

Long-Lasting Decrease in Viremia in Macaques Chronically Infected with Simian Immunodeficiency Virus SIVmac251 after Therapeutic DNA Immunization[∇]

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Rhesus macaques chronically infected with highly pathogenic simian immunodeficiency virus (SIV) SIVmac251 were treated with antiretroviral drugs and vaccinated with combinations of DNA vectors expressing SIV antigens. Vaccination during therapy increased cellular immune responses. After the animals were released from therapy, the virus levels of 12 immunized animals were significantly lower ($P = 0.001$) compared to those of 11 animals treated with only antiretroviral drugs. Vaccinated animals showed a persistent increase in immune responses, thus indicating both a virological and an immunological benefit following DNA therapeutic vaccination. Several animals show a long-lasting decrease in viremia, suggesting that therapeutic vaccination may provide an additional benefit to antiretroviral therapy.

Antiretroviral treatment has changed the prognosis of human immunodeficiency virus (HIV) infection, as patients can remain free of symptoms for extended periods of time. However, continuous antiretroviral treatment is associated with toxicity and the emergence of resistant viral strains. Therapy must be continued indefinitely, since virus replication resumes rapidly upon treatment interruption (5, 22, 23) due to the persistence of HIV in stable reservoirs and also due to continuous residual virus replication (3, 4, 34). Thus, additional approaches to control viral propagation are needed. Strengthening the host immune response is a possible strategy in the management of HIV infection (reviewed in reference 11). Few therapeutic vaccine modalities have been tested in animal models and in humans. Substantial control of viremia has been reported in humans and macaques with early HIV/simian immunodeficiency virus (SIV) infection (9, 13, 30, 37) upon therapeutic vaccination or even by a period of antiretroviral therapy (ART) alone. Early therapy was hypothesized to work by limiting the damage to the immune system. Despite evidence of immunogenicity of therapeutic vaccination, some studies did not show substantial virologic benefit (10, 18). Therapeutic vaccination of persons treated with ART early after infection showed evidence of an induction of immune responses, but the

magnitude and dynamics of virus rebound after therapy discontinuation was similar in both vaccinated and unvaccinated subjects (18). Results of a randomized placebo control trial (QUEST [10]) of HIV-infected individuals treated during primary infection and therapeutically vaccinated with a vaccinia recombinant vector (ALVAC-HIV) or in addition with inactivated virus particles (Remune) showed an induction of HIV-1-specific cellular immunity, although it did not lead to better virological control of HIV-1 after the discontinuation of ART. Compared to those with early infection, results have been even more variable after intervention during chronic infection. Some reports in macaques suggest that immune therapy during chronic infection was only transiently effective in controlling viral load (VL) and boosting immune responses (14, 15, 36). Successful therapeutic dendritic cell vaccination, in the absence of ART, has also been reported; *in vitro* treatment of macaque and human antigen-presenting cells with antigen, followed by reinfusion of the cells, resulted in a long-lasting decrease in viral load (16, 17). Several immunotherapy studies suggest that the restoration of the immune system and more efficient immunization procedures may improve virus control (38). A recent study combining ALVAC-HIV with lipopeptides and interleukin-2 (IL-2) administration showed immunological and virological benefits after vaccination compared to results with the control group (12).

The use of DNA-based vaccines in therapeutic immunization is attractive since DNA vaccines can be easily combined with other vaccine modalities and adjuvants, and can be administered repeatedly, which is an advantage over viral vector-

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TABLE 1. History and treatment of the animals in the study

Animal no.	Prior prophylactic vaccination	Infection prior to ART (wk)	ART (wk)	Post-ART follow-up (wk)	SIV DNA vectors	MamuA*01 status
795L		29	23	33	<i>gag, env</i>	+
797L		29	23	34	<i>gag, env</i>	+
538L		15	20	93	<i>gag, env</i> , RTNV	+
539L		15	20	59	<i>gag, env</i> , RTNV	
965L		20	13	90	<i>gag, env</i> , RTNV	
968L		20	14	74	<i>gag, env</i> , RTNV	
57M		34	20	40	<i>gag, env</i> , polNTV	
920L	<i>gag, env</i>	34	20	70	<i>gag, env</i> , polNTV	
923L	<i>gag, env</i>	34	20	70	<i>gag, env</i> , polNTV	
922L	<i>gag, env</i>	34	20	19	<i>gag, env</i> , polNTV	
926L	<i>gag, env</i>	70	19	35	<i>gag, env</i> , polNTV	
626L	<i>gag, env</i>	70	19	35	<i>gag, env</i> , polNTV	+
882L		16	25	41		
890L		16	25	49		
909L		16	25	49		
208M		16	25	49		
3077		24	34	36		+
3139		24	34	36		
3116		24	34	36		
3143		24	34	36		
921L	<i>gag, env</i>	34	20	45		+
924L	<i>gag, env</i>	34	20	14		
925L	<i>gag, env</i>	34	20	14		
Median		24	20	40		

based vaccines. Improved protocols for prophylactic DNA vaccination have shown promising results against SIV and SHIV either alone or in combination with other vaccine modalities (reviewed in reference 27). We have previously reported a significant reduction in acute and chronic viremia after SIVmac251 challenge of macaques vaccinated with a combination of SIV *gag* and *env* expression vectors modified to produce secreted and intracellular forms of SIV antigens (29). Here, we show that SIVmac251-infected rhesus macaques improve control of the virus upon immunization with DNA vectors expressing these SIV antigens during ART treatment. This vaccination induces long-lasting, virus-specific immune responses, resulting in a significant reduction of high-level viremia after ART termination. Interestingly, DNA vaccination of infected animals promoted a Th1-biased response, with a more prominent induction of cellular immune responses.

MATERIALS AND METHODS

Animals. Indian rhesus macaques (*Macaca mulatta*) were housed and handled in accordance with the standards of the Association for the Assessment and Accreditation of Laboratory Animal Care International. Screening for 10 major histocompatibility complex class I and II alleles was performed by PCR (D. Watkins, Wisconsin Regional Primate Center). The animals were infected by pathogenic SIVmac251 mucosally, except for animals 795L, 882L, 890L, 909L, and 208M, which were infected by the intravenous route. During the drug treatment period (13 to 34 weeks; median, 20 weeks) (Table 1), animals were given a combination of three drugs [(*R*)-9-(2-phosphonylmethoxypropyl) adenine (PMPA), 20 mg/kg of body weight injected subcutaneously once daily; didanosine (ddI), 5 mg/kg injected intravenously once daily; and stavudine, 1.2 mg/kg orally twice daily]. Five of the macaques (animals 920L, 922L, 923L, 926L, and 626L, in the vaccine group) (Table 1) and three in the control group (animals 921L, 924L, and 925L) were prophylactically vaccinated with the same SIV *gag* and *env* DNA vectors before SIV infection, as part of a previous study (29).

DNA vectors. The plasmids used for DNA vaccination contain the cytomegalovirus promoter without an intron, the bovine growth hormone polyadenylation

site, and the kanamycin-resistant gene. The RNA (codon)-optimized expression vectors for *gag* and *env* were generated by introducing multiple silent point mutations not affecting the sequence of the encoded proteins, as previously described for HIV-1 *gag* and *env* (20, 31–33) using synthetic DNAs. The secreted and intracellularly degraded variants of the SIV antigens were generated by the fusion of either IP10-MCP3 (2) or of a beta-catenin-derived peptide (amino acids 18 to 47) (1), respectively, at the N terminus of Gag and Env, as previously described (29). All animals received mixes of DNA vectors producing secreted and intracellular forms of *gag* and *env*. In addition, as indicated in Table 1, most animals received vectors expressing intracellular forms of a fusion protein polNTV consisting of Pol, Nef, Tat, and Vif proteins or alternatively a vector expressing a fusion (RTNV) of Rev-Tat-Nef (ReTaNeF [7, 8]) and Vif proteins. Several point mutations were introduced into *pol* to inactivate protease (D25A, T26A, and G27A), reverse transcriptase (D186A and D187A), RNaseH (E478A), and integrase (D64A, D116A, and E152A). In addition, the myristoylation signal of Nef was removed. These changes parallel changes we have introduced into HIV expression vectors.

Therapeutic immunization. Highly purified, endotoxin-free DNA plasmid preparations were produced using the QIAGEN kit (Hilden, Germany). Animals 795L and 797L received a total of 6 mg of plasmids expressing native *env* and *gag*, the secreted forms of *env* and *gag*, and the intracellular degraded forms of *gag* (1 mg each). Animals 538L, 539L, 965L, and 968L received a total of 10 mg DNA comprising 2 mg each of the intracellular degraded and secreted forms of SIV DNAs expressing *gag* or *env* and 2 mg polNTV. These DNAs were prepared at a concentration of 1 mg/ml in phosphate-buffered saline and injected (0.5 ml per injection) at separate sites intramuscularly. The remaining animals were immunized with mixtures of 8 mg of SIV DNA vectors in combination with 2 mg IL-15 DNA (animals 57M, 920L, 923L, and 922L) or IL-12 DNA (animals 926L and 626L), formulated as previously described (6) using 2 mg/ml DNA.

Humoral responses. Antibody production was measured in serial dilutions of plasma by enzyme-linked immunosorbent assay against SIVmac251 lysate spiked with gp120. The plates were analyzed at 450 nm. The binding antibody titers are reported as the reciprocal of the highest positive dilution.

Viral load analysis. SIV RNA copy numbers were determined by nucleic acid sequence-based isothermal amplification assay using SIVmac251-specific primers (28). During ART, an assay with a cutoff value of 20,000 RNA copies/ml was used and values below the cutoff were assigned the value of 10,000. Most of the chronic and post-ART period samples below this cutoff, if available in sufficient quantity, were analyzed by more sensitive assays, either a nucleic acid sequence-

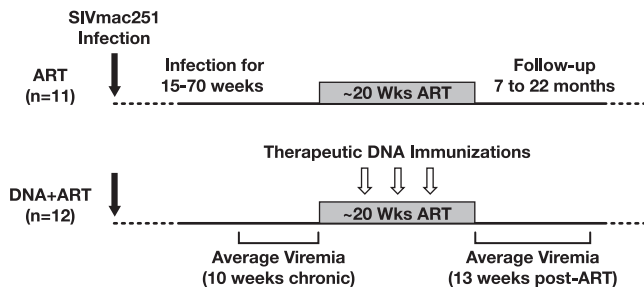


FIG. 1. Therapeutic vaccination of animals chronically infected by SIVmac251. Animals in the vaccine group (DNA+ART; $n = 12$) received three to four immunizations during therapy and were evaluated for several months after ART termination. Animals in the control group ($n = 11$) received only ART. The average viremia for the 10-week period before ART and for the 13-week period after ART were compared for each animal in the vaccine and control groups.

based isothermal amplification assay having a cutoff value of 2,000 copies/ml (28) or a real-time, quantitative reverse transcription-PCR assay with a cutoff value of 100 RNA copies/ml of plasma (35).

ELISPOT assay. The enzyme-linked immunospot (ELISPOT) assay was performed as previously described (21, 29). Eighty-eight peptides spanning gag p39 and 100 peptides covering gp120env were used as two separate pools. Specific spots to a given peptide pool were calculated by subtracting the cutoff value and adjusted to spot-forming cells per million peripheral blood mononuclear cells. The cutoff value was defined as the average number of spots in the negative control measured in triplicate plus two standard deviations (SD).

Statistical analysis. We compared the median change in average viral load (log transformed, base 10) from the chronic to the post-ART period between vaccinated and control animals using a Wilcoxon rank sum test. Before combining previously vaccinated animals with naïve animals, we examined whether differences between previously vaccinated and naïve animals existed by testing for an interaction between vaccination group and prevaccination status in a two-way analysis of variance with an interaction term, with the outcome being the difference in average viral load between the chronic and post-ART periods. Further, we performed analysis of covariance to adjust for any differences in pretreatment viral load between groups.

The animals were assigned randomly into the vaccine and control groups. The groups had similar values for mean pretreatment \log_{10} -transformed viral load (mean, 5.855; standard deviation, 0.7078; and mean, 5.768; SD, 0.9490 for the vaccine and control groups, respectively). After the exclusion of animals that did not fulfill the study criteria, there was a small difference in the means of the two groups (\log_{10} -transformed mean, 5.606; SD, 0.3977; and mean, 5.970; SD, 0.5889; for the vaccine and control groups, respectively). Analysis of covariance showed that the decrease in viral load after DNA vaccination remained significant after adjusting for the small difference in pretreatment virus loads. The statistical

analyses performed indicate that the therapeutic DNA vaccination benefit does not depend on the specific inclusion criteria or the differences in the history of the animals in the study.

Analyses of immune responses were conducted with a linear mixed model (26), which allows for testing period effects while accounting for the fact that repeated observations on the same subject may be correlated. We examined whether the mean varied across periods using a conditional F test. Prior to combining naïve and prevaccinated groups, we examined whether the pattern in changes in mean values across periods changed by prevaccination status by also using a conditional F test.

Survival curves for high versus low benefit (defined as above or below the median reduction, respectively) were compared using a log-rank test. Because of the small sample sizes (six in each group), a permutation testing procedure was used for calculating the P value. Specifically, the reference distribution under the null hypothesis was generated by randomly permuting high- versus low-benefit status and generating 20,000 chi-square statistics corresponding to the log-rank test. The reported P value (0.03) was the proportion of times the generated chi-square values were greater or equal to the observed chi-square value. All P values were two sided. All test results with P values of <0.05 were considered statistically significant.

RESULTS

DNA vaccination lowers virus loads after release from ART.

Twenty-three Indian rhesus macaques were chronically infected with pathogenic SIVmac251. The animals were enrolled in smaller groups as they became available from other studies and had been infected for periods ranging from 15 to 70 weeks prior to the start of ART. The animals were treated with a combination of three drugs (PMPA, ddI, and stavudine) for 13 to 34 weeks and subsequently released (Fig. 1). Animals included in the study had persistent chronic viremia above 10^4 RNA copies per milliliter of plasma and showed decreased plasma virus load to undetectable levels for at least one-third of the time during ART. Twelve animals were vaccinated intramuscularly with DNA during ART, whereas 11 animals were treated with only ART (control group). The DNA vaccine consisted of a mixture of plasmids expressing modified forms of Gag and Env antigens as well as additional SIV antigens (29). Table 1 summarizes information on the study animals, indicating the length of time of infection (median, 24 weeks), ART treatment (median, 20 weeks), post-ART follow-up period (median, 40 weeks), the types of DNA used, and the time and number of immunizations.

The plasma viral loads from the day of infection to the end of the follow-up period for all animals are shown in Fig. 2.

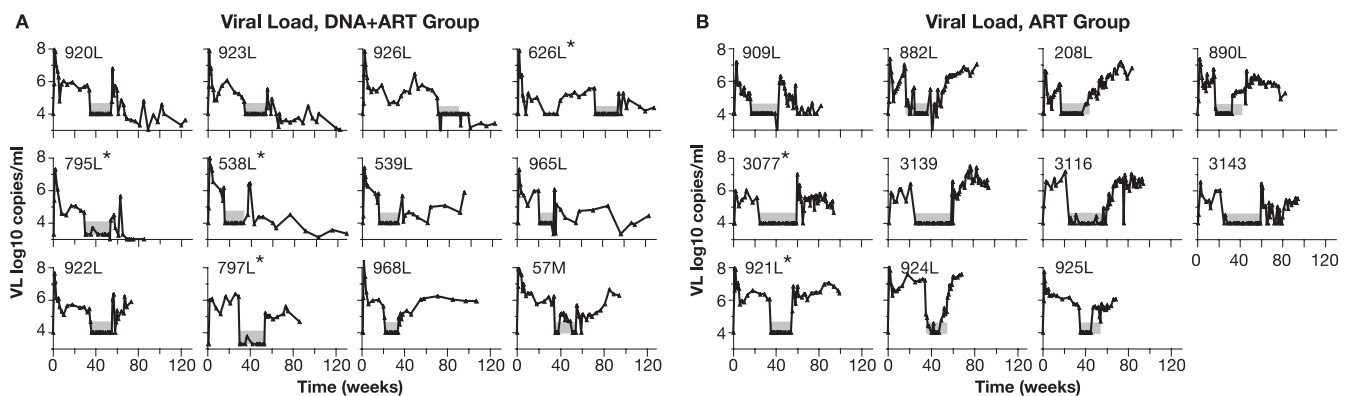


FIG. 2. Virus load in plasma of all macaques in the study, from infection to the end of follow-up period. Gray bars indicate the period under ART. (A) Twelve animals treated with ART+DNA vaccination. (B) Control group treated with only ART. Stars next to animal numbers indicate animals positive for MamuA*01.

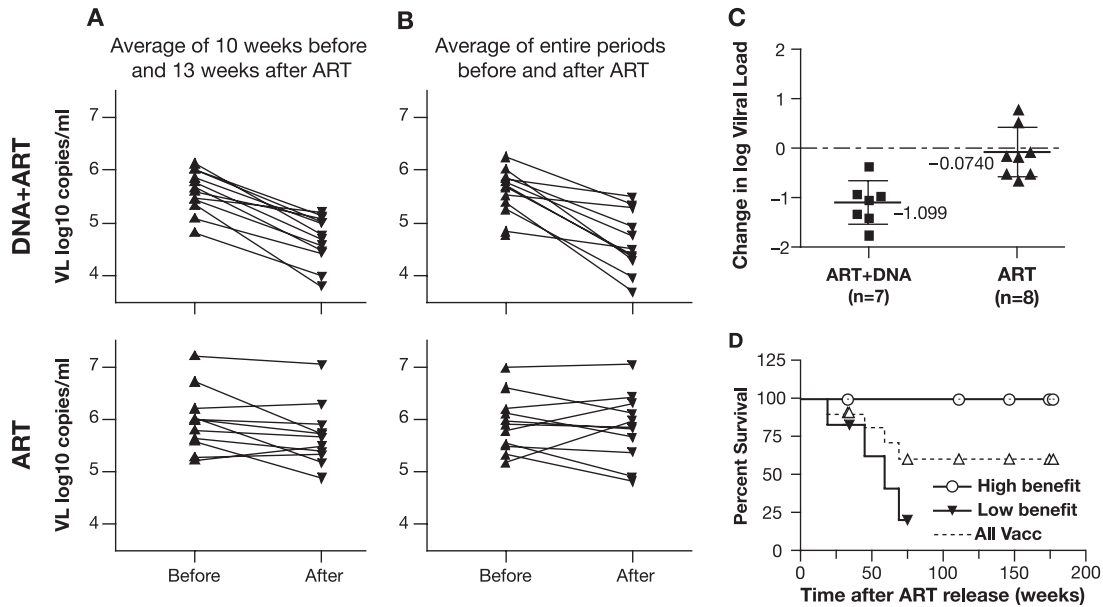


FIG. 3. (A) Comparison of average virus load over fixed periods of the 10 weeks preceding and the 13 weeks following ART therapy. Average viremia before and after therapy is shown for the ART+DNA group (top) and the ART group (bottom). (B) Comparison of average virus load for the entire chronic period before therapy versus the entire period post-ART. (C) Change in viral load in previously naïve animals after ART+DNA (seven animals) or ART (eight animals). These animals did not receive any vaccination prior to enrollment in the present therapeutic vaccination protocol. The difference between the groups is significant ($P = 0.002$; Wilcoxon rank sum test). (D) Survival curves for the two subgroups of DNA-immunized animals. The 12 animals were ranked by the benefit in VL after therapy release using the difference in VL between chronic phase and the 3 months after release value (~13 weeks). The animals were separated in high- and low-benefit groups of six, defined as above or below the median reduction in VL. Circle and closed triangle symbols indicate the time of termination of observation for animals in the high- and low-benefit groups, respectively (censored). The difference between the two groups is significant using a log-rank test ($P = 0.03$). The dashed line indicates the survival curve of all the animals in the ART+DNA group (All Vacc).

During ART, all animals decreased virus load to below the cutoff value for the assay for at least one-third of the time (Fig. 2A and B). Animals were kept on ART for at least 20 weeks, except for two animals that showed signs of drug toxicity (965L and 968L), for which ART was terminated earlier. The animals shown in Fig. 2A received DNA immunizations as outlined in Table 1, whereas the animals shown in Fig. 2B received only ART. After release from therapy, virus rebounded rapidly in most of the animals. Despite the initial rebounds, viral loads decreased dramatically in many DNA-vaccinated animals a few weeks after ART termination (Fig. 2A). Seven of the 12 vaccinated animals (920L, 923L, 926L, 626L, 795L, 538L, and 965L) showed significant long-term decreases in the levels of viremia; five of these animals (920L, 923L, 926L, 795L, and 538L) suppressed the virus to levels close to or below the detection level for several months. In contrast, in most of the control animals (Fig. 2B), viral loads returned to levels similar to those prior to therapy. The inability of ART alone to induce long-lasting benefits in viral load is in agreement with the results of other investigators in both macaques and humans, where therapy interruption resulted in virus rebound to levels similar to those prior to ART (5, 22, 23).

For statistical comparisons, we determined the average viremia during the 10 weeks immediately preceding ART and during the first 13 weeks after ART termination, for which data were available for all animals in the study. The comparison of the change in average viremia for the individual animals in vaccine and control groups is shown in Fig. 3A. All animals in

the vaccine group showed lower average viremia after ART release compared to that in the chronic phase. The mean differences in the log₁₀-transformed viral load measurements (mean VL after ART for each animal minus mean VL before ART) were -0.93 for the vaccine group and -0.28 for the control group. These differences were highly statistically significant ($P = 0.001$; Wilcoxon rank sum test) and demonstrate a benefit of DNA therapeutic immunization in macaques chronically infected with SIVmac251.

A previous study has suggested that the benefits of immunotherapy may be transient (36). To study the long-term effects of DNA vaccination, we performed additional analyses comparing the differences in viral load using the entire chronic and post-ART periods (Fig. 3B). In this comparison, the mean differences in viral load were -1.05 log₁₀ for the vaccine group and -0.068 for the control group ($P = 0.0004$; Wilcoxon rank sum test). Thus, a bigger difference between vaccinees and controls was found upon considering a longer period after therapy termination. This can be explained by the long periods of low viremia seen in many vaccinated animals after the initial period of virus rebound. As shown in Fig. 2, the majority of the animals in the vaccine group and some animals in the control group had one to three virus rebounds shortly after therapy termination. Thus, the inclusion of a longer follow-up period decreased the value of average viremia in the post-ART period.

Five of the animals in the vaccine group (animals 920L, 922L, 923L, 926L, and 626L) (Table 1) and three in the control

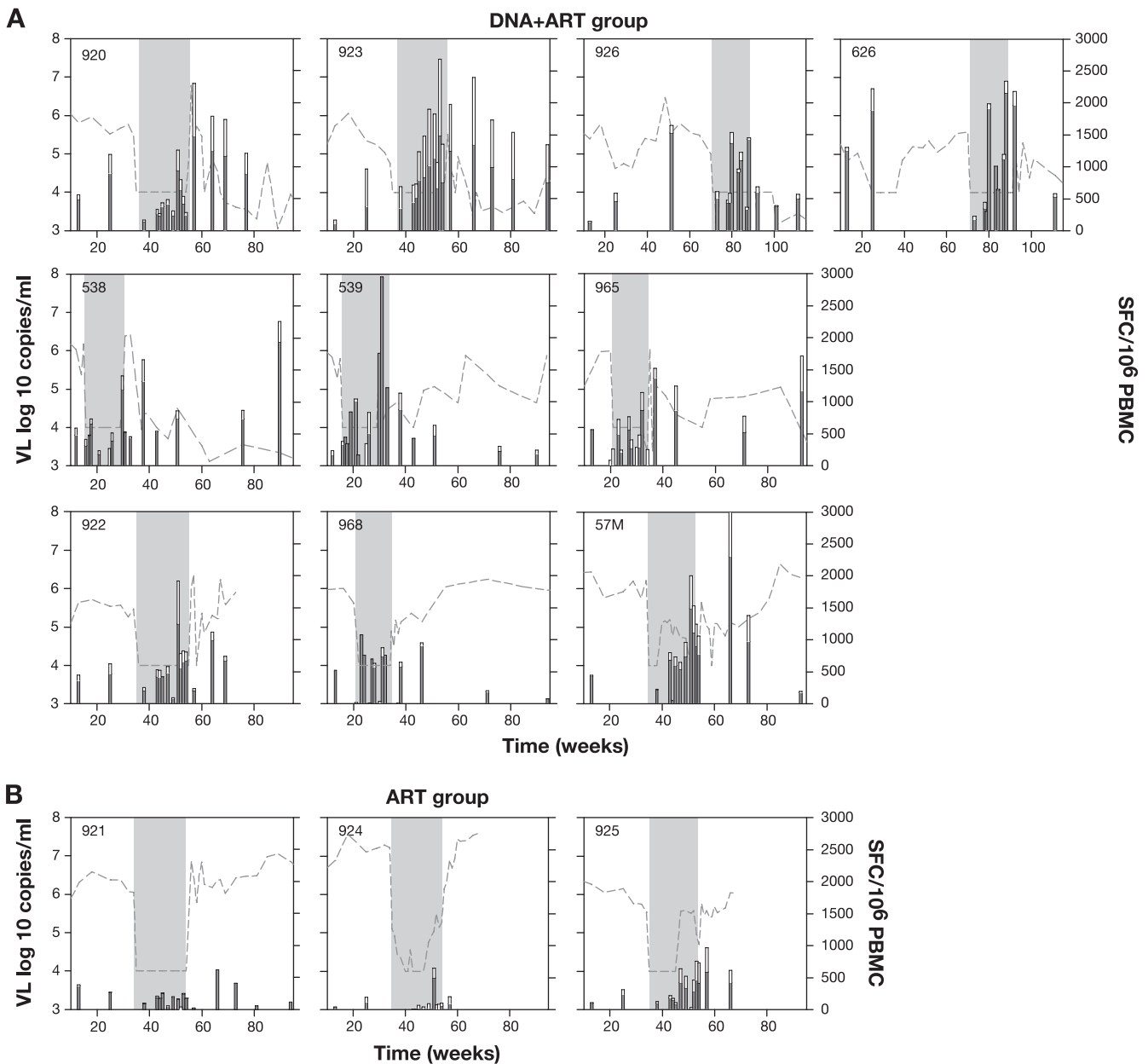


FIG. 4. ELISPOT analysis for 10 ART+DNA animals (A) and 3 ART-only controls (B). Gray and open stacked bars represent ELISPOT values (right scale) for *gag* and *gp120env*, respectively, for the indicated dates. Dotted lines indicate log₁₀ virus load (left scale). Shaded area indicates the period on ART.

group (animals 921L, 924L, and 925L) were prophylactically vaccinated with the same SIV *gag* and *env* DNA vectors before SIV infection as part of previous studies (29). At the time of ART initiation, these animals had been infected for 34 or 70 weeks and had stable chronic virus load levels (range, 4.8 to 7.1 log₁₀) (Fig. 2). We used a two-way analysis of variance (test for interaction) to examine whether prophylactic DNA vaccination influences the immunotherapy outcome. This analysis did not reveal a different effect of previous vaccination status (test of interaction was not significant; $P = 0.97$), suggesting that the benefit derived from therapeutic immunization is independent of the previous prophylactic vaccination. Therefore, combining

the previously vaccinated animals in the two groups described in this study was appropriate. Alternatively, even if we consider only the animals without any previous prophylactic vaccination (seven vaccinees and eight controls) (Fig. 3C), the difference in viral load between the groups also remains significant. The mean changes in VL were -1.10 and -0.074 for the vaccine and control groups, respectively, when considering the entire period of follow-up. Using a Wilcoxon rank sum test, the differences between these groups, which included only naïve challenged animals, were statistically significant (P values of 0.008 and 0.002 for the 13 weeks post-ART period and for the entire post-ART period, respectively). Some therapeutically vacci-

nated animals in this study received, in addition, DNA vectors expressing macaque IL-12 (926L and 626L) or IL-15 (57M, 920L, 922L, and 923L). There was no difference in VL outcome between animals receiving IL-12 or IL-15 compared with those of the other vaccinated animals ($P = 0.53$; Wilcoxon rank sum test). This preliminary analysis is based on a comparison of a few animals receiving low levels of cytokine DNA and must await further experiments with more animals for final conclusions on the effects of cytokine DNA inclusion during therapeutic vaccination.

Four animals in the vaccine group (795L, 797L, 538L, and 626L) and two animals in the control group (3077 and 921L) carried the MamuA*01 allele (Table 1), which has been associated with a milder disease course after challenge with SIVmac in some studies (19, 24). Analysis of virus loads did not reveal any statistical difference in the decrease of VL for the four MamuA*01 animals compared to the eight others in the vaccine group, using a Wilcoxon rank sum test ($P = 0.81$).

In conclusion, this analysis indicates that the decrease in viremia derived from immunotherapy did not depend on previous prophylactic vaccination or the MamuA*01 haplotype. The results also indicate that infected animals with previous histories of prophylactic vaccination may get an independent benefit in terms of viral load reduction upon therapeutic immunization.

Survival of DNA-vaccinated macaques. The animals that benefited most in virus load reduction also showed increased survivals compared to those of the animals that show less benefit. The vaccinees were ranked according to VL benefit and split in two groups of six macaques. Six of 12 vaccinees had decreases in viremia above the average of $1.05 \log_{10}$ (high-benefit group). Five of these animals (538L, 926L, 920L, 965L, and 923L) were alive at the end of the study, after 111 to 177 weeks of follow-up post-ART. In contrast, four of the six animals with low virological benefit (low-benefit group) died and two were removed from the study after 34 and 74 weeks of observation, respectively. The survival curves for these two groups are shown in Fig. 3D. The survival curve of all 12 vaccinees is also shown. It is interesting that the group of animals that have a strong long-lasting benefit in VL also shows increased survival (P was 0.03 using a log-rank test as described in Materials and Methods).

Immunological analysis. To monitor the effects of DNA immunization on cellular immunity, we measured the frequency of antigen-specific, gamma interferon-producing T cells in peripheral blood mononuclear cells by ELISPOT assay after stimulation by overlapping peptide pools for *gag* and *gp120env* over time (see Fig. 4 and 5A). Most of the DNA-vaccinated animals showed strong and increasing responses during the vaccination period (Fig. 4). These responses were maintained in several animals (920L, 923L, 926L, and 538L) despite low levels of viremia. Figure 5A shows the peak ELISPOT response to *gag* and *gp120env* for 10 vaccinated animals for which serial measurements were available, divided into four periods: chronic phase, ART before vaccination, ART and DNA vaccination (ART+DNA), and follow-up after ART termination. The frequency of antigen-specific T cells decreased immediately upon drug treatment, as expected from the decreasing viral load. Interestingly, ELISPOT values increased immediately upon vaccination and persisted at high

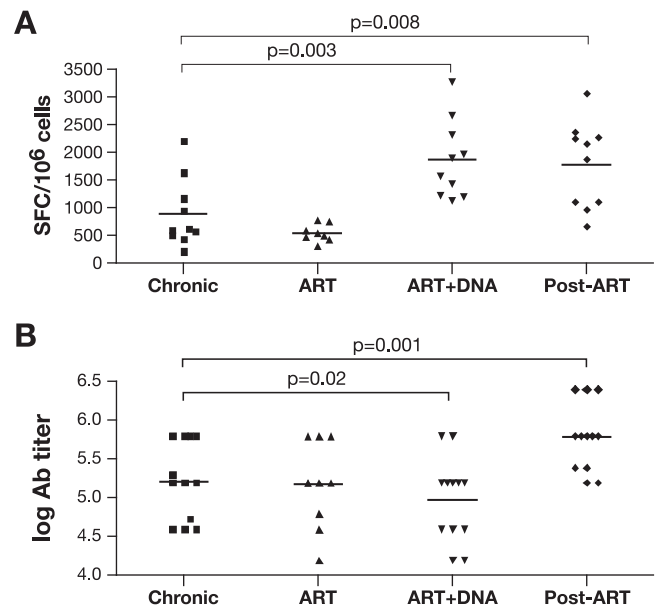


FIG. 5. Comparisons of cellular and humoral immune responses. (A) Peak ELISPOT responses to *gag* and *env* for 10 vaccinated animals divided into four periods: chronic phase, ART before vaccination, ART and DNA vaccination, and follow-up post-ART. (B) Peak antibody levels against SIV (reciprocal titers) during the same time periods. Brackets indicate comparisons showing statistical difference.

levels after ART termination. The ELISPOT values for *gag* and *gp120env* were compared during these four periods using a linear mixed model (26). The overall global test of a changing mean peak level over the four periods was highly significant ($P < 0.0001$). Increases from chronic to ART+DNA period (mean change, 980 SFC/ 10^6 cells; $P = 0.003$) and from chronic to post-ART period (mean change, 895; $P = 0.008$) were highly significant.

ELISA measurements showed that the animals had high antibody levels against SIV (Fig. 5B), which, in contrast to ELISPOT (Fig. 5A), did not increase during therapeutic vaccination. After ART termination, the antibody levels increased to higher levels. Application of a linear mixed model as above gave a significant global test of a changing mean over the four periods ($P < 0.001$). There was a nonsignificant decrease from the chronic period to the ART period (mean change, -0.11 ; $P = 0.21$), followed by a significant decrease in the ART+DNA period (mean change from chronic, -0.23 ; $P = 0.02$), and a significant increase in the post-ART period (mean change from chronic, 0.56 ; $P = 0.001$). Therefore, unlike cellular immunity, the antibody levels did not increase during therapeutic vaccination, indicating a polarized Th1 response to DNA vaccination in the infected macaques. Despite this, a significant antibody increase after release from therapy indicates a change in the humoral immune response after vaccination, despite the lower VL after immunotherapy. Three animals in the ART group were also studied in parallel by the same assays. These animals showed a progressive drop in antibody titers during ART and did not show increased immune responses after release.

DISCUSSION

This study demonstrates that chronically infected macaques with substantial viremia mount more efficient immune responses after ART and DNA vaccination, which results in decreases in viral load by approximately 1 log on average. The animals varied widely in their responses; those showing strong and long-lasting virological benefits may also have prolonged survival. It will be important to understand the reasons for this heterogeneity in order to design better therapy approaches. Several animals in the vaccine group were able to suppress viremia close to the detection limits of the assay. In contrast, ART alone did not result in any significant viral load decrease in these chronically infected animals, which is in agreement with data from several studies on structured therapy interruption in monkeys and humans.

It is interesting that the initial viral rebound upon termination of ART was subsequently suppressed, presumably by the immune system, in the majority of therapeutically immunized macaques. The increase in cellular immune responses measured by ELISPOT in this period agrees with the hypothesis that viral rebound leads to increased cytotoxic T-lymphocyte activity and the elimination of the infected cells. In several animals, we observed maintenance of the high frequency of *gag*- and *env*-specific T-cell responses in spite of low viral loads. This is in contrast to the expected decrease in the level of immune responses upon a decrease in viremia and suggests that the immune system of the therapeutically immunized animals reached a different steady state. This observation is supported by the negative correlation of viral load with ELISPOT values during the post-ART period.

Our analysis focused on the comparison of all DNA-vaccinated animals to the control group. Small variation in the schedule, DNA vectors, and the inclusion of small amounts of cytokine DNA in some vaccinated animals could not change the basic conclusions. The inclusion of some chronically infected but previously prophylactically vaccinated animals in both the control and vaccine groups could be questioned because such animals may have more intact immune systems. Using two-way analysis of variance, we show that there is no evidence of different outcome in the animals with prior prophylactic vaccination, suggesting that the benefit derived by ART and therapeutic immunization is independent of previous history. To further address this concern, in a separate analysis presented in Fig. 3C, we considered only naïve animals infected by SIV without any history of previous treatments. The basic conclusion of our study, that there is a long-lasting virological benefit in therapeutically vaccinated macaques, is also verified by this analysis. Several studies support the notion that therapeutic immunization may augment immune responses against HIV or SIV, but the beneficial effect on VL decrease and disease progression remains controversial. Our data agree with recent reports indicating a virological benefit after therapeutic immunization (12, 16, 17).

It could be argued that animals enrolled in an immunotherapy study like the one presented here are preselected since rapid progressors are excluded. We (unpublished data) and others (9) noted that animals with very high acute viremia not decreasing substantially during the chronic phase respond poorly to an ART regimen (PMPA, emtricitabine, ddI, stavu-

dine). Thus, the animals enrolled represent macaques with immune systems that may be less damaged by the virus during the acute phase. Therefore, one cannot exclude the possibility that individual differences among infected macaques may affect the outcome of the study. Although such parameters are still under investigation, the positive and long-lasting benefit in 7 of 12 animals is an encouraging observation which warrants detailed comparisons of animals with differential benefit, so that the factors responsible for better outcomes are discovered. The sustained cellular immune response after therapeutic DNA vaccination may indicate an important clue for additional studies. We have found a better preservation of central memory antigen-specific SIV lymphocytes in animals with long-lasting virologic benefit (our unpublished data).

It has been proposed that decreases of 1.5 to 2 logs in virus load caused by prophylactic vaccination may be important for achieving decreases in transmission and slowing disease development, providing a useful vaccine, despite the absence of sterilizing immunity (25). Long-lasting reduction in chronic virus loads in already infected animals may also provide a meaningful benefit, as suggested by the survival difference of the high responders versus low responders to therapeutic vaccination (Fig. 3D). Comparison of strong responders to non-responders may provide ways to identify correlates of protection. In conclusion, DNA vaccination during ART was able to elicit effective antiviral cellular responses in chronically infected macaques, resulting in long-term decrease of viremia. Therefore, repeated vaccination during antiretroviral therapy may provide better control of virus propagation, which is associated with better disease control. Unlike viral recombinant vectors expressing HIV/SIV antigens, DNA therapeutic vaccination can be delivered repeatedly without focusing the immune response to other antigens. In addition, DNA vaccination provides efficient access to the major histocompatibility complex class I pathway and should be considered for further development and optimization. It is anticipated that improvements in DNA delivery to the tissues, obtained with techniques such as electroporation and effective doses of molecular adjuvants, can dramatically improve the magnitude of responses to DNA vaccination.

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