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DNA immunization in combination with effective antiretroviral drug therapy controls viral rebound and prevents simian AIDS after treatment is discontinued

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Abstract

DNA immunization in conjunction with antiretroviral therapy was evaluated in SIV-infected rhesus macaques treated with [R]-9-[2-phosphonylmethoxypropyl]adenine (PMPA). Macaques were immunized monthly with DNA vaccines expressing either SIV *gag/tat* or SIV *gag/tat* and 19 CD8+ T cell epitopes during 7 months of therapy. Half the animals from each group were additionally immunized before infection. Only 60% of the animals (4 controls, 20 vaccinated) responded to PMPA (ART responders). All 4 ART responder controls demonstrated viral rebound or CD4 decline after PMPA was withdrawn. In contrast, 17 of 20 vaccinated ART responders contained viral rebound for over 7 months after PMPA was withdrawn. Viral control correlated with stable CD4 counts, higher lymphoproliferation and an increase in the magnitude and breadth of the CD8+ T cell response. Immunizing before infection or with multi-epitopes enhanced these effects. These results demonstrate that DNA immunization during antiretroviral therapy may be an effective strategy to treat HIV infection.

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Keywords: Immunotherapy; Antiretroviral therapy; DNA vaccine; SIV AIDS

Introduction

Despite considerable progress, an effective vaccine against HIV is not yet available, and clinical management of AIDS currently depends on long-term treatment with a cocktail of antiretroviral drugs (highly active antiretroviral therapy; HAART). Although HAART has proven to be effective in decreasing symptoms and prolonging life in patients infected with HIV-1 (UNAIDS, 2000), the long-term benefits are limited by the emergence of drug resistant strains (Pillay et al., 2000), complex dosing schedules, toxicity (Carr et al., 1998; Vigouroux et al., 1999), and the failure to deplete viral reservoirs (Chun et al., 1997; de Jong et al., 1997; Staszewski et al., 1998).

Abbreviations: ART, antiretroviral therapy; HAART, highly active antiretroviral therapy; PMPA, [R]-9-[2-phosphonylmethoxypropyl]adenine; HBcAg, hepatitis B virus core antigen; PMED, particle-mediated epidermal delivery; PBMC, peripheral blood mononuclear cells; LPR, lymphoproliferative response; CTL, cytotoxic T lymphocyte.

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Recent strategies to improve long-term treatment of HIV-1 infection have focused on immunotherapeutic intervention as an adjunct to HAART. HAART reduces plasma viremia and partially restores immune function in HIV-infected patients (Autran et al., 1997, 1999; Emery and Lane, 1997; Valdez et al., 2000). Immune restoration during HAART is characterized by a rise in CD4+ T cell counts (Autran et al., 1997, 1999; Emery and Lane, 1997), reconstitution of antibody and T cell responses to recall antigens (Kim et al., 2001; Valdez et al., 2000), and partial restoration of HIV-specific antibody responses (Kim et al., 2001). HIV-specific CTL and CD4+ T helper cells play a critical role in controlling HIV replication and determining viral set point (16–18, 20, 21, 24, 27). However, depletion and/or anergy of HIV-specific CD4+ T cells, which are required to sustain CD8+ T cell antiviral activity (Janssen et al., 2003; Matloubian et al., 1994; Shedlock and Shen, 2003; Sun and Bevan, 2003; Zajac et al., 1998), occurs during the earliest stages of infection (Douek et al., 2003) and HAART is unable (Casazza et al., 2001; Ghanekar et al., 2001; Kalams et al., 1999; Valdez, 2002) or under certain conditions, provides only partial restoration of these responses (Lopez et al., 2004). These findings suggest that improving HIV-specific T cell responses during HAART by immunotherapeutic intervention may be an effective strategy to improve treatment of HIV infection. In particular, immunizing infected patients with vaccines that boost HIV-specific CTL and T helper cell responses after HAART has reduced the virus burden may restore these responses to levels that can better control HIV during HAART or even in the absence of HAART.

DNA vaccines induce virus-specific CD4+ and CD8+ T cell responses (Donnelly et al., 1997), and have been extensively studied in the rhesus macaque model for AIDS. Challenge studies have shown that DNA vaccines administered either alone or in combination with recombinant viral vaccines can provide complete or partial protection against avirulent and homologous, pathogenic AIDS viruses (Amara et al., 2001; Barouch et al., 2000; Boyer et al., 1997; Kent et al., 1998; Robinson et al., 1999). In addition, we have previously shown that delivery of DNA vaccines directly into the cells of the skin using a particle mediated delivery device or gene gun can also provide significant protection against challenge with a primary, heterologous SIV isolate (Fuller et al., 2002). Since effective treatment of an established HIV infection will need to target diverse viral variants, this result suggests that DNA vaccination may be a promising approach for immunotherapy as well. Here, we investigated two DNA vaccines in rhesus macaques infected with a heterologous primary isolate of SIV for their ability to augment virus-specific T cell responses and control viremia after discontinuation of monotherapy with the nucleoside inhibitor, [R]-9-[2-phosphonylmethoxypropyl]adenine (PMPA).

Results

Study design

An effective immunotherapeutic vaccine will likely need to induce multiple specific CTL responses to enable targeting of diverse viral isolates. We constructed an epitope-based DNA vaccine encoding 19 Mamu-A*01-restricted SIV_{mac239}-specific CTL epitopes using an approach wherein the epitope sequences are inserted into the gene encoding hepatitis B core antigen to enhance epitope immunogenicity (Lesinski et al., 2001). The epitopes were selected by their ability to bind the Mamu-A*01 MHC class I molecule and be recognized in SIV-infected macaques (Table 1) (Allen et al., 2001). The HBc-epitope vaccine was combined with whole gene DNA vaccines encoding SIV_{mac239} *gag* and *tat* to provide SIV-specific T cell help (*gag/tat*/multi-epitope vaccine). This vaccine and separately, the *gag/tat* DNA vaccine alone (*gag/tat* vaccine) were evaluated for immunogenicity and therapeutic efficacy in the SIV rhesus macaque model for AIDS. Both DNA vaccines were administered using particle-mediated epidermal delivery (PMED), a method that has been previously shown to be highly effective for inducing immune responses in both nonhuman primates (Fuller et al., 1997a, 2002; Kent et al., 1998) and humans (McConkey et al., 2003; Roberts et al., 2005; Rottinghaus et al., 2003; Roy et al., 2000).

Sixteen animals were immunized both before and after infection to test the effects of vaccine-priming on post-infection immunotherapy (Fig. 1, Groups A and B). A second group of sixteen animals were immunized only post-infection during ART to test the effect of immunizing after infection was established (Fig. 1, Groups C and D). Eight animals in each group were immunized with the *gag/tat*/multi-epitope DNA vaccine (Groups A and C), and the remaining 8 received the *gag/tat* vaccine (Groups B and D). Mamu-A*01-positive monkeys

Table 1
Conservation between SIVmac239 CTL epitopes in the *gag/tat*/multi-epitope DNA vaccine and the heterologous primary isolate SIV/DeltaB670 or similar H9 consensus sequence as indicated

CTL epitopes in multi-epitope DNA vaccine	Sequence	Conservation of epitopes[a]
Gag_CM9	CTPYDINQM	Y → S (H9)
Tat_SL8[b]	STPESANL	1st S → D, N → S
Env_SI9	SWPWQIEYI	100%
Vif_QA9	QVPSLQYLA	100%
Pol_LV10	LGPHYTPKIV	H → N (H9)
Env_ST10	SPPSYFQTHT	Highly variable
Env_CL9	CAPPGYALL	100%
Pol_YI9	YTPKIVGGI	100%
Pol_EA11	EAPQFPHGSSA	SSA → SDA (H9)
Gag_VT10	VNPTLEEMLT	V → M (H9)
Pol_SV9	STPPLVRLV	100%
Pol_QF10	QMPRQTGGFF	Highly variable
Vif_VI8	VTPDYADI	I → T
Tat_TL8*	TTPESANL	1st T → D, N → S
Pol_IL10	IYPGIKTKHL	100%
Env_TL9	TVPWPNASL	AS → DT
Pol_MI8	MTPAERLI	I → V
Pol_QV9	QVPKFHLPV	K → R
Gag_LA9	LAPVPIPFA	Highly variable
Pol_GM10	GSPAIFQYTM	Y → H

[a] → indicates the amino acid differences between CTL epitopes included in the vaccine and SIV/DeltaB670 or H9 consensus sequences.

[b] Since recognition of Tat_SL8 and Tat_TL8 by PBMC from infected macaques is indistinguishable, they are considered the same epitope.

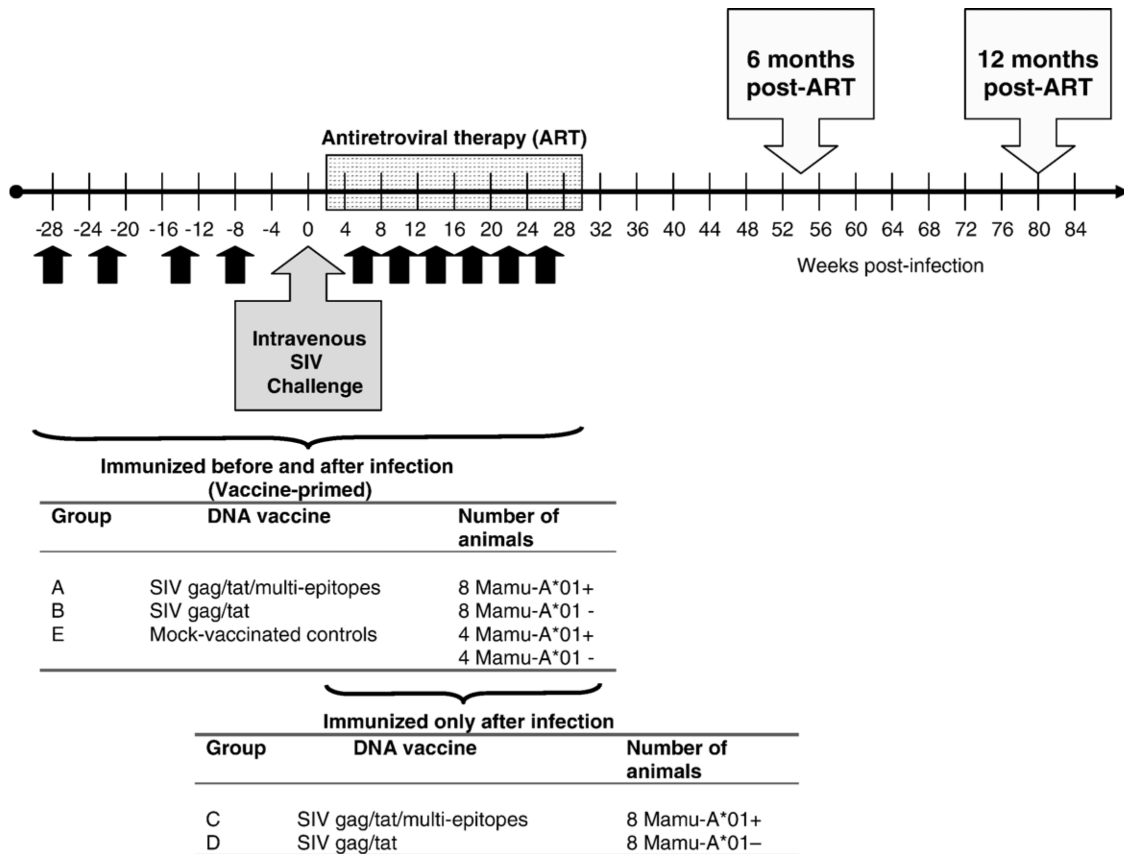

Fig. 1. Study design. Arrows indicate each DNA immunization. Each dose consisted of 32 μg of DNA of either the SIV *gag/tat*/multi-epitope or *gag/tat* DNA vaccine. Animals were inoculated intravenously with 10 $TCID_{50}$ of SIV/DeltaB670. Antiretroviral therapy (ART) consisted of 20 mg/kg/day of the nucleophosphonate analog R-9-(2-phosphonylmethoxypropyl) adenine (PMPA) and was administered without interruption during weeks 2–30 post-infection. PMPA was withdrawn at week 30 to assess the effects of vaccination on viral control. Vaccine efficacy was evaluated at 6 and 12 months post-PMPA.

were used for testing the *gag/tat/*multi-epitope vaccine because expression of this molecule is required for recognition of the epitopes and Mamu-A*01-negative monkeys were used to test the *gag/tat* vaccine. To control for possible influences of the Mamu-A*01 allele, the ART-treated control group consisted of 4 Mamu-A*01-positive and 4 Mamu-A*01-negative animals (Group E). Mock vaccinations in this group consisted of immunizations before and after infection with the empty HBcAg carrier vector.

Pre-infection immune responses in vaccine-primed animals

Vaccine-primed animals received 4 DNA immunizations at 6–8 week intervals prior to infection. To assess the immunogenicity of the vaccines, SIV-specific CD8+ and memory T cell response were analyzed by ELISPOT and lymphoproliferation (LPR) assays, respectively, in monkeys immunized prior to infection (Fig. 2).

SIV gag and tat-specific LPR were measured by stimulating with recombinant SIV p28 and purified viral SIV *tat* protein. The *gag/tat*/multi-epitope and *gag/tat* vaccines induced comparable LPR in Mamu-A*01-positive (Fig. 2A) and -negative macaques (Fig. 2B), respectively. SIV-specific CD8+ T cell responses were measured by depleting PBMC of the CD4+ T cell subset prior to ELISPOT analysis. In Mamu-A*01-positive macaques, CD8+ T cell responses were measured against 8 representative CTL epitopes included in the multi-epitope vaccine using specific peptides for stimulation. In Mamu-A*01-negative macaques, CD8+ T cell responses were measured against overlapping peptide pools from SIV Gag and Tat. Both DNA vaccines induced significant levels of SIV-specific CD8+ T cells (Figs. 2C and D). Immunization of Mamu-A*01-positive macaques with the *gag/tat*/multi-epitope vaccine induced significant CD8+ T cell responses to multiple epitopes in all 8 macaques, and strong responses against the immunodominant Gag_CM9 and Tat_SL8 epitopes (Allen et al., 2001) (Fig. 2C). Immunization of Mamu-A*01-negative macaques with the SIV *gag/tat* vaccine induced predominantly a Tat-specific CD8+ T cell response (Fig. 2D). Maximum CD8+ T cell responses were similar in both vaccine groups, but the *gag/tat/*multi-epitope vaccine appeared to provide more consistent induction of CD8+ T cell responses among all 8 animals. Among the animals immunized with the *gag/tat* DNA vaccine, 3 macaques (M11999, M12299, M12599) showed CD8+ T cell responses that were at or below the positive threshold (25 SFC/10^6 PBMC) for this assay. However, these same 3 animals exhibited significant proliferative responses against whole Gag and Tat proteins (Fig. 2B) indicating that the *gag/tat* DNA vaccine effectively primed for an immune response in each

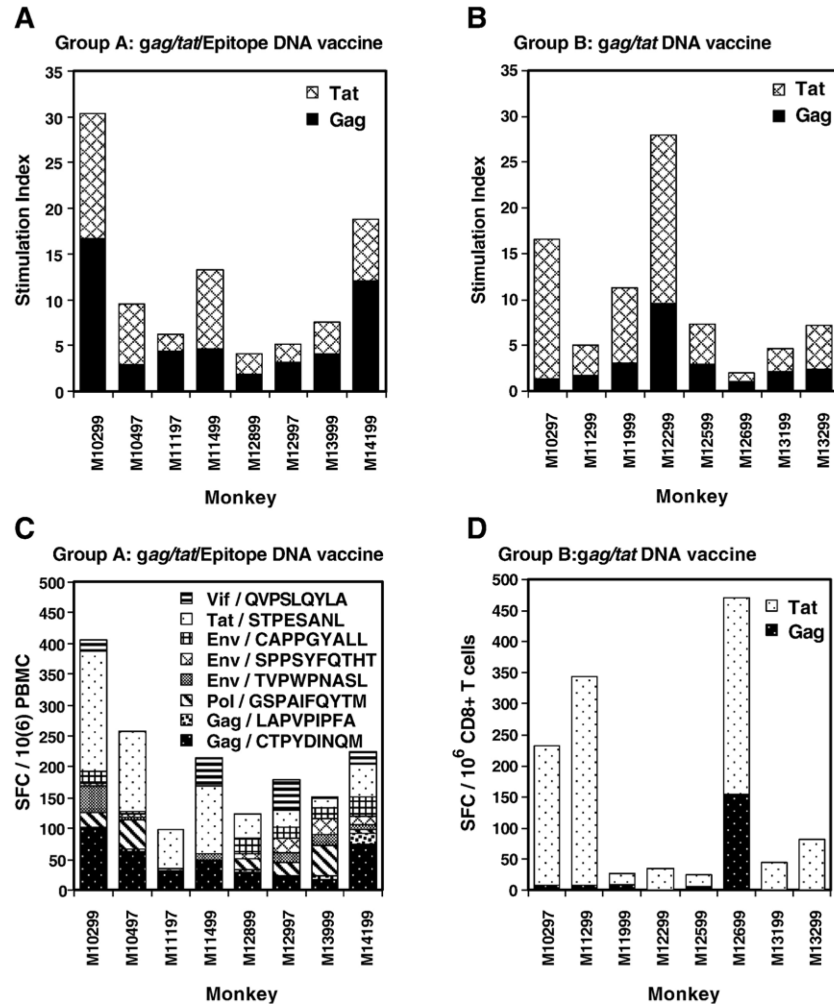

Fig. 2. SIV-specific T cell responses in vaccine-primed rhesus macaques following 4 pre-infection DNA immunizations. (A) Lymphoproliferative responses in Mamu-A*01-positive monkeys immunized with the SIV*gag/tat/*multi-epitope DNA vaccine (Group A). (B) Lymphoproliferative responses in Mamu-A*01-negative macaques immunized with the SIV*gag/tat* DNA vaccine (Group B). (C) CD8+ T cell responses in Mamu-A*01-positive macaques primed with the SIV gag/tat/multi-epitopes DNA vaccine (Group A). (D) CD8+ T cell responses in Mamu-A*01-negative macaques primed with SIV gag/tat DNA vaccine (Group B). CD8+ T cell responses were measured by ELISPOT and data shown are net values after background subtraction. In all cases, background levels were less than 25 SFC/10^6 PBMC.

macaque but the magnitude and the type of response varied between animals, an outcome that is not unexpected in an outbred species such as the rhesus macaque. Together, these data demonstrate that both DNA vaccines induced significant levels of SIV-specific CD8+ and CD4+ T cell responses in the monkeys that were vaccine-primed prior to infection.

SIV infection and anti-retroviral therapy

Seven weeks after the final pre-infection DNA immunization, all animals and 4 additional untreated infection controls, were inoculated intravenously with the primary isolate SIV/DeltaB670 (Fig. 1). SIV/DeltaB670 is a highly pathogenic and causes AIDS in untreated rhesus macaques with a mean time to death of 11 months (Seman et al., 2000). This strain was chosen because the genetic diversity between this isolate and the SIV_{mac239} and $SIV_{17E\text{-}Fr}$ strains used to construct the DNA vaccines, mimics the intraclade diversity observed for HIV (Amedee et al., 1995). Despite this diversity, alignment of the CTL epitope sequences in the *gag/tat*/multi-epitope DNA vaccine to those from the dominant variant found in SIV/DeltaB670 or the closely related SIVmmH9 consensus sequence revealed that most of the epitopes were highly conserved in the challenge strain. Fourteen of the 19 epitopes either contained only a single amino acid substitution or were 100% conserved (Table 1). Monkeys were infected by intravenous inoculation because we had previously shown that a PMED DNA vaccine provided protection from an intrarectal challenge with this virus in nearly 60% of the vaccinated macaques (Fuller et al., 2002) and it was imperative to establish a disseminated infection in all animals.

ART with the nucleophosphonate analog R-9-(2-phosphonylmethoxypropyl) adenine (PMPA) (Tsai et al., 1995) was initiated 2 weeks after infection, administered daily during the 6 monthly therapeutic DNA immunizations, and then withdrawn to assess the effects of vaccination on viral control (Fig. 1). We chose to use a less aggressive monotherapy than the

potent combination HAART drug cocktail typically used for treatment of HIV because a previous study employing a triple combination of PMPA, d4T, and DDI initiated at the same time point resulted in significant viral containment in mock-vaccinated control animals once therapy was discontinued, a result that precluded statistical analysis of the augmentive effects of immunization (Hel et al., 2000). In contrast, an earlier experiment in our laboratory demonstrated that PMPA alone when initiated during acute infection with SIV/DeltaB670 did not prevent viral rebound after the drug was discontinued (data not shown). Furthermore, macaques infected with SIV/DeltaB670 demonstrate a shortened survival time when compared to humans infected with HIV (Mellors et al., 1996; Riddler and Mellors, 1997; Sauermann et al., 2000; Seman et al., 2000; Staprans et al., 1999; Watson et al., 1997), suggesting more rapid development of immune dysfunction during acute infection in the macaque model. We therefore reasoned that initiating ART with a single drug during acute infection would be more similar to initiating HAART with multiple drugs in HIV-infected humans during later stages of infection.

Control of virus burden during and after ART

Viral loads were measured during therapy and for over 1 year after discontinuation of PMPA. SIV/DeltaB670 infection resulted in comparable peak viral loads in all groups by 2 weeks post-infection (Fig. 3). We previously showed that DNA vaccination prior to infection suppressed acute viremia in animals challenged intrarectally with SIV/DeltaB670 (Fuller et al., 2002). In the present study, however, animals immunized before and after infection (Groups A and B) demonstrated similar viral loads at 2 weeks post-infection as animals that were not vaccinated prior to infection (Groups C, D, PMPA controls, and naive controls) (Fig. 3) suggesting

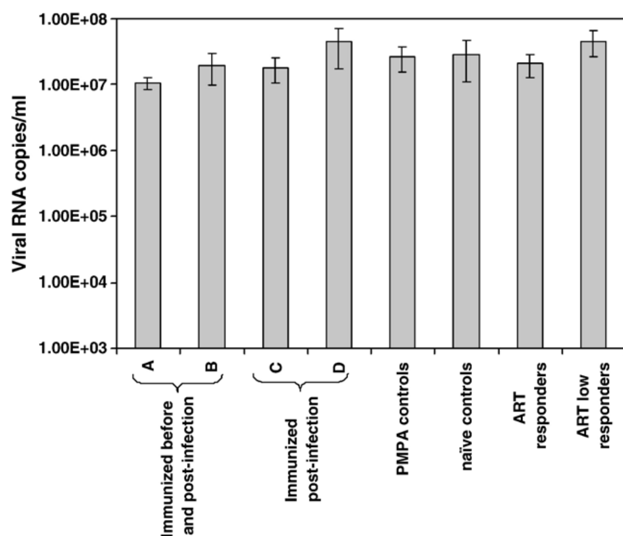

Fig. 3. Mean peak acute viral loads and SEM per group at 2 weeks post-infection. Mean viral loads were determined in each vaccine and control group and in ART responders and ART low responders that were not vaccinated prior to infection (from Groups C–E).

that prophylactic DNA immunization may be less effective in controlling acute SIV replication following an intravenous infection.

The effects of vaccination on viral control were examined in terms of the ability to contain viral rebound at a level equal to or lower than 10^4 viral RNA copies/ml for a period of at least 6 months after PMPA was withdrawn. This level was selected because it is comparable to the mean viral load observed in a group of 3 rare long-term nonprogressors from a separate study in our laboratory that were infected with a lethal dose of SIV/DeltaB670 but maintained normal CD4 T cell numbers and clinical health for over 2–5 years in the absence of drug treatment (data not shown). Other studies have similarly shown that durable antiretroviral or immune suppression of SIV at a level equal to or below 10^4 RNA copies/ml prevents or delays progression to AIDS in rhesus macaques (Fuller et al., 1997b; Hel et al., 2000; Lifson et al., 2000; Patterson et al., 2004; Silvera et al., 2000). Since the goal of vaccine therapy is improvement in clinical health, we reasoned that vaccine-induced containment of viremia at this level after PMPA was withdrawn would similarly prevent or delay disease progression.

Naive controls infected with SIV/DeltaB670 developed viral set points ranging from 10^6 to 10^7 viral RNA copies/ml (Fig. 4A). In the PMPA-treated animals (Fig. 4B), viral loads ranged from undetectable levels to over 10^7 viral copies/ml during PMPA treatment and increased to levels that ranged from 2.5×10^4–10^7 viral RNA copies/ml in 7 of the 8 mock-vaccinated controls within the first 6 months after PMPA was removed. Among the 32 vaccinated animals, 17 (53%) contained viral loads at levels below 10^4 RNA copies/ml (undetectable levels—9600 viral RNA copies/ml) for at least 6 months after PMPA was withdrawn and 13 (41%) of these animals continued to control plasma viremia at this level for 13 months (the duration of the study) in the absence of drug treatment (Figs. 4C–F).

However, PMPA treatment suppressed viral load in only 60% (24 of 40) of the animals (Figs. 4B–F, red plots). These animals (ART responders) demonstrated a progressive 3–6 log-fold reduction in viral load during PMPA treatment that ranged from below the limits of detection (10 copies/ml) to 5000 copies/ml by the end of the 6-month treatment period (week 30). The remaining 16 animals responded relatively poorly to ART (ART low responders) demonstrating, at most, a 2-fold log reduction in viral load in response to therapy and viral loads that were 10^5–10^7 copies/ml during and after the PMPA treatment period (Figs. 4B–F, black plots) and similar to that observed in the naive controls throughout the study (Fig. 4A).

ART responders and low responders demonstrated comparable viral loads at 2 weeks post-infection (Fig. 3) indicating PMPA responsiveness was not related to the degree of viral replication present at the time drug was initiated. There was also no significant difference in the number of A*01-positive (14/20) and -negative animals (11/20) that responded to PMPA treatment indicating that ART responsiveness was not related to the A*01 allele ($P = 0.514$ by Fisher's Exact Test).

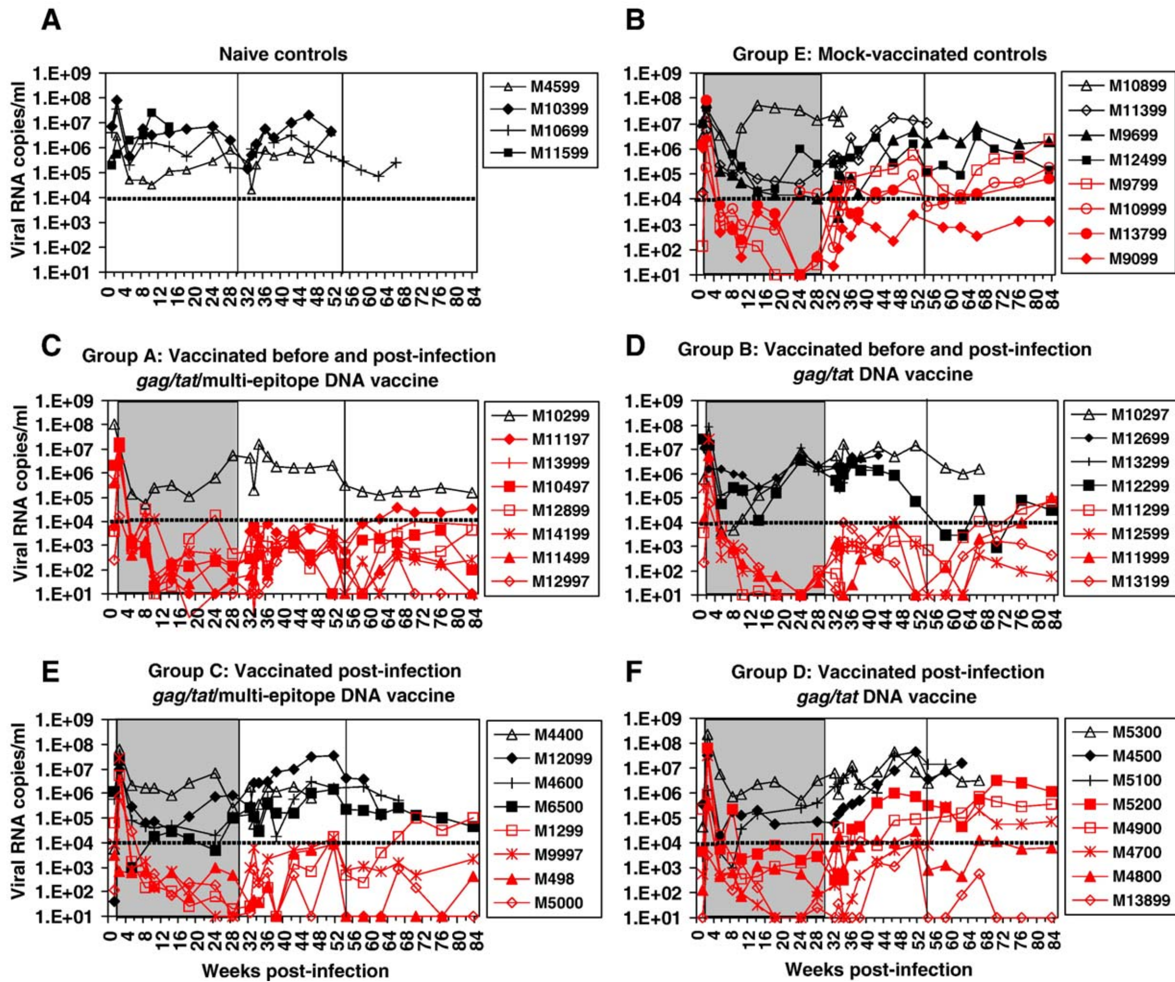

Fig. 4. Virus loads in vaccinated and control groups. (A) Untreated naïve controls; (B) mock-vaccinated PMPA-treated controls; (C, D) macaques vaccinated before and post-infection; (E, F) macaques vaccinated only post-infection. For panel B only, the open symbols indicate Mamu-A*01-negative controls and the closed symbols indicate Mamu-A*01-positive controls. The shaded areas indicate the period of continuous PMPA treatment. The solid vertical lines indicate the 6-month post-PMPA evaluation time point (week 54). Virus loads below the dashed line indicates viral containment at or below 10^4 viral RNA copies/ml, as determined by real time PCR. Red plots: antiretroviral (ART) responders or animals exhibiting viral load suppression below 10^4 RNA copies/ml during PMPA therapy. Black plots: ART low responders or animals exhibiting viral loads above 10^4 RNA copies/ml during PMPA therapy. The limit of sensitivity for the viral load assay is 10 viral RNA copies/ml.

As previously suggested by others (Franchini et al., 2002; Gotch et al., 1999; Hel et al., 2000; Lederman, 2001), we hypothesized that vaccine immunotherapy is likely to be most effective during ART-induced suppression of viremia. We therefore separately evaluated the effects of vaccination in ART responders and low responders.

A comparison of ART responders and low responders in Fig. 4 shows that viral containment post-PMPA occurred only in the ART responders (red plots). Viral rebound was controlled for at least 6 months after PMPA was withdrawn in 17 of 20 vaccinated ART responders compared to 1 of 4 ART responders in the mock-vaccinated control group ($P = 0.035$ by Fisher's Exact Test). The *gag/tat/*multi-epitope vaccine prevented immediate viral rebound in all 7 ART responders immunized before and after infection (group A, Fig. 4C) and in all 4 ART responders immunized only after infection (group C, Fig. 4E). The *gag/tat* vaccine also contained viral rebound post-PMPA in all 4 ART responders that were immunized both before and post-infection (group B, Fig. 4D), but appeared to be less effective when administered only post-infection providing viral control for at least 6 months post-PMPA in only 2 of 5 ART responders in this group (group D, Fig. 4F).

Disease progression

To determine if vaccination influenced disease progression, we analyzed CD4+ T cell loss in as an indication of progression to AIDS (Fig. 5). A stable CD4+ T cell count was defined as maintenance of CD4 counts within 80% of the baseline value, a level that is similar to the degree of variability we have observed in healthy uninfected, naive macaques (unpublished observations). As expected, 3 of the 4 naive controls showed progressive loss of CD4+ T cells to levels that were below 50% of baseline values and all 3 were sacrificed within 64 weeks post-infection due to AIDS (Fig. 5A). The 4th naive control animal was a rapid progressor that succumbed to AIDS within 14 weeks of infection.

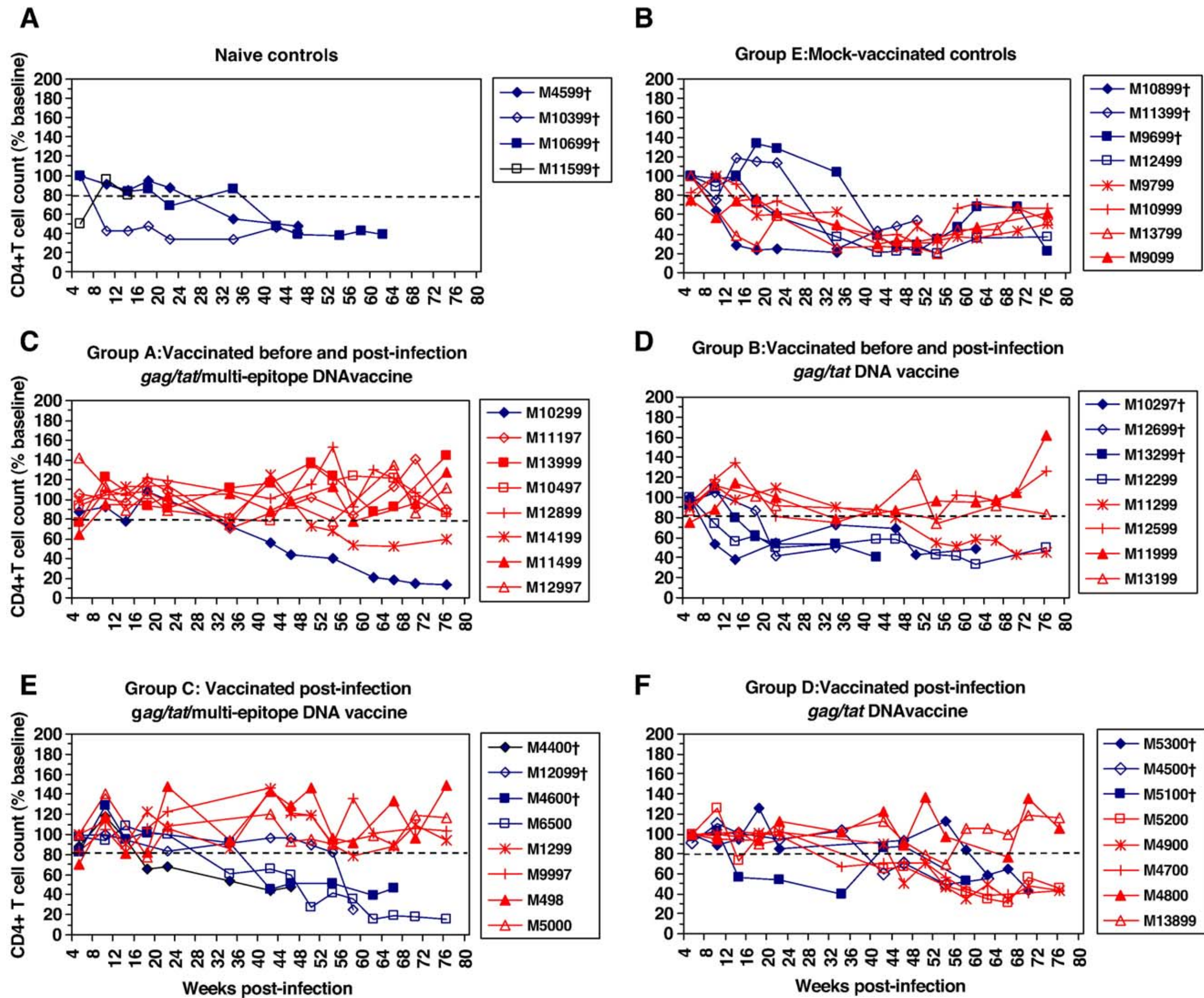

Fig. 5. CD4+ T cell counts. Shown are % baseline levels in (A) naïve controls; (B) mock-vaccinated PMPA controls; (C, D) macaques vaccinated before and post-infection; (E, F) macaques vaccinated only post-infection. Red plots: ART responders; Black plots: ART low responders.

In PMPA-treated animals, similar CD4+ T cell declines were evident in most ART low responders (Figs. 5B–F, black plots). By the end of the study, 15 of the 16 ART low responders demonstrated at least a 50% decline in CD4 counts, and 12 of these animals were sacrificed before the end of the study due to end-stage AIDS. Similarly, all 4 ART responders in the control group (Fig. 5B, red plots) exhibited CD4+ T cell loss after PMPA was withdrawn. CD4 counts declined to levels that were less than 80% of baseline measurements in all 4 animals within 3 months after PMPA was discontinued and remained below this threshold throughout the study. One of the ART responders (M9099) in the control group had low viral loads throughout the study but still exhibited significant CD4 decline. The cause for this observation at present is not clear but suggests that the bulk of viral replication in this monkey may not have been detectable in the peripheral blood.

In contrast, 15 of the 20 vaccinated ART responders maintained stable CD4 counts and remained clinically healthy for over 1 year after PMPA was withdrawn without further intervention (Figs. 5D–F, red plots). Four of the 5 vaccinated ART responders that exhibited CD4 loss were immunized with the *gag/tat* vaccine (M11299, Fig. 5D; M4700, M4900, M5200, Fig. 5F) and had all demonstrated viral rebound after PMPA was discontinued (see Figs. 4D and F). Late viral rebound was also observed in 2 additional vaccinated animals between 8 and 13 months (weeks 62–84) post-PMPA (M11999, Fig. 4D and M1299, Fig. 4E), but these animals maintained stable CD4+ T cell counts for the duration of the study (Figs. 5D and E), suggesting that temporary vaccine-induced containment of viral load may have longer-term benefits in preventing or delaying progression of disease.

Vaccine efficacy

Since control of viremia and long-term prevention or delay of disease progression is the goal of therapy, the effects of vaccination were analyzed in the context of containment of viral burden at or below 10^4 viral copies/ml plasma for a period of at least 6 months (weeks 30–54) in addition to prevention of CD4 decline for nearly 1 year after discontinuing ART (weeks

30–80). Vaccine efficacy was analyzed in the ART responders because vaccination was effective only in animals that responded to PMPA. All 4 ART responders in the control group demonstrated viral rebound and/or CD4 decline after PMPA was withdrawn (Table 2), whereas 15 of the 20 (75%) vaccinated ART responders (groups A, B, C, and D) contained viremia at or below 10^4 viral RNA copies for at least 6 months and maintained CD4 counts for 1 year after PMPA was withdrawn ($P = 0.012$).

Separate analysis of each vaccine group showed that 2 of the 4 vaccine strategies provided significant therapeutic benefit in ART responders when compared the controls (Table 2). The *gag/tat*/multi-epitope vaccine prevented viral rebound for at least 6 months and CD4 decline for 1 year in 6 of 7ART responders immunized both before and after infection (group A, $P = 0.015$) and all 4 ART responders immunized only post-infection (group C, $P = 0.029$). The *gag/tat* vaccine prevented viral rebound for at least 6 months in all 4 ART responders immunized before and after infection, but only 3 of the 4 animals maintained stable CD4 counts for 1 year after PMPA was withdrawn (group B, $P = 0.143$). The one animal in this group that exhibited CD4 decline also showed viral rebound 36 weeks after PMPA was withdrawn (see Fig. 4D, M11299). The *gag/tat* vaccine was also less effective when administered only after infection with only 2 of 5 animals showing viral control and stable CD4 counts, an outcome that was not significantly different from that observed in the mock-vaccinated controls (group D, $P = 0.444$).

Animals immunized with the *gag/tat*/epitope vaccine (groups A and C) demonstrated an improved virological outcome during the first 6 months post-PMPA when compared to animals immunized with the *gag/tat* vaccine (groups B and D). All eleven ART responders immunized with the *gag/tat*/multi-epitope vaccine (groups A and C) contained viremia for at least 6 months as compared to 5 of 9 animals immunized with the *gag/tat* vaccine (groups B and D) ($P = 0.026$ by Fishers exact test).

A comparison between the two immunization regimens shows that all 11 ART responders immunized before and after infection (groups A and B) contained viral rebound for at least 6 months after PMPA was discontinued as compared to 6 of the 9 animals immunized only after infection (groups C and D). This difference did not quite reach statistical significance ($P = 0.076$ by Fisher's Exact Test), in part, because of the smaller number of ART responders in the group immunized only post-infection. However, the results indicate an apparent trend toward vaccine-priming providing a benefit that will need to be confirmed in further studies that include a larger numbers of macaques per group or an ART regimen that induces a higher responder rate.

*Influence of the Mamu-A*01 MHC class I allele on vaccine efficacy*

Several studies have shown that the Mamu-A*01 allele is associated with reduced viral load and slower disease progression in SIV_{mac251}-infected animals (Mothe et al., 2002; Muhl et al., 2002; Pal et al., 2002) suggesting that the therapeutic effects of vaccination in the Mamu-A*01-positive animals used in this study may be due to an inherent MHC-related advantage. In this study, we observed no difference in peak and set point virus loads (Fig. 4B) or CD4+ T cell counts (Fig. 5B) between the 4 Mamu-A*01-positive and 4 Mamu-A*01-negative animals in the PMPA treated controls throughout the duration of the study. This finding is not due to the low numbers of animals in this group because similar analysis of the larger cohort of Mamu-A*01-positive and Mamu-A*01-negative vaccinated and unvaccinated animals that were poor ART responders (and therefore not affected by vaccination) also failed to reveal a significant difference due to haplotype in viral loads ($P > 0.120$) or CD4+ T cell counts ($P > 0.224$) (by Wilcoxon rank sum test at each time point, data not shown). Taken together, these results provide evidence that the Mamu-A*01 allele is not associated with lower viral loads or slower disease progression in SIV/DeltaB670-infected rhesus macaques and further, that therapeutic effects observed in the vaccinated animals in this study were not influenced by the presence or absence of the Mamu-A*01 class I allele.

Table 2
Effect of vaccination in ART responders: control of viremia and CD4+ T cell decline after discontinuing PMPA

Group: DNA vaccine	Immunization regimen	Number ART responders	VL[a] < 10^4 (6 months)	VL[a] < 10^4 (12 months)	Stable CD4+ T cell count (12 months)	Efficacy[b]
Controls	Mock-vaccinated	4/8	1/4	1/4	0/4	
A: *gag/tat*/multi-epitopes	Vaccine-primed[c]	7/8	7/7	6/7	6/7	$P = 0.015$
B: *gag/tat*	Vaccine-primed	4/8	4/4	2/4	3/4	$P = 0.143$
C: *gag/tat*/multi-epitopes	Post-infection[d]	4/8	4/4	3/4	4/4	$P = 0.029$
D: *gag/tat*	Post-infection	5/8	3/5	2/5	2/5	$P = 0.444$
All ART responders (Groups A, B, C, D)		20/32	18/20	13/20	15/20	$P = 0.012$

[a] Number of ART responders per group that controlled viremia at a level that was equal to or below 10^4 viral RNA copies/ml for the indicated period of time after discontinuing PMPA.

[b] Vaccine efficacy was defined as containment of viral loads for at least 6 months and maintenance of stable CD4+ T cell counts for 12 months after PMPA was withdrawn. *P* values were determined by comparing the outcome in each vaccinated group to that of the controls in the two-sided Fisher exact probability test. In all cases, $P < 0.05$ was considered significant.

[c] Vaccine-primed: Immunized both before and post-infection during ART.

[d] Post-infection: Immunized only post-infection during ART.

Post-infection SIV-specific immune responses

An effective immunotherapeutic vaccine should enhance and sustain virus-specific T cell responses. Lymphoproliferative responses (LPR) against whole Gag and Tat proteins were measured during therapy as a marker of virus-specific memory T cell responses. SIV-specific CD8+ T cell responses were also measured in each animal by ELISPOT following stimulation of CD4+ T cell-depleted PBMC with overlapping peptide pools from SIV Gag and Tat and 8 representative CTL epitopes encoded by the *gag/tat*/multi-epitope vaccine. The 8 epitopes selected for this assay were conserved in the challenge strain (Table 1) and are frequently recognized in SIV-infected monkeys (Allen et al., 2001). Epitope-specific CD8+ T cell responses were also measured during the first 3 months post-infection by tetramer staining but this method was not used for the duration the study because we found that it was less sensitive than ELISPOT for detection of responses against the less dominant epitopes in the vaccine (data not shown).

SIV-specific T cell responses were separately measured in ART responders and low responders. In ART responders, comparable LPR were initially detected in both control and vaccinated groups during acute infection (Fig. 6A). However, responses in the mock-vaccinated controls progressively declined to low levels by week 30 while LPR in all 4 groups of vaccinated ART responders continued to increase to levels that were 3–5-fold higher than that seen in the mock-vaccinated controls. Among all 4 vaccine groups, there were no significant differences in the mean LPRs measured at each time point.

Fig. 6B shows that significant SIV-specific CD8+ T cell responses were initially detected during acute infection in ART responders in the PMPA-treated control group, but these responses declined by week 28 in concert with ART-induced viral suppression with only a modest increase at week 64. In contrast, ART responders immunized with the SIV *gag/tat*/multi-epitope vaccine developed elevated SIV-specific CD8+ T cell responses during therapy that were sustained at higher levels than that observed in the mock-vaccinated animals for over 1 year after PMPA was withdrawn. There were no significant differences in CD8+ T cell responses detected at any time point between animals immunized both before and after infection (group A) or those immunized only after infection (group C). ART responders immunized with the *gag/tat* vaccine before and after infection (group B) or only after infection (group D) exhibited no differences in CD8+ T cell responses from that of

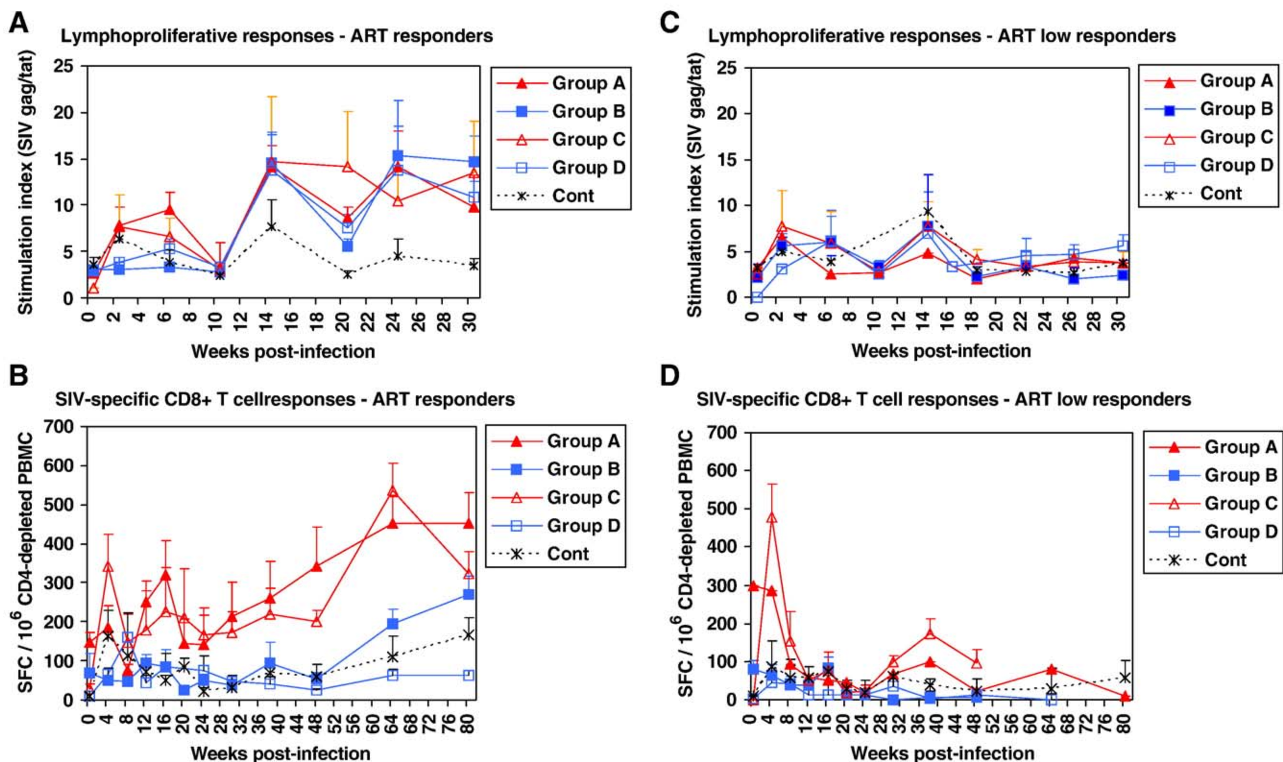

Fig. 6. Post-infection SIV-specific LPR and CD8+ T cell responses in ART responders and low responders. (A) LPR in ART responders; (B) CD8+ T cell responses in ART responders; (C) LPR in ART low responders; (D) CD8+ T cell responses in ART low responders. Closed symbols: groups immunized before and post-infection (Groups A and B), Open symbols: groups immunized only post-infection (Groups C and D). Dashed line: Mock-vaccinated PMPA controls Each value represents the mean response and standard error (SEM) in each group. In Panel A, the mean LPR measured in ART responders at each of the following time points was significantly higher in the vaccine group than in the control group: Group A ($P = 0.047$ at week 6; $P < 0.007$ at weeks 14, 20, 24; $P = 0.033$ at week 30), Group B ($P < 0.030$ at weeks 14–30), Group C ($P = 0.047$ at week 14; $P < 0.030$ at weeks 20, 24), Group D ($P < 0.030$ at weeks 20–30). In Panel B, the mean number of spot forming cells (SFC) measured in ART responders at the following time points was significantly higher in the vaccine group than in the control group: Group A ($P < 0.012$ at weeks 12, 16; $P = 0.006$ at week 30; $P < 0.024$ at weeks 48–80), Group C ($P < 0.030$ at weeks 24, 30 and 48–80). Differences at each time point were analyzed by the Wilcoxon rank sum test.

the control group throughout the study. However, mean CD8+ T cell responses increased in the group immunized before and after infection after week 64 concurrent with late viral rebound in two animals from this group (see Fig. 4D, M11299 and M11999). Interestingly, ART responders immunized with either vaccine did not demonstrate temporal boosts in the CD8+ T cell response as a result of successive DNA dosings (Fig. 6B). Rather, CD8+ T cell responses were maintained at higher levels throughout the study than that observed in the ART responder controls. This result suggests that the therapeutic immunizations may not have increased the magnitude of the virus-specific CD8+ T cell response *per se*, but rather, prevented decline of the memory response.

In ART low responders, all 4 DNA vaccine strategies uniformly failed to boost LPR (Fig. 6C) or CD8+ T cell responses (Fig. 6D). This result is not due to an inability of ART low responders to respond to the vaccines or infection since ART low responders immunized prior to infection demonstrated significant responses to vaccination prior to infection (see Fig. 2, group A: M10299; group B: M10297, M12699, M13299, M12299), and LPR and CD8+ T cell responses measured at 2 weeks post-infection were not significantly different between ART responders and low responders ($P > 0.482$ for LPR and $P > 0.790$ for CD8+ T cell responses by Wilcoxon rank-sum test). Taken together, these results suggest that the uncontrolled SIV replication in the ART low responders likely impaired their T cell function and ability to respond to vaccination prior to initiation of the therapeutic immunizations.

The influence of vaccination on the breadth of the CD8+ T cell response was examined in Mamu-A*01-positive macaques. The ELISPOT assay was used to measure individual responses to each of the 19 epitopes encoded by the multi-epitope vaccine in Mamu-A*01-positive animals at 10, 28, and 56 weeks post-infection. Significant CD8+ T cell responses were detected in each animal against one or more epitopes, but the repertoire was limited to 8 CTL epitopes that were previously shown to be the most consistently recognized in unvaccinated SIV-infected macaques (Allen et al., 2001). Responses against the remaining 11 epitopes encoded by the vaccine were either undetectable or detected at low levels at only one or two time points (data not shown). These results suggest that immunization enhanced the CD8+ T cell response against multiple epitopes but did not alter the relative hierarchy of epitope dominance that develops during infection.

The breadth of the CD8+ T cell response in each Mamu-A*01-positive animal was further examined by measuring the average response that developed against each of the 8 dominant epitopes during the treatment period (weeks 14–18), and after PMPA was withdrawn (weeks 38–54 and weeks 72–84) (Table 3). The breadth of the responses differed markedly between the different groups. In ART responder controls, the CD8+ T cell response was limited to only 1–2 epitopes during the early (weeks 14–18) and middle phases (weeks 38–54) of the study and directed primarily against the immunodominant Gag_CM9 epitope. This limited CD8+ T cell response was also observed in both vaccinated and control ART low

Table 3
Epitope specificity in Mamu-A*01-positive ART responders

Time p.i. [a]	Epitope	*gag/tat*/multi-epitope vaccine (Pre- and post-infection)							*gag/tat*/multi-epitope vaccine (Post-infection)				Controls	
		M 10497	M 11197	M 11499	M 12899	M 12997	M 13999	M 14199	M 0498	M 1299	M 5000	M 9997	M 9099	M 13799
Weeks 14–18	Gag_CM9	++++ [b]	+	+	+	+++	+	+	+	+	+	++	+	+
	Gag_LA9				+			++				+		
	Pol_GM10		+		+	+	+	++		+		+		
	Env_TL9	++	+	+		++		+		+		+		
	Env_ST10	+				+		+				+		
	Env_CL9	+		+		+		+				+		
	Tat_SL8	+		+		+		+				++		
	Vif_QA9	+					+	+				+		
Weeks 38–54	Gag_CM9	++++	++	+	+	+	++	++	+	++	+	+	+	+
	Gag_LA9									+				
	Pol_GM10	+	+	+			+	+	+	+	+	+		
	Env_TL9	+	+			+	+							
	Env_ST10	+	+			+			+	+		+		
	Env_CL9	++++		+	+		+	+				+		
	Tat_SL8	+	+	+	+	+	+	+	+	+	+	+		+
	Vif_QA9	+	+							+		+		
Weeks 72–84	Gag_CM9	++++	+++	++	+++	+	+++	++++	+	++	+	++	++	+
	Gag_LA9				+						+	+		
	Pol_GM10				+	+			+	+	+	+	+	
	Env_TL9	+			+									
	Env_ST10				+	+								
	Env_CL9	+	+	+	+		+	+	+			+		
	Tat_SL8				+			+		+	+	+	+	
	Vif_QA9	++++	+	++	+		+	++	+	+	+	+	++++	+

[a] Time post-infection.
[b] + = 25–99; ++ = 100–199; +++ = 200–299; ++++ > 300 SFC/10^6 PBMC.

responders (data not shown). In contrast, vaccinated ART responders demonstrated a broader CD8+ T cell response during this period that included, in addition to Gag_CM9, 3–7 less dominant epitopes. Differences in the breadth of the CD8+ T cell response during acute infection (weeks 14–18) were also evident in ART responders that were vaccine-primed with the *gag/tat*/multi-epitope vaccine (Group A) when compared to those immunized with the same vaccine only post-infection (Group C). All 7 vaccine-primed ART responders developed multi-specific CD8+ T cell responses against 3–7 epitopes while 2 of the 4 ART responders immunized only post-infection demonstrated a more limited response against only a single epitope during this period (Table 3, weeks 14–18). This result is consistent with previous studies showing that DNA immunization before infection can increase the breadth of the SIV-specific CD8+ T cell responses during acute infection (Barouch et al., 2001; Radaelli et al., 2003). During the post-PMPA phases (weeks 38–52 and 72–84), the breadth of the CD8+ T cell response in all 4 ART responders immunized only after infection expanded to 3–8 epitopes and was comparable to that seen in the vaccine-primed animals, indicating that immunization with the *gag/tat*/multi-epitope DNA vaccine after infection was established may have altered the existing repertoire of virus-specific CD8+ T cell responses.

Discussion

This study demonstrates for the first time that DNA immunization when administered as an adjunct to transient antiretroviral therapy either before and post-infection or only post-infection can induce durable immune control of a virulent, primary AIDS virus after drug is withdrawn. Further, this effect was associated with vaccine-induced T cell responses that were both greater in magnitude and broader in epitope specificity than that induced by viral infection alone.

However, the efficacy of therapeutic DNA vaccination was dependent on the virologic response to ART because containment of viral rebound was observed only in vaccinated animals that responded to PMPA. This observation is similar to a previous study where enhancement of virus-specific T cell responses in SIV-infected macaques by a recombinant poxvirus vaccine was dependent on suppression of viremia by a more potent triple drug regimen (Hel et al., 2000). In that study, however, significant viral containment was also observed in the mock-vaccinated control animals after HAART was discontinued, an outcome that compromised evaluation of the effects of vaccination on viral control. A more recent study demonstrated reduction in virus loads following therapeutic poxvirus immunizations in chronically-infected macaques, but this effect was transient (Tryniszewska et al., 2002). The results reported here represent an important advance from these earlier findings because DNA vaccination induced persistent control of viral burden in the majority of ART responders for over 1 year after discontinuing drug therapy (e.g., the full duration of the study). Moreover, these effects were achieved in combination with a less rigorous ART regimen employing only a single drug and in a setting where virus burden remained above the threshold of detection.

The observation that ART low responders failed to respond to DNA immunization even though the vaccine was administered only 56 days post-infection suggests that considerable immune impairment had already taken place in these animals during acute infection. At least partial preservation of immune function would be expected in ART responders (Autran et al., 1999; Lederman, 2001; Valdez, 2002). It is therefore not surprising that these animals were better able to respond to vaccination. This observation suggests that in a setting where infection is associated with a naturally lower viral set point and slower impairment of immune function, HAART may not be required for effective immunotherapy. A previous study employing antigen-loaded autologous dendritic cells as a therapeutic vaccine demonstrated significant virus load reduction in Chinese origin rhesus macaques without the need for HAART (Lu et al., 2003). Since SIV infection of this subspecies is associated with a 2–3 log lower viral load and slower disease progression than the Indian origin rhesus macaques used in our study, a more competent T cell response is likely retained in Chinese origin macaques in the absence of additional drug-induced virus suppression (Ling et al., 2002a, 2002b; Marthas et al., 2001; Trichel et al., 2002). Together, these results suggest that the outcome of therapeutic immunization may not depend on the response to ART per se, but rather the relative competence of the immune system to respond to antigen stimulation when therapy is introduced.

Macaques immunized with CTL epitopes in addition to *gag* and *tat* developed a higher and broader repertoire of CD8+ T cell responses, but comparable levels of SIV-specific LPR, when compared to macaques immunized only with the *gag*/*tat* DNA vaccine. Interestingly, only the *gag/tat*/multi-epitope vaccine was effective when administered only post-infection. This result supports the concept that increasing the magnitude and/or breadth of the virus-specific CD8+ T cell response after an infection has been established can improve viral control and clinical outcome. This result further suggests that therapeutic immunization with an epitope-based DNA vaccine is an effective strategy to achieve this goal.

We found no evidence that the Mamu-A*01 allele provides a protective effect on viral load or CD4+ T cell loss in animals infected intravenously with SIV/DeltaB670. This result strongly indicates that the improved outcome observed in the Mamu-A*01-positive animals was due to the effects of the *gag/tat*/multi-epitope vaccine and not genetic background. Our findings that the Mamu-A*01 allele does not influence viremia or disease progression in SIV/DeltaB670-infected macaques are in contrast with several studies reporting a significant Mamu-A*01-related protective effect on viral load and disease progression in macaques infected with SIV_{mac251} (Mothe et al., 2003; Muhl et al., 2002; Pal et al., 2002). This suggests that the influence of genetic background on SIV infection may depend on the viral stock employed. Consistent with this possibility, Pal et al. (2002) also did not observe a Mamu-A*01-related protective effect on viremia in rhesus macaques infected with the closely related SIV_{SME660} viral stock, although in that study, CD4+ T cell counts were found to be higher in the Mamu-A*01-positive animals.

DNA immunization prior to infection did not reduce acute viral load following intravenous challenge. This result is in contrast to a previous finding in our laboratory where 4 of 7 animals immunized with 7 doses of a particle-mediated DNA vaccine encoding SIV *gag/pol/env* were protected from intrarectal challenge with the same viral strain (Fuller et al., 2002). Particle-mediated epidermal DNA immunization induces mucosal immune responses in the gut associated lymphoid tissue (GALT) (Chen et al., 2001; Fuller et al., 2002) and the different outcomes in these two studies may be due to the induction of mucosal immune responses that can provide an additive protective barrier against intrarectal but not intravenous exposure to the virus. Furthermore, in the previous study, the 3 animals that were not protected developed plasma viral loads that were indistinguishable from the controls, suggesting that the peripheral blood immune responses were less effective in controlling the virus once it penetrated the mucosal barrier. Consistent with this possibility, a previous study showed that animals that developed class I restricted CTL responses in the GALT as a result of rectal exposure to a low dose of replication competent virus had sterilizing protection against a high-dose rectal challenge with SIV while those with undetectable responses in the GALT demonstrated plasma viral loads that were similar to that of the controls (Murphey-Corb et al., 1999). Although differences in the number of doses and/or composition of the two vaccines cannot be ruled out as a cause for the different outcomes of the two studies, these data suggest that particle-mediated DNA immunization may afford better mucosal than systemic protection against SIV.

Our data indicate that immunizing prior to infection enhances the effects of post-infection vaccine immunotherapy. Since our study did not include a group immunized only before infection, we cannot exclude the possibility that vaccination prior to infection and PMPA therapy alone could be sufficient to provide this benefit. However, immunization only post-infection with the SIV *gag/tat*/epitope provided a therapeutic benefit in ART responders, providing strong evidence that the DNA immunizations administered post-infection provided additional benefit in animals that were immunized both before and post-infection. Interestingly, the *gag/tat* vaccine induced strong LPR but only modest CD8+ T cell responses. Nevertheless, immunizing with this vaccine both before and after infection prevented viral rebound for at least 6 months in all 4 ART responders. This result suggests that less potent vaccines may still provide some benefit if administered both before and after infection. This observation may prove important in ongoing and future prophylactic vaccine trials in humans where candidate vaccines may nevertheless fail to prevent the establishment of infection (Letvin et al., 2002). Vaccinated, infected individuals from these trials may be excellent candidates for subsequent immunizations post-infection, perhaps even in the absence of HAART. Such an approach may be particularly useful in developing countries where widespread use of costly drug regimens may not be possible.

In summary, an effective therapeutic vaccine should ideally replenish virus-specific CD4+ T cells and maintain or boost virus-specific CD8+ T cell responses. Our studies are consistent with this hypothesis and further indicate that these vaccines should induce responses against a broad repertoire of CTL epitopes. The ability of these DNA vaccine strategies to provide a significant therapeutic benefit in the rigorous model system employed in these studies provides compelling evidence that DNA immunization is a promising approach for treating HIV infection in humans and reducing long-term dependence on complex drug regimens. What remains now is the development of strategies that provide similar benefits in those individuals that respond poorly to antiretroviral drugs.

Materials and methods

Rhesus macaques

Macaques were maintained in accordance with the NIH Guide to the Care and Use of Laboratory Animals under the approval of the University of Pittsburgh Institutional Animal Care and Use review committee. The University of Pittsburgh is accredited by the American Association for the Accreditation of Laboratory Animal Care International. Rhesus macaques were identified as Mamu-A*01 positive by PCR-SSP and by direct sequencing as previously described (Knapp et al., 1997).

HBcAg-multi-epitope DNA vaccine

The hepatitis B core antigen (HBcAg) carrier expression vector PJV7198 (PowderJect Vaccines, Inc., Middleton, WI) expresses a modified HBcAg under the control of the CMV immediate early promoter. It contains a unique *Bsp*120I restriction site within the immunodominant loop of HBcAg between amino acids 80 and 81, and a unique *Not* I restriction site at the C-terminus, facilitating insertion of epitope sequences at either site (Lesinski et al., 2001). To construct chimeric HBcAg-epitope DNA vaccines, PJV7198 was digested with either *Bsp*120I or *Not*I (New England Biolabs, Beverly, MA). Oligonucleotides encoding codon-optimized SIV CTL epitopes and flanked by *Bsp*120I or *Not*I were synthesized, annealed, and ligated into PJV7198 at either the immunodominant or C-terminus regions of the HBcAg gene. A total of Twenty-one SIV-specific CTL epitopes (Allen et al., 2001) (Table 1) were inserted into 9 HBc-epitope plasmids. Each plasmid contains 1–3 epitopes separated by 2 alanines that were inserted into either the internal or C-terminus region of the HBcAg gene as follows:

Plasmid	CTL epitopes	Insert position
1. pHBc-SIV-CM9	Gag_CM9	Internal
2. pHBc-SIV-SL8	Tat_SL8	Internal
3. pHBc-SIV-SI9	Env_SI9	C-terminus
4. pHBc-SIV-A	Vif_QA9, Pol_LV10, Env_ST10	C-terminus
5. pHBc-SIV-B	Env_CL9, Pol_YI9, Pol_EA11	C-terminus
6. pHBc-SIV-C	Gag_VT10, Pol_SV9	Internal
7. pHBc-SIV-D	Pol_GF10, Vif_VI8, Tat_TL8	Internal
8. pHBc-SIV-E	Pol_IL-10, Env_TL9, Pol_MI8	C-terminus
9. pHBc-SIV-F	Pol_QV9, Gag_LA9, Pol_GM10	C-terminus

SIV gag/tat DNA vaccine

The SIV gag/tat DNA vaccine is a cocktail of two plasmids, pcSIVgag and pcSIVtat (PJV 7271) (PowderJect Vaccines, Inc. Madison, WI), that encode the full-length SIV_{mac239} p55 *gag* and *tat* genes under the control of the CMV immediate early promoter. Both plasmids were constructed and tested for in vitro expression of their respective antigens and in vivo immunogenicity as previously described (Vogel et al., 2003).

DNA immunizations

Plasmid DNA was precipitated onto 1–3 μm gold particles as previously described (Roy et al., 2000) at a rate of 2.0 μg DNA per 1.0 mg of gold. Each of the 9 HBc-SIV epitope plasmids and the SIV *gag* and *tat* DNA vaccines were coated onto separate gold particles and then mixed prior to administration. Animals were sedated with ketamine (Parke-Davis, Ann Arbor, MI) (10 mg/kg), the abdominal and inner leg fur was clipped,, and DNA-coated gold particles were accelerated into the skin of both the abdominal and inguinal lymph node regions using the PowderJect® XR1 gene delivery device (PowderJect Vaccines, Inc., Middleton, WI) to deliver the DNA directly into the cells of the epidermis. DNA/gold was delivered at a helium pressure of 500–600 pounds per square in. Each delivery consisted of 1.0 mg of gold and 2.0 μg of DNA. A dose of 32 μg of total DNA per immunization was achieved by administering the DNA vaccines into 16 sites. DNA immunizations administered prior to infection were spaced 6–8 weeks apart, and post-infection therapeutic vaccinations were spaced 4 weeks apart.

Viral challenge

Macaques were fasted for 12 h, sedated with ketamine and challenged intravenously with 1 ml of RPMI containing 100 $TCID_{50}$ of cryopreserved SIV/DeltaB670. Clinical status was monitored by complete blood cell counts and physical examination that consisted of palpating peripheral lymph nodes and spleen and monitoring for signs of opportunistic infections. CD4+ T cell counts were measured by flow cytometry on fresh or frozen PBMC as described (Martin et al., 1993).

Anti-retroviral therapy (ART)

Rhesus macaques received daily subcutaneous injections of the anti-retroviral (R]-9-[2-phosphonylmethoxypropyl]adenine) (PMPA) (Gilead Biosciences, Foster City, CA) at a dosage of 20 mg/kg starting 2 weeks after infection. ART was continued without interruption for 28 weeks and then discontinued to monitor the effects of vaccination on viral rebound and progression to disease.

Plasma viral loads

Virion-associated RNA in plasma was quantified by real-time PCR in a Prism 7700 (Applied Biosystems, Inc., Foster City, CA) using primers specific for the viral long terminal repeat as described (Fuller et al., 2002). This assay has a sensitivity threshold of 10 copies/reaction and is linear over an 8-log range of template copy number. Control amplifications of samples omitting reverse transcriptase yielded negative results. RNA copy numbers from the unknown plasma samples were calculated from a similarly amplified external standard and expressed as RNA copies/ml plasma.

Proliferation assay

Rhesus macaque PBMCs were isolated by Ficoll density gradient centrifugation and washed 3 times with RPMI 1640 (Life Technologies, Gibco BRL) supplemented with Human AB+ serum (BioWhittaker, Walkersville, MD) (R10AB). PBMC (200,000/well) were aliquoted into 96-well flat-bottomed plates in triplicates in 100 μl R10AB. SIV Gag p28 recombinant protein (Trinity Biosciences, Frederick, MD) and purified SIV Tat viral protein were added at 0.2 μg/well in 100 μl R10AB. Medium containing no antigen was added to negative control wells and 5 μg/ml Conconavalin A (Sigma, St. Louis, MO) was added to positive control wells. Plates were incubated for 6 days before adding 1 μCi tritiated thymidine to each well. After a 16- to 18-h incubation in the presence of radionucleotide, cells were harvested onto 96-well Unifilter GC plates (Packard Bioscience, Meridien, CT) and counted on a Topcount scintillation counter (Packard Bioscience). Counts per minute (CPM) were averaged for triplicate wells and stimulation indices were calculated by dividing the average CPM obtained from test wells by the average CPM from the negative control wells.

IFN-γ ELISPOT assay

SIV-specific CD8+ T cells secreting IFN-γ were enumerated using the previously described ELISPOT protocol (Roy et al., 2000) modified for the macaque system. PVDF filter 96-well plates (Millipore, Bedford, MA) were coated with 10 μg/well of anti-IFN-γ mAb MD-1 (U-Cytech-BV, The Netherlands) overnight at 4 °C. The plates were then washed 4 times with sterile PBS (BioWhittaker) and blocked with RPMI-10% fetal calf serum for 1 h at 37 °C. Blocking solution was discarded and 10 μM synthetic peptide (QCB, Hopkinton, MA) and/or Gag and Tat pepsets (Chiron, Emeryville, CA) consisting of 15-mer peptides overlapping by 11 amino acids were added to each well. 5 μg/ml Concanavalin A was added to positive control wells and 10 μM of an irrelevant peptide or no peptide was added to negative control wells. PBMC were isolated from heparinized blood by Ficoll gradient (Histopaque-1077, Sigma) and CD4+ T cells were depleted with antibody-coated magnetic beads (Dynal, Oslo, Norway). CD4+ T cell-depleted PBMC were added at a rate of to 4×10^5 or 2×10^5 each well. Following 24–48 h incubation at 37 °C, 5% CO_2, the cells were removed and 200 μl/well of deionized water was added to lyse the remaining PBMC. The plates were then washed 4 times with PBS, and 1 μg/well of rabbit anti-IFNγ polyclonal biotinylated detector antibody (U-Cytech-BV) added. After 2 h at

room temperature or overnight at 4 °C, the plates were washed 5 times, incubated for 1 h with 50 μl/well of 1:1000 solution of streptavidin–alkaline phosphatase (BioRad, Hercules, CA), washed 5×, and 50 μl of BCIP/NTP membrane substrate (BioRad) was added to develop spots. Spot forming cells (SFC) were enumerated with ImagePro Plus software (Media Cybernetics, Silver Spring, MD). Responses significantly higher than background levels (twice the SFCs from untreated PBMC plus 10 spots) were considered positive.

Prior to ELISPOT analysis, the effectiveness of the CD4+ T cell depletion was measured in 3 representative animals by flow cytometry. Following depletion, cells in the isolated subset were stained in the dark in 100 μl FACS buffer with 10 μl anti-rhesus CD3 FITC and 10 μl anti-rhesus CD4 APC conjugated antibodies (Becton Dickinson Biosciences, San Jose, CA) for 30 min at room temperature. The cells were then washed with FACS-buffer and fixed with 450 μl of 2% paraformaldehyde. Data were acquired on a Becton Dickinson FACSCalibur instrument and analyzed using CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA). In all 3 samples, staining of CD4+ T cells following the depletion was less than 0.02% (data not shown).

Statistical analysis

Data from the vaccinated and control groups were compared by the Wilcoxon rank sum test at each time point. Vaccine efficacy was analyzed by the Fisher exact probability test. All P values reported are two-sided. In all cases, $P < 0.05$ was considered significant. Except where noted, the statistical analysis used to determine each indicated P value is described in the corresponding figure legend or table.

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