a 36-week period and challenged 4 weeks after the third immunization. All six animals became infected with the challenge virus, with viral RNA concentrations peaking between 5×10^6 and 5×10^8 copies/ml during the first 3 weeks after challenge. These values declined after this period (Figs. 5A and 6A).

The six animals had developed ELISPOT responses by 3 weeks post-challenge as shown in Figs. 5B and 6B. At this point, we elected to determine whether administration of vaccine DNA in the presence of active virus replication would result in enhancement of virus replication, or a more rapid decline in virus replication. We therefore arbitrarily divided the animals into two groups of three and administered the (M) DNA to one group (Fig. 5). The DNA was administered at weeks 6 and 13 post-challenge.

The second group received no post-challenge immunizations (Fig. 6).

Plasma viral RNA concentrations in the six animals remained high for only 3 weeks after which the concentrations declined about 1000-fold (Figs. 5A, 6A), irrespective of whether or not the animals were re-immunized. All six animals developed complete control of the challenge virus, becoming viral RNA negative between weeks 19 and 86. There was no significant difference between the two groups in the rate of viral RNA decline in plasma (Figs. 5A, 6A).

The CD4$^+$ T cell counts in the six animals declined following challenge but have remained above 500 cells/ml at 2 years post-challenge (data not shown).

Discussion

Previously, we reported that a plasmid DNA vaccine Δ*rt*SHIV$_{KU2}$ containing SIV *gag–nef* and HIV-1 *env*, regulated by the SIV promoter, induced protection in macaques from AIDS, without need for pre-challenge boosts with vectors expressing viral proteins (Singh et al., 2005). Although we did not detect infectious virus either in transfected HEK cells or in PBMCs of Δ*rt*SHIV$_{KU2}$-injected macaques, that construct still had *integrase*, *vif*, and 3′*LTR* sequences and thus retained the potential for integration and re-acquisition of infectivity by recombination. We undertook the study reported here to determine if further deletion of *vif* and *integrase* genes and substitution of the 3′LTR with SV40 poly A sequences could be performed without affecting the immunogenicity or efficacy of the DNA in inducing protection against AIDS caused by a heterologous virus. Since *env*, *gag*, *tat*, *rev*, *vpu* and *nef* have all been shown to be important for induction of protection against AIDS (Amara et al., 2001, 2002b; Ensoli and Cafaro, 2000; MacGregor et al., 2002; Singh et al., 2005), we retained these genes in both of the constructs used in this study. We tested both for infectivity and kinetics of viral protein production in transfected cell cultures and showed that the DNA expressed all of the viral proteins with kinetics similar to that of parental virus SHIV$_{KU2}$, but as expected, they did not produce infectious particles.

SHIV89.6P$_{-CY}$ was chosen as the challenge virus because it has dual tropism for the CXCR4 and CCR5 co-receptors used by HIV-1. Furthermore, this virus contains several of the HIV-1 proteins that make it relevant to study of HIV-1 pathogenesis. In its normal pathogenesis, SHIV89.6P causes rapid elimination of naive CD4$^+$ T cells present in peripheral blood and lymph nodes, and later, the memory CD4$^+$ T cells present in the gut-associated lymphoid tissues, spleen and lungs. In unpublished studies, animals that were partially protected against this virus were spared the precipitous loss of the naive CD4$^+$ T cells but succumbed to the slower disease with maintenance of high viral RNA concentrations in plasma. In the study reported here, the seven unvaccinated macaques challenged with this virus developed the typical precipitous loss of naive CD4$^+$ T cells from the blood, and this was maintained in four of the seven animals. One animal
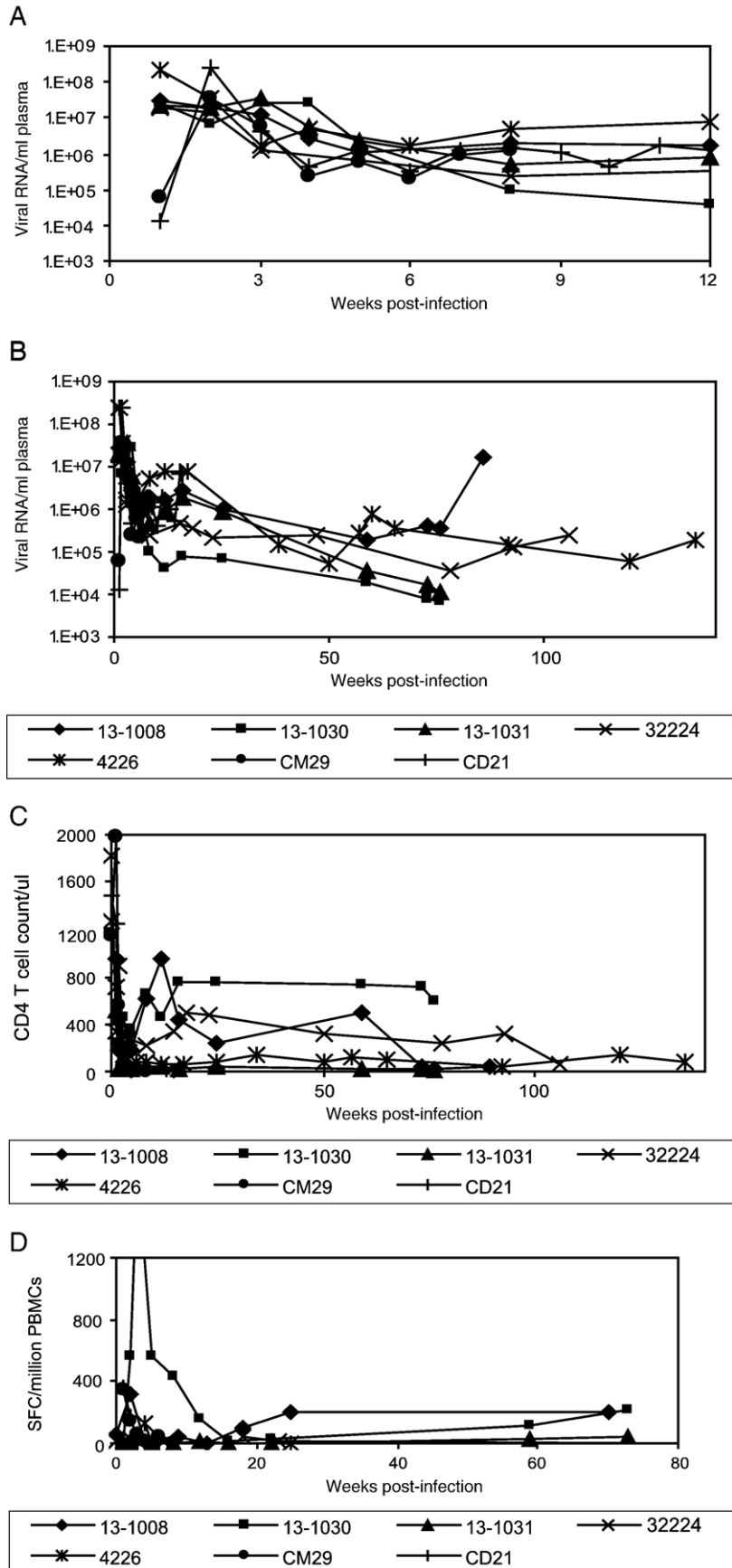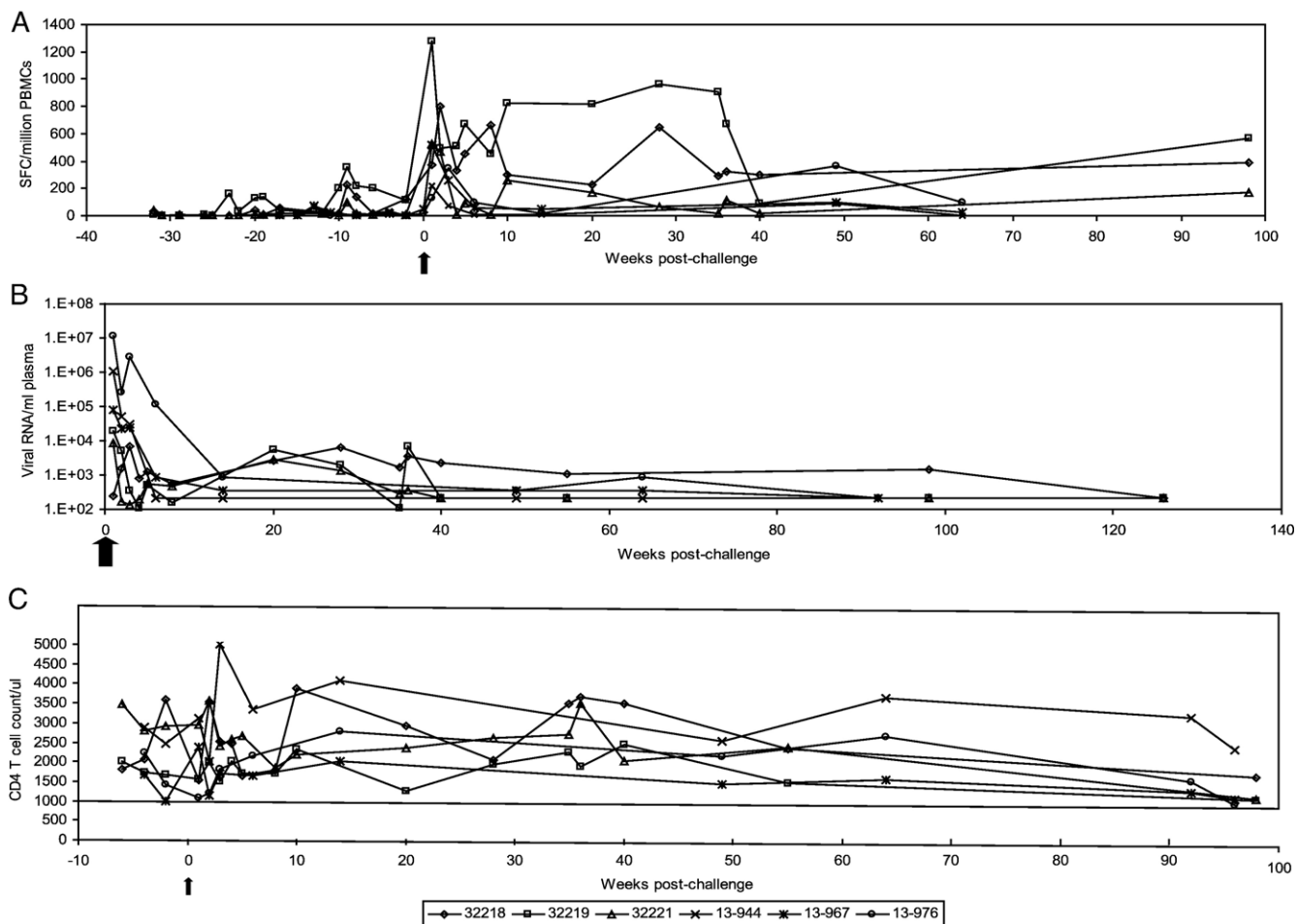
Fig. 4. (A) Pre- and post-challenge ELISPOT responses in macaques immunized with vaccine (M) DNA are presented as spot-forming cells (SFC) per million PBMC at weeks post-inoculation. (B) Plasma viral concentrations in macaques immunized with vaccine (M) DNA are presented as viral RNA copy numbers per ml plasma at weeks post-inoculation. (C) CD4+ T cell counts in vaccine (M) animals indicated by number of cells per μl blood. A horizontal line is shown at the level of 1000 CD4 cells per μl. For all graphs, the solid arrow represents the time of virus challenge.

maintained a count of 600 cells/ml blood. In contrast, all of the vaccinated animals resisted loss of naive CD4+ T cells, and at the end of the study in which all 12 challenged vaccinates remained healthy, all animals have greater than 796 cells/ml blood and negligible viral RNA in plasma at 2 years p.i.

The vaccines induced ELISPOT responses against peptides primarily in the Gag (SIV and HIV-1) in all of the twelve of the macaques that were immunized with the DNAs. The responses increased 5- to 20-fold following challenge and were associated with suppression of replication of the challenge virus. Decrease in virus replication was continuous. In the six animals immunized with the (M) DNA, viral RNA in plasma became undetectable by RT-PCR in four of the six by weeks 6–35, and in the other two, by week 92–126. There was a minimal loss of CD4+ T

cells during this period. These protective effects were a dramatic contrast to the virus control group.

Since it would be important to have a vaccine that could induce cross-clade protection, we decided to test immunogenicity of a construct expressing HIV-1 Gag–Nef in addition to the heterologous Env. This construct, (H) DNA, was used to immunize six macaques. Although all six became viremic following challenge with SHIV89.6P, three that were observed for the next several weeks developed a similar level of control of the challenge virus as the six animals that were immunized with the (M) DNA. In order to determine whether the vaccine could be administered safely to animals that were still viremic, and if vaccine would decrease viremia more rapidly, two doses of (M) DNA were administered sequentially to three of the six vaccinates. First, there was no evidence that the vaccine

Fig. 3. (A, B) Plasma viral RNA concentrations determined by real-time RT-PCR are presented as viral RNA copy numbers per ml plasma at weeks post-inoculation in virus control animals. Plasma viral RNA set points remained >10⁴ copies per ml throughout the study period in all seven animals. (C) CD4+ T cell counts assayed in a Beckton-Dickinson FACSCalibur flow cytometer and are represented as CD4+ T cells per μl blood. (D) ELISPOT cells were detected per million PBMCs at the indicated time points.
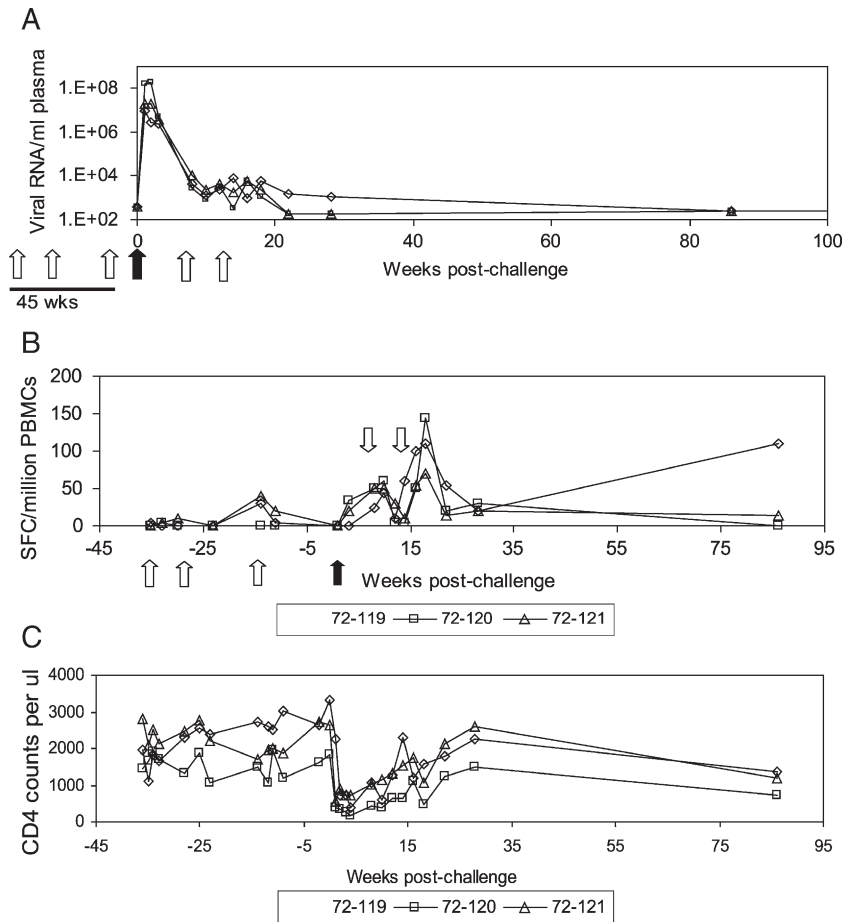
Fig. 5. (A) Plasma viral RNA concentrations in three macaques immunized with (H) DNA, challenged with SHIV89.6P$_{-CY}$, and given two post-challenge immunizations with (M) DNA. Immunizations are indicated by open arrows and challenge by solid arrow. (B) ELISPOT responses in the same macaques, represented by spot-forming cells per million PBMCs. Immunizations are indicated by open arrows, and challenge by solid arrow.

caused enhancement of virus replication. Furthermore, the three that received the post-challenge immunization completely controlled replication of challenge virus with approximately the same kinetics as the three that did not receive post-challenge immunizations. None of the animals developed AIDS nor did they lose CD4$^+$T cells since all six maintained cell counts above 500 per ml and showed undetectable viral RNA in plasma by week 135 post-challenge. Since most of the ELISPOT responses in immunized macaques were directed against Gag, protection induced by DNA encoding HIV-1 G*ag–Nef* against SHIV89.6P virus that contains an SIV *gag–nef* illustrated the breadth of efficacy of this type of vaccine DNA. Recent studies have shown that HIV-1 Gag has conserved cross-clade CD8 and CD4 epitopes that are recognized by humans and macaques (Amara et al., 2005). Efficacy of the (H) DNA, encoding *gag–nef* genes that were different from the challenge virus, corroborates this report.

In summary, data shown in this report indicate that a Δ4SHIV$_{KU2}$ DNA construct that expresses six proteins of the lentivirus under the lentiviral promoter has the potential for eliciting protection against challenge virus containing heterol-

ogous *env*, *gag*, and *nef* genes and that this protection could be induced by using the DNA alone without need for supplemental boosts with viral proteins.

Materials and methods

Cells and viruses

Human embryonic kidney 293 (HEK293) cells were used to assess protein expression capability of the Δ4SHIV$_{KU2}$ construct. These cells were also used to determine kinetics of production of viral Gag protein. Jurkat cells were used to test infectivity of this construct and were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10 mM HEPES buffer, pH 7.3, 2 mM glutamine, 5 μg/ml of gentamicin, and 10% fetal bovine serum. HEK293 cells were maintained in DMEM supplemented with 10 mM HEPES buffer, pH 7.3, 2 mM glutamine, 5 μg/ml of gentamicin, and 10% fetal bovine serum.

SHIV89.6P was obtained from Dr. Norman L. Letvin and was passage-adapted in our laboratory in cynomolgus monkeys (SHIV89.6P$_{-CY}$). The virus adapted in cynomolgus
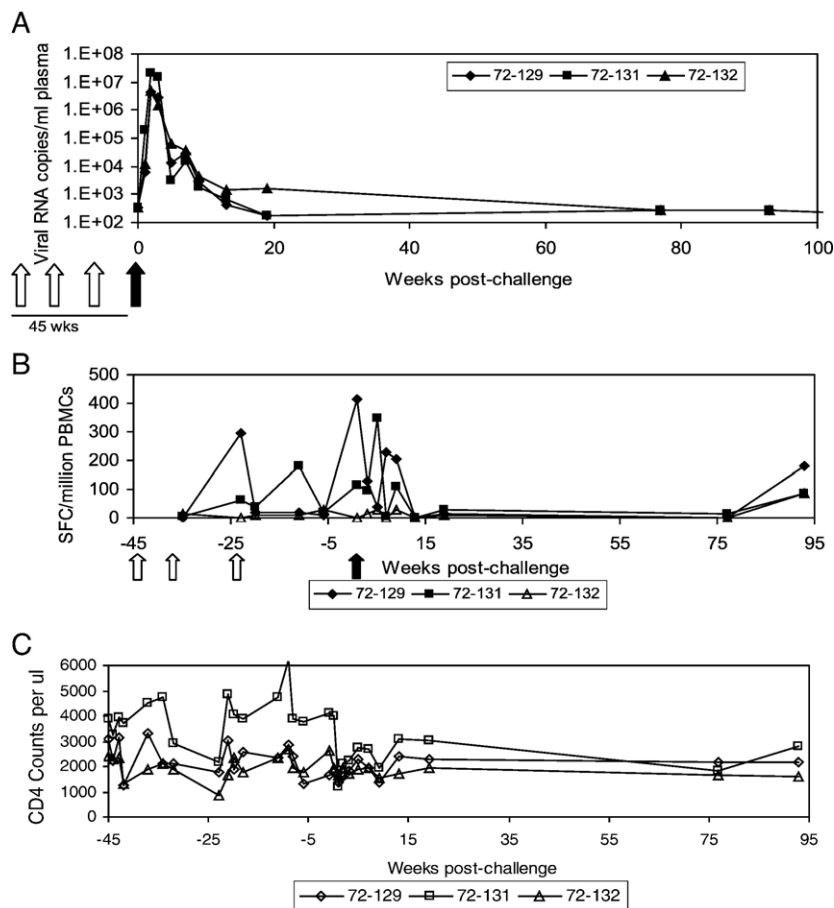
Fig. 6. (A) Plasma viral RNA concentrations in three macaques immunized with (H) DNA followed by challenge with SHIV89.6P$_{-CY}$. Immunizations are indicated by open arrows, and challenge by solid arrow. (B) ELISPOT responses in the same macaques. Immunizations are indicated by open arrows, and challenge by solid arrow.

monkeys has been used to challenge vaccinated macaques. This cynomolgus-adapted virus (SHIV89.6P$_{-CY}$) can utilize the CCR5 co-receptor very well in culture, as shown by assays on Ghost cell Hi-5 cells (data not shown; Smith et al., unpublished). Stocks of the viruses were prepared in concanavalin-A-treated, CD8 T cell-depleted macaque PBMC cultures and stored frozen at −80 °C and in liquid nitrogen.

Construction of Δ4SHIV$_{KU2}$(M) plasmid DNA that contained HIV-1 (HXB2) env and SIV gag and nef and Δ4SHIV$_{KU2}$(H) that contained the same genes of (M) except for the gag and nef of HIV-1$_{SF2}$

The construction procedures of SHIV$_{KU2}$ plasmid DNA have been described earlier (Liu et al., 1999), and sequences have been submitted to GenBank (GenBank data base accession number AY751799). Construction of Δ*rt*SHIV$_{KU2}$ has been described previously Singh et al., 2005. This plasmid was used to derive Δ4SHIV$_{KU2}$ (M) that contains HIV-1 (HXB2) *env* and SIV *gag–nef* and another construct Δ4SHIV$_{KU2}$ (H) that contains HIV-1$_{SF2}$ *gag–nef* (Fig. 1).

The deletion of *integrase* and *vif* from Δ*rt*SHIV$_{KU2}$ was performed in two steps. First, a *Bst*EII site was created immediately before the start codon of *vpx* gene at nucleotide position 5042. A PCR fragment was amplified from this plasmid using the forward primer 5′-**GGTCACC**ATGTCA-GATCCCAGGGAGAG-3′ at the position 5042–5068 (the bold letters represent the created *Bst*EII restriction site) and reverse primer 5′-CTCTTAATTT**GCTAGC**TATCTG-3′ at the position 7125–7146 (the bold letters represent the *Nhe*I site of Δ*rt*SHIV$_{KU2}$). This produced a fragment of 2089 bp that was used below. In second reaction, a 3817 bp fragment starting from *Bst*EII site in *pol* gene at nucleotide position 3316 to *Nhe*I site at position 7132 was replaced by the 2089 bp fragment from the first step. Next, a series of PCR and cloning steps were performed to substitute the 3′ LTR with SV-40 poly A sequences. Briefly, a 2337 bp PCR fragment was amplified from Δ*rt*SHIV$_{KU2}$ using the forward primer 5′-**GCTAGC**AAATTAAGAGAAC-3′ at the position 7131–7149 (bold letters represent the original *Nhe*I site) and reverse primer 5′-**GTCGAC**TCAGCGAGTTTCC-3′ at the position 9452–9470 (bold letters represent the created *Sal*I site). This fragment contained the *nef* and part of the *env* of Δ*rt*SHIV$_{KU2}$. Next, the 2743 bp fragment starting from the

*Nhe*I site at position 7132 encompassing part of *env*, *nef*, and 3′ LTR was substituted by the 2337 bp PCR fragment lacking the 3′LTR from the previous step.

The sequences for the vector were derived from plasmids pET-9a (Novagen, San Diego, CA) and pCMV-Tag2 (Stratagene, La Jolla, CA). The 1.8 kb *Bam*HI–*Xmm*I fragment of pET-9a was substituted by the 547 bp *Bam*HI–*Ssp*I fragment of pCMVtag2, such that the resultant 2.5 kb plasmid contained the SV40 polyadenylation signal sequences and kanamycin resistance gene in the final clone.

Construction of $\Delta 4SHIV_{KU2}$ (H) plasmid DNA that contained HIV-1$_{SF2}$ gag–nef

To replace SIV *gag* and *protease* genes with those of HIV-1$_{SF2}$, PCR was performed using forward primer 5′-AATCTC-TAGCAGT**GGCGCC**CGAAC-3′ at position 640 (the bold letters represent the original *Nar*I site of HIV-1$_{SF2}$) and reverse primer 5′-GCT**GGTGACC**CTTTCCATCCCTG-3′ at position 3007 downstream of HIV-1$_{SF2}$ protease coding sequences (bold letters represent the *Bst*EII site created by site-directed mutagenesis). This 2368 bp *Nar*I–*Bst*EII PCR fragment was used to replace the corresponding 2486 bp *Nar*I–*Bst*EII fragment of $\Delta 4SHIV_{KU2}$ (M). These positions are mentioned with reference to GenBank database accession number K02007).

To substitute SIV *nef* with that of HIV-1$_{SF2}$, a series of PCR and cloning steps were used. Briefly, an intermediate containing part of SHIV$_{KU2}$–gp41 was made using the forward 5′-GTGAAC**GGATCC**TTGGCAC-3′ (bold letters represent the original *Bam*HI site) and reverse 5′-**GATATC**T-TATAGCAAAATCCTTTCC-3′ (bold letters represent the created *Eco*RV site) primers. The resulting PCR fragment was cloned into pGEM-T (Promega). The HIV-1$_{SF2}$ *nef* was amplified by PCR in a 1.4 kb fragment containing a *Not*I site at the 5′ end and *Sal*I at the 3′ end, using the forward 5′-**GCGGCCGC**ATGGGTGGCAAGTGGTC-3′ and reverse 5′-**GTCGAC**TCAGCAGTCTTTGTAGTAC-3′ primers. The HIV-1$_{SF2}$ *nef* was then fused at the end of SHIV$_{KU2}$-gp41. The 1 kb *Bam*HI–*Sal*I fragment of this clone was used to replace that of $\Delta 4SHIV_{KU2}$ (M). The resultant construct $\Delta 4SHIV_{KU2}$ (H) contains the *gag*, *pro*, partial *rt*, and *nef* of HIV-1$_{SF2}$.

Transfection studies

Transfection of HEK293 cells with the (M) and (H) DNAs was carried out using a cationic polymer polyethylenimine (ExGen 500, MBI Fermentas, Hanover, MD) according to the manufacturer's instructions. Approximately, $1-3 \times 10^5$ cells/well in six-well plates were transfected with 4.75 µg of vaccine DNA and 15.5 µl of ExGen 500, corresponding to 6 equivalents. Culture supernatant fluids were assayed on days 1 and 2, and every 2 days thereafter, until day 14, to assess kinetics of synthesis and duration of production p27 or p24. Another set of transfected cultures was used for immunoprecipitation of different viral proteins.

Quantitation of p27/p24 in the supernatant fluids of transfected cultures

Supernatant fluids were analyzed by a capture enzyme-linked immunosorbent assay (ELISA) kit (Coulter Laboratories, Hialeah, FL) for quantification and duration of secretion of the two Gag proteins. A standard curve was prepared for each assay according to manufacturer's instructions, and p27/p24 concentrations were determined from the OD$_{450}$ using linear regression analysis.

In vitro infectivity assay

Supernatant fluids from transfected HEK293 cells were harvested on days 2, 4, and 6 after transfection, and 1 ml of the fluid was inoculated into three cultures of Jurkat cells. The inoculated cultures were incubated at 37 °C and observed for the development of fusion cytopathic effects (CPE). At 10 days, supernatant fluids were tested for the presence of viral p27, and cell lysates were analyzed for viral DNA by PCR. Infectious SHIV$_{KU2}$ DNA-transfected Jurkat and HEK293 cells served as positive controls in this experiment.

Analysis of inoculated Jurkat cells for viral DNA

DNA was extracted using Qiagen DNA reagents (Valencia, CA) from Jurkat cells 2 days after transfection and from PBMCs of immunized macaques a few days after each immunization. DNA copy numbers were determined by real-time PCR using SIVmac239-derived *gag* primers and Taqman probe (Hofmann-Lehmann et al., 2000; Smith et al., 2002) and the Taqman Universal PCR Mastermix (Applied Biosystems, Foster City, CA) in duplicate 25 µl reactions in the ABI PRISM 7700 Sequence Detection System. Proviral copy numbers were normalized to the quantity of total cellular DNA used in the reaction. DNA real-time conditions were as described Smith et al., 2002. Serial 10-fold dilutions of cloned SHIV *gag* plasmid over six orders of magnitude were used as standards. The minimum detectable level of proviral DNA was 30 copies.

Radio-immunoprecipitation assay

We analyzed transfected cultures by standard radio-immunoprecipitation of ^{35}S-labeled proteins using normal macaque and human sera as controls and serum from an SIV-infected macaque and an HIV-infected person as sources of antiviral antibodies. Autoradiographs were analyzed for the presence of different bands corresponding to Env gp160, gp120, gp41, and Gag p55 and p27/p24.

Immunization of macaques with (M) DNA and challenge with SHIV89.6P$_{-CY}$

Nineteen cynomolgus macaques were used in this study. The animals were prescreened serologically for SIV and STLV and individually housed at an Association for Assessment and Accreditation of Laboratory Animal Care-International

accredited facilities of the University of Kansas Medical Center and maintained in accord with the guidelines of the *Guide for the Care and Use of Laboratory Animals* (National Research Council, National Academic Press, Washington, DC, 1996). Animals were divided in three groups. The first group of six macaques received 5 mg of (M) DNA, IM, twice, 11 weeks apart. The vaccine plasmid DNA was suspended in 1 ml of PBS and injected at two sites. The second group of six macaques was injected three times with the same amount of (H) DNA. Three macaques from this group received two post-challenge boosts of 5 mg of (M) DNA at 8 weeks and 13 weeks. Seven animals in the third group served as non-vaccinated virus controls. All animals were challenged with SHIV89.6P$_{-CY}$ using 1 ml of stock virus containing 10^4 TCID, given IV. This virus uses CCR5 well in Ghost cell assays and, as expected, can replicate in both normal human donor and Δ32CCR5 human PBMCs (M. Smith et al., unpublished). It has been widely used as the challenge virus in macaques vaccinated with plasmid DNA and other agents. While this virus can cause rapid CD4 loss after macaque infection, thus providing a rapid assessment of vaccine efficacy, we use it with the caveat that it may not recapitulate all aspects of solely CCR5-tropic HIV-1 in primary human infection. Animals were analyzed for CMI, CD4$^+$ T cell counts, and viral RNA copies in plasma.

Processing of blood and serum samples

Peripheral blood collected in EDTA was centrifuged to separate plasma and buffy coat. Plasma samples were immediately stored at −80 °C for later measurement of viral RNA concentrations using real-time RT PCR. PBMCs were purified from buffy coats by centrifugation through Ficoll-Paque density gradients. Portions of the cells were used for flow cytometry (FACS), detection of viral DNA by PCR, and ELISPOT assays, respectively.

Assessment of plasma viral RNA concentration using quantitative real-time RT-PCR analysis

Plasma viral RNA concentrations were measured from RNA extracted from 800 to 1000 µl of plasma samples as previously described (Mackay et al., 2002; Smith et al., 2002). RNA samples were subjected to real-time RT-PCR using *gag* primers and a 5′FAM- and 3′TAMRA-labeled Taqman probe measuring SIVmac239 *gag*, as described by Hofmann-Lehmann et al. (2000) and Smith et al. (2002).

FACS analysis

PBMCs from vaccinated and control macaques were subjected to FACS analysis at different time intervals for quantitation of CD3$^+$, CD4$^+$, and CD8$^+$ T-cells as described earlier (Kumar et al., 2001). Briefly, 5 µl of a 3-color anti-CD3$^+$, -CD4$^+$, and -CD8$^+$ antibody mix (Becton-Dickinson, Ruther-ford, NJ) was added to 100 µl of whole blood and incubated for 60 min in the dark. Lysing solution (Becton-Dickinson) was then added, and the samples were incubated for another 20 min at room temperature. Stained cells were fixed with 1% formalin and analyzed in a Becton-Dickinson FACSCalibur flow cytometer.

ELISPOT assay

We used a quantitative ELISPOT assay that measured IFN-γ production by PBMC responding to groups of overlapping 15-mer peptides, with 11 amino acid overlaps, spanning the entire molecule of selected viral proteins. We used the following peptide groups represented in SHIV and constructs: HIV-1-Env (MN) (Cat. No. 6451), consensus HIV-1 Tat (Cat. No. 5138), consensus HIV-1 Rev (Cat. No. 6445), SIVmac239 Gag (Cat. No. 6204), SIV Nef (Cat. No. 6206), HIV-1 Gag (Cat No. 7872–7904), and HIV-1 Nef (Cat No. 5139–5187), all kindly provided by the AIDS Research and Reagent Program. Pools of approximately 20 peptides were used in the assays. Env was divided into 10 and Gag into 5 pools. There were a total of 23, 27, and 21 peptides, respectively, for the Tat, Rev, and Nef, and each was made into a single pool. Peptide pools were aliquoted and stored at −80 °C at a concentration of 1 mg/ml. The assay protocol was reported in detail in an earlier publication (Letvin et al., 1997). Spots were counted with a stereo-microscope and reported as the number of spots/10^6 PBMC.

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