Published in final edited form as:
Vaccine. 2010 February 23; 28(8): 1962–1974. doi:10.1016/j.vaccine.2009.10.099.

Repeated DNA Therapeutic Vaccination of Chronically SIV-Infected Macaques Provides Additional Virological Benefit

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Abstract

We have previously reported that therapeutic immunization by intramuscular injection of optimized plasmid DNAs encoding SIV antigens effectively induces immune responses able to reduce viremia in antiretroviral therapy (ART)-treated SIVmac251 infected Indian rhesus macaques. We subjected such therapeutically immunized macaques to a second round of therapeutic vaccination using a combination of plasmids expressing SIV genes and the IL-15/IL-15 receptor alpha as molecular adjuvant, which were delivered by the more efficacious *in vivo* constant-current electroporation. A very strong induction of antigen-specific responses to Gag, Env, Nef, and Pol, during ART (1.2-1.6% of SIV-specific T cells in the circulating T lymphocytes) was obtained with the improved vaccination method. Immunological responses were characterized by the production of IFN-γ, IL–2, and TNFα either alone, or in combination as double or triple cytokine positive multifunctional T cells. A significant induction of $CD4^+$ T cell responses, mainly targeting Gag, Nef, and Pol, as well as of $CD8^+$ T cells, mainly targeting Env, was found in both T cells with central memory and effector memory markers. After release from ART, the animals showed a virological benefit with a further ~1 log reduction in viremia. Vaccination with plasmid DNAs has several advantages over other vaccine modalities, including the possibility for repeated administration, and was shown to induce potent, efficacious, and long-lasting recall immune responses. Therefore, these data support the concept of adding DNA vaccination to the HAART regimen to boost the HIV-specific immune responses.

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Introduction

Although the introduction of highly active antiretroviral therapy (HAART) resulted in a remarkable decrease of AIDS related deaths, the current pharmacological regimens to treat HIV infection fail to eradicate the virus and are associated with several problems, including drug toxicity, and development of resistance with viral rebound. In this context, new strategies to control viral replication and to restore immune functions are needed. During the period of antiretroviral therapy (ART), due to efficient control of viral replication, the virus specific cellular immune responses in the periphery are strongly reduced. After interruption of ART treatment, there is a rapid increase of viremia within 10 days. The introduction of new therapeutic interventions that boost immune responses would be beneficial for the clinical management of HIV-infected individuals. Towards this goal, studies were designed to boost the immune system of SIV-infected macaques during ART using DNA immunization. It was previously observed that vaccination during ART using DNA plasmids (Lisziewicz *et al.*, 2005; Lori *et al.*, 2005; Fuller *et al.*, 2006; Lisziewicz *et al.*, 2007; von Gegerfelt *et al.*, 2007; Halwani *et al.*, 2008; Zur Megede *et al.*, 2008), pox-virus vectors (Hel *et al.*, 2000; Tryniszewska *et al.*, 2002), antigen-pulsed dendritic cells (Lu *et al.*, 2003) or peptide-pulsed blood (De Rose *et al.*, 2008) in SIVmac251-infected macaques was able to evoke SIV-specific recall immune responses. After release from ART, variable results regarding virological benefit were reported from no control (Zur Megede *et al.*, 2008), temporal control (Hel *et al.*, 2000; Tryniszewska *et al.*, 2002; Fuller *et al.*, 2006), to long-lasting control (Lori *et al.*, 2003; Lu *et al.*, 2003; Lisziewicz *et al.*, 2005; von Gegerfelt *et al.*, 2007; De Rose *et al.*, 2008). Using DNA only as vaccine, two reports showed successful immunological and virological benefit, which are intramuscular injection (von Gegerfelt *et al.*, 2007) and topical administration of DNA-based Dermavir (Lori *et al.*, 2003; Lisziewicz *et al.*, 2005). Importantly, the ability to induce immune responses able to reduce viremia could therefore offer an opportunity to use vaccination as an additional component to antiretroviral therapy. The use of DNA only as vaccination method is a promising immunization strategy that has advantages (production, stability, repeated use) over other vaccination modalities. Therapeutic vaccination in humans against HIV-1 has given mixed results. Some studies have reported an immunological and sometimes also a virological benefit, whereas others did not (Rosenberg *et al.*, 2000; Markowitz *et al.*, 2002; Lu *et al.*, 2003; Lu *et al.*, 2004; Kinloch-De Loes *et al.*, 2005; Levy *et al.*, 2005; Tubiana *et al.*, 2005; Andrieu and Lu, 2007; Hardy *et al.*, 2007; Connolly *et al.*, 2008; Pialoux *et al.*, 2008; Wilson *et al.*, 2008). Several studies suggest that vaccination during highly active antiretroviral therapy (HAART) induces HIV-specific recall responses. The efficacy of therapeutic vaccines may be variable, and it is hypothesized that more consistent immunological and virological benefits could be achieved by improving the vaccination approaches.

We focused on DNA vaccination, since previous data in macaques have been encouraging and demonstrated strong immunogenicity, long-term decrease of viral load and a survival benefit in the animals with robust response to the therapeutic vaccination (Lori *et al.*, 2003; Lisziewicz *et al.*, 2005; von Gegerfelt *et al.*, 2007). Recent developments to improve DNA delivery include *in vivo* electroporation (Aihara and Miyazaki, 1998; Mathiesen, 1999; Rizzuto *et al.*, 1999; Selby *et al.*, 2000; Widera *et al.*, 2000; Mir, 2001;Wang *et al.*, 2004b; Prud'homme *et al.*, 2006; Draghia-Akli *et al.*, 2008), which showed to be more efficient than traditional intramuscular injection in SIV/HIV DNAs vaccinating rhesus macaques and induced significantly increased antigen-specific immunity (Selby *et al.*, 2000; Otten *et al.*, 2004; Otten *et al.*, 2006; Luckay *et al.*, 2007; Hirao *et al.*, 2008; Rosati *et al.*, 2008; Zur Megede *et al.*, 2008).

In this study, we examined whether repeated immunotherapeutic vaccination is of further virological benefit. We used macaques animals previously immunized during ART with

plasmid DNAs encoding SIV antigens (von Gegerfelt *et al.*, 2007) and, after release from ART, showed a long-lasting partial control of viremia without any signs of progression towards immunodeficiency. Of the eight control animals that were ART-treated only without receiving vaccination (von Gegerfelt *et al.*, 2007), none controlled viremia after ART release and they subsequently developed AIDS. In this report, we tested whether macaques that benefited from the first cycle of immunotherapy could further benefit from a 2nd round of immunotherapeutic immunization using the combination of improved plasmid DNAs and DNA delivery by electroporation. Here, we report, the induction of strong and potent increases in SIV-specific immune responses in the immunized macaques followed by a further significant virological benefit after release from ART.

Materials and Methods

Animals

The Indian rhesus macaques (*Macaca mulatta*) included in the study were housed and handled in accordance with the standards of the Association for the Assessment and Accreditation of Laboratory Animal Care International. Screening for MHC alleles was performed by PCR (D. Watkins, Wisconsin Regional Primate Center). Monkey 538L was Mamu A*01 positive and B*17 negative, whereas the monkeys 920L and 965L were both negative for Mamu A*01 and B*17. These SIVmac251 infected animals were previously involved in a therapeutic SIV DNA vaccination study (von Gegerfelt *et al.*, 2007). Approximately 3 years after release from ART, the animals were enrolled into the present study and subjected to a 2nd round of therapeutic vaccination. During this second antiretroviral treatment period (31 weeks), the animals received the following therapeutic regimen: 20 mg/kg ((R)-9-(2-phosphonylmethoxypropyl) adenine (PMPA), and 50 mg/kg FTC, both injected subcutaneously once daily, and 5 mg/kg Didanosine (ddI), injected intravenously once daily. After two weeks, ddI was discontinued, and the dose of PMPA was reduced to 10mg/kg/day.

DNA vectors

All plasmids used in the study contain the human CMV promoter without an intron, the bovine growth hormone polyadenylation site, and the kanamycin resistance gene, pCMV.kan (Rosati *et al.*, 2005). The RNA/codon optimized genes (Schwartz *et al.*, 1992a; Schwartz *et al.*, 1992b; Nasioulas *et al.*, 1994; Schneider *et al.*, 1997) for *gag*, *pol*, and *env* were generated upon introduction of multiple silent point mutations not affecting the sequence of the encoded proteins. The animals were vaccinated with a mixture of DNAs producing secreted and intracellularly degraded variants of the SIV Gag generated by N-terminal fusion with either IP10-MCP3 (Biragyn *et al.*, 1999) (MCP3-p39gag, 21S) or with a beta-catenin-derived peptide (aa 18-47) (Aberle *et al.*, 1997) (CATEgagDX, 2S) as previously described (Rosati *et al.*, 2005; von Gegerfelt *et al.*, 2007). The authentic SIVmac239 Env protein sequence (native Env, 99S) and a fusion of Env to IP10-MCP3 replacing its native signal peptide with IP10-MCP3 (MCP3-Env, 73S) were used. The optimized *pol* with inactivating mutations in PRT, RT and INT was inserted into pCMVLAMP.kan between the human LAMP-1 luminal domain and the LAMP-1 transmembrane and cytoplasmic (TM/cyt) tail domain (Chikhlikar *et al.*, 2004), generating the plasmid LAMPpol (103S). Plasmid LAMP-NTV (147S) expresses a LAMP-Nef-Tat-Vif fusion protein. LAMP targets the antigen to lysosomes and lysosome-like compartments, which in antigen presenting cells also contain MHC molecules and was shown to affect the trafficking and immunogenicity of HIV-1 gag (Valentin *et al.*; Marques *et al.*, 2003; De Arruda *et al.*, 2004; Chikhlikar *et al.*, 2006). The rhesus macaque (rm) IL-15 plasmid (AG65) contains the optimized IL-15 DNA sequence expressing a stable mRNA encoding an IL-15 that has the native signal peptide replaced by the tPA signal and propeptide (Jalah *et al.*, 2007). Plasmid rmIL15Rα (AG120) expresses the optimized rhesus IL-15 receptor alpha (Bergamaschi *et al.*, 2007) chain.

Therapeutic immunization

Highly purified, endotoxin-free DNA plasmid preparations were produced using the Qiagen kit (Hilden, Germany). The 1 ml DNA mixture contained 100 μg of each SIV plasmid and 200 μg of the cytokine plasmids, respectively (a total of 1 mg of plasmid DNA). These DNAs were injected intramuscularly (0.5 ml per injection) at the left and right thighs using *in vivo* electroporation by the CELLECTRA® adaptive constant-current electroporator (VGX Pharmaceuticals, Inc., The Woodlands, TX).

Flow cytometric analysis

Flow cytometric analysis was performed as described (Rosati *et al.*, 2008). To determine the number and phenotype of SIV-specific cells, isolated PBMCs were incubated at a density of 10^6 cells/ml in RPMI-1640 supplemented with 10% FBS, 100 U/ml penicillin/streptomycin and 2 mM L-glutamine, in the presence of pools of 15-aa peptides overlapping by 11 aa, derived from SIV Gag, Pol, Env, Nef, and Tat at a final concentration of 1 μg/ml for each peptide. Cells were treated overnight with monensin to prevent protein secretion, and cell surface staining was performed using the following antibody cocktail: CD3-APCCy7, CD4-PerCPCy5.5, CD45RA-PE, CD28-biotin and Streptavidin-APCCy5.5 (BD Pharmingen, San Jose, CA) and CD8-AF405 (Caltag, Carlsbad, CA). Cells were washed twice, fixed, and permeabilized with Cytofix/Cytoperm (BD Pharmingen). Staining for intracellular cytokine detection was performed using the following antibody mixture: IFN-γ-FITC, IL-2-APC and TNF-α-PECy7 (BD Pharmingen). In some experiments, surface staining was performed with a different antibody cocktail including CD95-FITC, CCR7-APC, CD3-APCCy7, CD4-PerCPCy5.5, CD45RA-PE, CD8-AF405, CD28-Biotin and Streptavidin-APCCy5.5, and the presence of antigen specific cells was monitored in permeabilized cells by intracellular staining with anti-IFN-PECy7 mAb. This second staining allows the comparison of memory cell populations defined by CD28/CD45RA or CD28/CD95 expression. After intracellular staining, the cells were washed twice and the samples were analyzed in a FacsAria or LSRII flow cytometers (BD Pharmingen). All data analysis was performed using the FlowJo platform (Tree Star, Inc., Ashland, OR).

Humoral immune responses

Antibody production against Gag and Env was measured in serial dilutions of plasma by separate ELISA assays. The plates were analyzed at the absorbance of 450 nm (A_{450}). The negative cutoff value was twice the mean A_{450} values obtained with non-immune sera. All samples with A_{450} value higher than the cutoff were considered positive. The binding antibody titers are reported as the reciprocal of the highest positive dilution.

Viral load analysis

SIV RNA copy numbers were determined by a real-time nucleic acid sequence-based isothermal amplification (NASBA) assay using SIVmac251-specific primers (Romano *et al.*, 2000) with a threshold of detection of 50 copies/ml.

Results

Study design

We used SIVmac251-infected Indian rhesus macaques (538L, 920L, 965L), which were previously subjected to one round of therapeutic vaccination (von Gegerfelt *et al.*, 2007). During the previous study, the animals received three DNA immunizations, using intramuscular needle injection, which resulted in a strong virological benefit as demonstrated by decreased viral loads. These animals were kept and were monitored following the 1st cycle of therapeutic immunization (3.1, 3.7 and 3.8 years for monkey 920L, 965L and 538L,

respectively), and their viral loads remained below the levels measured prior to treatment, demonstrating a long-term benefit of therapeutic DNA vaccination.

In the present study, we report data obtained after a 2nd round of therapeutic vaccination (ART/DNA) outlined in Fig. 1A. The animals were subjected to ART for 31 weeks using PMPA, FTC and ddI as described previously (von Gegerfelt *et al.*, 2007). Starting at week 14 of ART (time point 0), the animals received 4 DNA vaccinations (week 0, 4, 8, and 15) by electroporation (EP) using the *in vivo* CELLECTRA® adaptive constant-current electroporator (VGX Pharmaceuticals, Inc.). Two weeks after EP4, ART treatment was terminated and the animals were monitored for another 16 wks (termination of the study).

The SIV DNA mixture contained optimized expression vectors for different SIV antigens; antigen sequences were modified to alter their intracellular trafficking by fusions to either MCP-3, catenin (CATE) or human lysosomal membrane associated protein 1 (LAMP) (Rosati *et al.*, 2005; von Gegerfelt *et al.*, 2007; Rosati *et al.*, 2008). The plasmid DNAs in vaccine mixture expressed Gag (MCP3gag, CATE-gag), Pol (LAMP-pol), Env (native and MCP3env), and the a Nef-Tat-Vif fusion to LAMP (LAMP-NTV) (Rosati *et al.*, 2005; von Gegerfelt *et al.*, 2007; Rosati *et al.*, 2008). A combination of rhesus IL-15 and IL-15Rα expression plasmids (Bergamaschi *et al.*, 2007; Jalah *et al.*, 2007) was used as a molecular adjuvant. During the course of this study, the animals were monitored for the development of SIV-specific immune responses and changes in the viral loads.

Virological benefit of the 2nd therapeutic vaccination

Figure 1B shows the viral load data of the vaccinated macaques prior, during and after ART. During the period preceding the start of second ART (13 weeks), the animals showed mean viral loads ranging from 3.6 to 5.1 log10. All animals responded immediately to ART treatment, resulting in persistent control of viremia (<50 copies/ml plasma). After ART release, we observed a pattern of fluctuating viral loads as previously observed (von Gegerfelt *et al.*, 2007). After the initial virus rebound, the viral load fluctuations were at levels lower than before ART/DNA, resulting in additional partial control of viremia. Importantly, the improved control of viremia was maintained during the 16 weeks of follow-up (Fig. 1B). At this point, the animals were enrolled in another study.

The mean viral loads during the 13 weeks before ART (PRE) and the 16 weeks after ART release (POST) were compared. Figure 1C shows the differential in viral loads (ΔVL) of the three animals as a result of the 2nd therapeutic vaccination. This analysis revealed a significant decrease (P=0.0356) in viremia with an average of ~1 log10 in viral load reduction. A similar benefit in viremia reduction was found by comparing viral loads during 39 weeks PRE and 16 weeks POST ART. In conclusion, the reduction in plasma viral loads demonstrates that the 2nd round of therapeutic vaccination provides an additional virological benefit in these animals.

Increase of cellular immune responses

We studied the development of SIV-specific cellular immune responses in the peripheral blood from the immunized macaques using flow cytometric analysis. Figure 2A outlines the strategy used for the flow cytometric analysis to detect antigen-specific IFN-γ positive T cells using as example PBMC from animal 965L at 3 weeks post EP3. $CD3^+$ T cells were identified within the main lymphocyte population, which was defined by forward and side scatter characteristics. The presence of SIV-specific T cells was determined by intracellular cytokine staining upon stimulation with peptide pools (15-aa peptides overlapping by 11 aa) or medium only, as negative control.

Figure 2B shows the frequency of IFN-γ secreting T cells of the vaccinated animals determined upon stimulation with peptide pools spanning five of the six SIV proteins used for vaccination (Gag, Pol, Env, Nef, and Tat). At the day of the first vaccination, none of the animals showed detectable cellular immune responses, consistent with the absence of replicating SIV due to successful ART treatment. The first DNA vaccination by electroporation (EP1) immediately induced strong immune responses in all animals, at the range of ~4,000-6,000 SIV-specific IFN-γ producing T cells/10^6 T cells. These high immune responses persisted for 4 weeks up to the day of EP2. After the 3rd vaccination, the SIV specific immune responses were further boosted to ~12,000-16,000 IFN-γ producing T cells/10^6 T cells or 1.2-1.6% of SIV-specific T cells among the total circulating T lymphocyte population. After EP4, we only analyzed the 2-week post vaccination sample, since the animals were released from ART at this point of time. As observed for EP1, subsequent vaccinations not only boosted the recall of cellular immune responses, but these immune responses persisted.

We noted that individual animals responded to the different vaccine antigens to different extents. The recall immune responses in 538L were mainly targeted to Gag, whereas 965L had similar Env and Gag responses. In contrast, animal 920L had primarily an Env response and a very poor immune response to Gag (peak 790 and mean 460 IFN-γ^+ T cells/10^6 T cells). The vaccination was unable to induce recall or *de novo* Gag-specific immune responses in this macaque. This observation suggests that the ability of 920L to respond to Gag may be exhausted, since this animal had shown higher Gag responses in the previous ART/DNA treatment (von Gegerfelt *et al.*, 2007). All animals showed significant immune responses to the Nef peptide stimulation, while the responses to the Pol peptide pool were lower. We did not detect any significant immune responses to Tat and the responses to Vif were not analyzed.

To evaluate the efficacy of the DNA delivery by *in vivo* electroporation, we compared the responses in these animals to the data obtained previously after direct intramuscular DNA injection (von Gegerfelt *et al.*, 2007). Immune responses after the 1st round of vaccination were measured by the ELIspot assay, therefore the comparison can only be approximate, comparing the cells producing IFN-γ per million (we could not repeat this measurements using intracellular cytokine staining because no frozen samples were available from the first DNA therapeutic vaccination). We noted that DNA vaccination via electroporation resulted in at least ~10× higher levels of cellular immune responses compared to the direct IM injection. Therefore, in agreement with observations by us and others (Otten *et al.*, 2004; Otten *et al.*, 2006; Luckay *et al.*, 2007; Hirao *et al.*, 2008; Rosati *et al.*, 2008; Zur Megede *et al.*, 2008), vaccination with SIV/HIV DNA plasmids using electroporation is a potent method to achieve high immune responses in macaques.

After release from ART, we found a persistence of long-lasting SIV-specific cellular immune responses (~4,000-14,000 IFN-γ^+ T cells per million T cells) without any significant changes in the distribution of the antigens recognized by the T cells for at least 2 months (Fig. 2B). While the levels of cellular responses in animals 538L and 920L remain similar during ART and after release, the cellular immune responses in animal 965L decreased immediately after release from ART. We noted that the animals with higher levels of antigen-specific T cell responses showed lower mean viral loads, indicating control of viremia by the vaccine-elicited immune responses.

Characterization of T cell subsets induced by DNA vaccination

We next analyzed the phenotype of the antigen-specific IFN-γ producing T cells in more detail. The gating strategy is shown in Figs. 3A and 3B for animal 965L (3 weeks post EP3). Subsets of memory T cells were identified based on the staining with CD28 and CD45RA: $CD3^+CD45RA^-CD28^+$ represents the population of central memory (CM) T cells and $CD3^+CD28^-$ represents effector memory (EM) T cells (Fig. 3A). Cells with an EM phenotype

were mainly $CD8^+$ T cells (91% in macaque 965L), while the majority of CM cells were $CD4^+$ T cells. These subsets were further examined for the presence of antigen-specific subpopulations as shown in the dot plots in Fig. 3B for the same animal (965L) and the data are summarized in Fig. 4 for all the animals.

A second strategy to define CM and EM cells was also used, employing the CD28 and CD95 markers in parallel to the CD28 and CD45RA markers in samples stained with all three antibodies (Fig. 5). We also analyzed the frequency of the $CD4^+$ and $CD8^+$ T cells within each population. The comparison of the antigen-specific cells using either definition ($CD3^+CD45RA^-CD28^+$ versus $CD3^+CD95^+CD28^+$ for CM and $CD3^+CD28^-$ versus $CD3^+CD95^+CD28^-$ for EM) showed no significant differences. Thus, both staining strategies give similar results for circulating T cells. These data indicate that either approach can be used to define CM and EM T cell subsets in macaque PBMC (Valentin *et al.*).

The responses against Gag (Fig. 4A) were dominated by $CD4^+$ T cells with central memory phenotype, whereas the predominant T cell responses against Env (Fig. 4B) consisted of $CD8^+$ T cells with effector phenotype. Low levels of $CD8^+$ cells with CM phenotype could be detected in all three animals in response to both Gag and Env peptide stimulation. Similar to Gag, Nef responses (Fig. 4C) were predominantly produced by $CD4^+$ T cells, which for 538L and 965L were almost exclusively cells with CM phenotype. The responses to Pol (Fig. 4D) were quantitatively the lowest and were predominantly $CD4^+$ with CM phenotype in 965L and 920L, and a mixture of $CD4^+$ and $CD8^+$ with CM and EM phenotype in macaque 538L. In conclusion, vaccination by *in vivo* electroporation during ART potently induced SIV-specific recall immune responses in different T-cell subsets. The nature of the response was also influenced by the specific antigen, as shown by comparison of the Gag and Env responses (predominantly CM CD4 for Gag versus EM CD8 for Env).

Vaccination by electroporation induces SIV-specific T cells producing IL-2 or TNFα

We further asked whether the antigen-specific T cells in these vaccinated animals produce other cytokines, such as IL-2 or TNFα (Fig. 6), in addition to IFN-γ production shown above (Figs. 2 through 5). Although we found high levels of IFN-γ-producing SIV-specific T cells after EP1 (Fig. 2B), we failed to detect SIV-specific T cells producing either IL-2 (Fig. 6A) or TNFα (Fig. 6B) until 3 weeks post EP3. From this time point on, a significant number of SIV-specific T cells producing IL-2 (Fig. 6A) and to a lesser extent TNFα (Fig. 6B) could be detected. We found these responses to be induced by all the peptide pools tested (Gag, Env, Pol, Nef, Tat). We observed that in comparison to IFN-γ (see Fig. 2B), the ratio of the responses to the individual antigens is changed for the IL-2 and TNFα producing cells, *i.e.* there are proportionally less responses to Gag and Env. As noted for the IFN-γ responses, animal 920L showed predominant Env-specific IL-2 and TNFα responses, further supporting the conclusion that this animal could no longer respond to Gag.

Vaccination by electroporation induces multifunctional SIV-specific T cells

Having established that vaccination by *in vivo* electroporation induces SIV-specific T cells able to produce significant levels of IFN-γ, IL-2 or TNFα, we further analyzed the population of antigen-specific T cells producing 2 or more cytokines. First, we analyzed double positive cells secreting IFN-γ in combination with IL-2 (Fig. 7A) and with TNFα (Fig. 7B), respectively. We found dual cytokine producing T cells induced by the Gag, Env and Nef peptide pools, respectively, in all three animals. We noted that the levels of $IFN\text{-}\gamma^+IL\text{-}2^+$ cells were significantly higher, reflecting the respective higher single-positive levels. The dual cytokine producing T cells responded mainly to Gag, Env, and Nef, and no significant responses were induced upon stimulation by the Pol and Tat peptide pools. As observed for the single cytokine

producing T cells, the nature of the responses varied among the animals with a major focus on Gag in animals 538L and 965L and on Env in animal 920L.

In addition to dual positive cells, DNA vaccination also induced SIV specific triple cytokine positive cells. Figure 8 shows a comparison of the levels of the double and triple cytokine producing SIV-specific (Gag, Env and Nef) T cells. For all animals, dual (IFN-γ plus IL-2; IFN-γ plus TNFα) as well as triple (IFN-γ, IL-2 and TNFα) positive cells were induced. Together, these findings indicate that vaccination by electroporation using the optimized DNA mixture is effective and potently induces SIV specific recall responses in T cells with multifunctional phenotypes. After ART release, the polyfunctional responses persisted in 2 of the 3 animals, but not in animal 965L that also had a significant drop of single IFN-γ responses (see Fig. 2B).

Humoral immune responses

We also monitored the humoral immune responses before, during, and after release from ART/DNA. Figure 9 shows the reciprocal levels of binding antibody against Env (Fig. 9A) and Gag (Fig. 9B), respectively. Our results show that the humoral immune responses decline during ART and the vaccination period, resulting in a reduction of ~0.5 log10 for Env (A) and ~1-2 log10 for Gag (B). These data indicate that immunization with DNA only is insufficient to maintain or boost the humoral immune responses, and this is likely due to the relative low levels of protein produced upon vaccination. After release from ART, the animals showed anamnestic humoral immune responses to Gag and Env reaching the pre-ART levels as response to the replicating SIV after ART release. Therefore, DNA electroporation maintained the T helper type 1 nature of DNA vaccination.

Discussion

DNA based immunization is an attractive vaccination approach because its production is simple and cost effective, it can be repeatedly administered and it can be combined with other vaccine modalities and molecular adjuvants. Although several trials of DNA vaccination in humans have shown encouraging, though variable results (Mwau *et al.*, 2004; Graham *et al.*, 2006; Catanzaro *et al.*, 2007; Eller *et al.*, 2007; Tavel *et al.*, 2007; Bansal *et al.*, 2008; Gorse *et al.*, 2008; Jaoko *et al.*, 2008; Kutzler and Weiner, 2008; Wang *et al.*, 2008; Wilson *et al.*, 2008), it appears that naked DNA delivery and expression is inefficient in primates compared nonhuman primates and rodents, which is one key drawback for using DNA vaccination. Different strategies are being developed to improve the efficiency of DNA gene delivery include the combination of antigen expressing plasmids with vectors producing cytokines, or the use of DNA as prime in combination with recombinant virus or protein boost [(Hartikka *et al.*, 2001; Fuller *et al.*, 2002; Lori *et al.*, 2003; Bertley *et al.*, 2004; Wang *et al.*, 2004a; Lisziewicz *et al.*, 2005; Dale *et al.*, 2006; Duerr *et al.*, 2006; Girard *et al.*, 2006; Hokey and Weiner, 2006; Liu *et al.*, 2006; Lori *et al.*, 2006; Lu, 2006; Mcmichael, 2006; Rodriguez-Chavez *et al.*, 2006; Brave *et al.*, 2007; Hinkula, 2007; Thorner and Barouch, 2007; Kutzler and Weiner, 2008; Manrique *et al.*, 2008; Wang *et al.*, 2008)]. The development of DNA delivery by *in vivo* electroporation is an important advance for DNA delivery (Aihara and Miyazaki, 1998; Mathiesen, 1999; Rizzuto *et al.*, 1999; Selby *et al.*, 2000; Widera *et al.*, 2000; Mir, 2001; Wang *et al.*, 2004b; Prud'homme *et al.*, 2006; Draghia-Akli *et al.*, 2008), and initial studies with DNAs producing HIV and SIV antigens have shown great improvement in gene expression as shown in this report and by others (Selby *et al.*, 2000; Widera *et al.*, 2000; Otten *et al.*, 2004; Otten *et al.*, 2006; Luckay *et al.*, 2007; Halwani *et al.*, 2008; Hirao *et al.*, 2008; Rosati *et al.*, 2008; Zur Megede *et al.*, 2008).

Using DNA only as vaccination modality, we had previously demonstrated a significant virological benefit in a group of SIVmac251 infected animals (von Gegerfelt *et al.*, 2007),

resulting in ~1 log10 drop in viremia. Importantly, the animals had maintained this reduced viral loads for more than 3 years. None of the control animals, which were subjected to ART treatment only without DNA vaccination, showed reduction of viral loads after release from ART (von Gegerfelt *et al.*, 2007) and subsequently developed AIDS and were no longer available. Based on the success of the first therapeutic vaccination (von Gegerfelt *et al.*, 2007), the current study was designed to examine the efficacy of a 2nd round of therapeutic immunization using macaques from that study. We decided to apply the 2nd therapeutic DNA vaccination by using the more potent *in vivo* electroporation as DNA delivery methodology, that further allowed detailed measurement of the development of SIV-specific immune responses.

We show that combination of optimized DNA vectors and *in vivo* electroporation induced high levels of SIV-specific cellular immune responses in the ART-treated SIV-infected animals. It is noteworthy that direct DNA intramuscular immunization into SIV-infected ART-treated rhesus macaques induced recall responses to all the antigens produced by the DNA mixture, but to a lower extent (von Gegerfelt *et al.*, 2007; Halwani *et al.*, 2008) when compared to the more effective *in vivo* electroporation, as shown in this report and by others (Widera *et al.*, 2000; Otten *et al.*, 2004; Otten *et al.*, 2006; Luckay *et al.*, 2007; Hirao *et al.*, 2008; Rosati *et al.*, 2008; Zur Megede *et al.*, 2008). Thus, *in vivo* electroporation of plasmid DNAs producing SIV antigens induces higher and longer-lasting primary as well as recall immune responses in macaques. We typically observed that immune responses peaked at 4 weeks post immunization. The large increase in immune responses revealed up to 1.6% of SIV-specific IFN-γ-producing T cells in the blood. Importantly, the efficient immunization method led to the induction of high levels of both $CD4^+$ and $CD8^+$ antigen-specific T cells. A large proportion of these SIV antigen-specific cells had markers of effector memory. Although DNA vaccination resulted in both $CD4^+$ and $CD8^+$ T cell memory and effector cells, we noted that the type of antigen affected the responses: Gag, Pol and Nef induced mainly $CD4^+$ T cell responses, whereas Env induced a higher $CD8^+$ T cell response. Importantly, the vaccination effects were long-lasting and also led to significant development of multifunctional SIV-specific immune responses. These findings demonstrate the potency of DNA vaccination in inducing broad and diverse cellular immune responses. In contrast to the changes in the cellular immune responses, we observed no increase in the humoral immune responses. On the contrary, we found a rather significant decline for both Gag and Env binding antibody titers during ART/DNA vaccination, as reported during the 1st immunotherapy cycle (von Gegerfelt *et al.*, 2007). Thus, DNA vaccination by *in vivo* electroporation in SIV-infected macaques continues to produce a polarized Th1 immune response. It is likely that the combination of DNA with a protein boost is necessary to activate higher antibody responses in chronically infected ART-treated animals.

Upon release from ART, the animals showed an initial virus rebound, followed by several viral load fluctuations, resulting in viral loads lower than before ART/DNA. We previously reported such fluctuations in virus levels that ultimately resulted in reduced viremia (von Gegerfelt *et al.*, 2007). This manifestation is indicative of active immune control. Although the underlying reason for the fluctuation is not known, we anticipate that the initial virus rebound is eliminated by the immune system and a new homeostasis is achieved, usually after 2-3 fluctuations, resulting in reduced virus loads compared to pre-ART levels.

It is important to note that the animals involved in this study were infected by SIV for long periods of time. ART treatment only was reported to lead to complete or partial virus control after ART discontinuation uniquely in animals treated very early after infection (Tsai *et al.*, 1998; Van Rompay *et al.*, 1999; Emau *et al.*, 2006), which is different than the experience with chronically infected macaques (Hel *et al.*, 2000; Tryniszewska *et al.*, 2002; Lisziewicz *et al.*, 2005; Fuller *et al.*, 2006; von Gegerfelt *et al.*, 2007) and humans (Chun *et al.*, 1999; Davey *et al.*, 1999; Garcia *et al.*, 1999; Neumann *et al.*, 1999; Ortiz *et al.*, 1999; Ortiz *et al.*, 2001;

Oxenius *et al.*, 2002), where the virus rebounds rapidly to levels similar to those prior to ART. This may be a critical difference with a recently reported study (Zur Megede *et al.*, 2008) in which the animals were treated with ART early after infection and DNA vaccination failed to show a virological benefit over the benefit achieved by ART only.

Important for the success of the immunotherapeutic vaccination is not only the quality of the DNA and the DNA delivery, but also the successful control of viremia during ART. Animals partially controlling viremia (median viral load of 4.9 log10) and not receiving ART during the DNA vaccination period did not show immunological or virologic benefit even with the more efficient EP DNA vaccination method. This observation is in agreement with previous studies (Hel *et al.*, 2000; von Gegerfelt *et al.*, 2007), which reported that animals that lost control of viremia due to development of drug resistance or non-adherence to drug treatment did not benefit from immunotherapy. Therefore, potent control of viremia using effective ART treatment is an essential part of the regimen in addition to the use of optimized DNA and efficient DNA delivery to induce high and long-lasting recall immune responses.

An important conclusion of our study is that rhesus macaques chronically infected with SIV benefited from a 2nd round of immunotherapy during ART. The combination of a cocktail of optimized SIV DNA plasmids and of efficient DNA delivery by *in vivo* electroporation was critical for achieving high levels of long-lasting recall immune responses. Furthermore DNA vaccination provided a virological benefit, since the therapeutically vaccinated macaques were able to further lower viremia upon release from ART. The 2nd round of immunotherapy demonstrated that an additional virological benefit can be obtained by repeated ART/DNA vaccination. In summary, our data provide support for a novel immunotherapeutic vaccination approach, which could be an addition to anti-retroviral drug therapy.

Acknowledgments

We thank D. Weiss, J. Treece, R. Pal, P. Markham and the staff at Advanced BioScience Laboratories, Kensington, MD for their expert help; N. Bischofsberger (Gilead Sciences, Inc.) for PMPA and FTC. We thank T. Jones for editorial assistance. This research was supported by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research.

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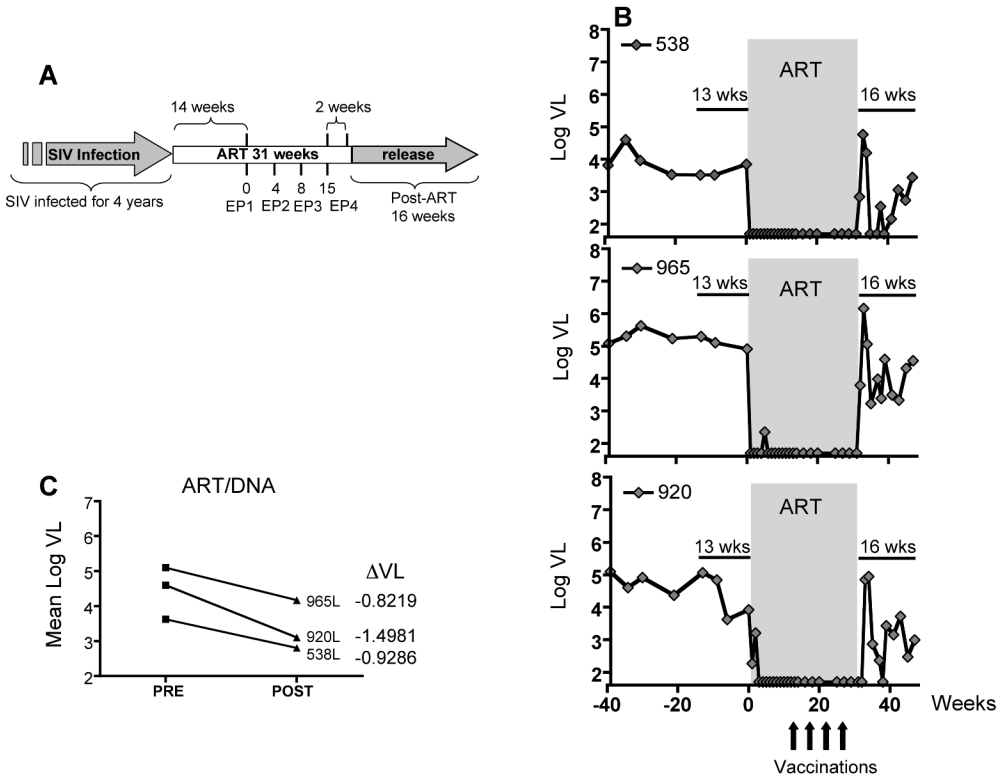
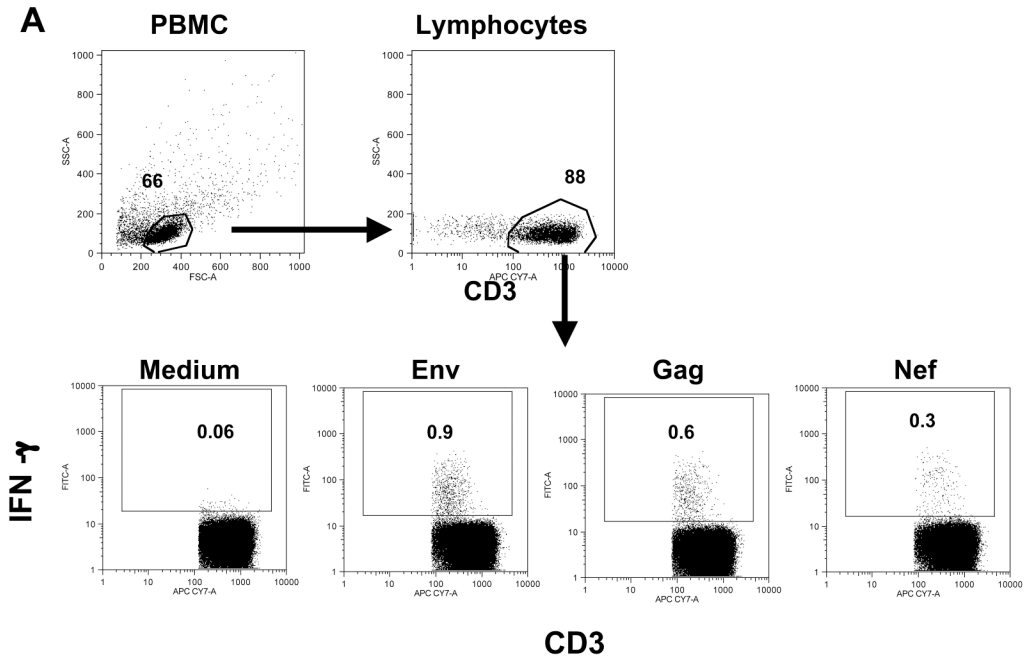


FIG. 1.

Virological benefit from 2nd round of ART/DNA. (**A**) Study Outline. SIV-infected macaques were subjected to a 2nd round of ART/DNA after more than 3 years of infection. Previously, we reported the data obtained from the 1st cycle of ART/DNA (von Gegerfelt *et al.*, 2007). After 3.1 to 3.8 years, the animals were subjected to a 2nd round of ART/DNA. The duration of the 2nd ART/DNA was 31 weeks and the animals were vaccinated by *in vivo* electroporation four times (EP1 to EP4) initiated at week 14 of ART. The animals were released from ART and monitored for another 16 weeks. (**B**) Viral loads of the three macaques during 2nd round of ART/DNA. Viral load data are shown for 39 weeks prior to ART (PRE), 31 weeks of ART/DNA and 16 weeks post release from ART (POST). (**C**) Mean virus loads and decrease in mean virus load (ΔVL) comparing 13 weeks PRE and 16 weeks POST treatment. The differences are statistically significant (paired T-test).



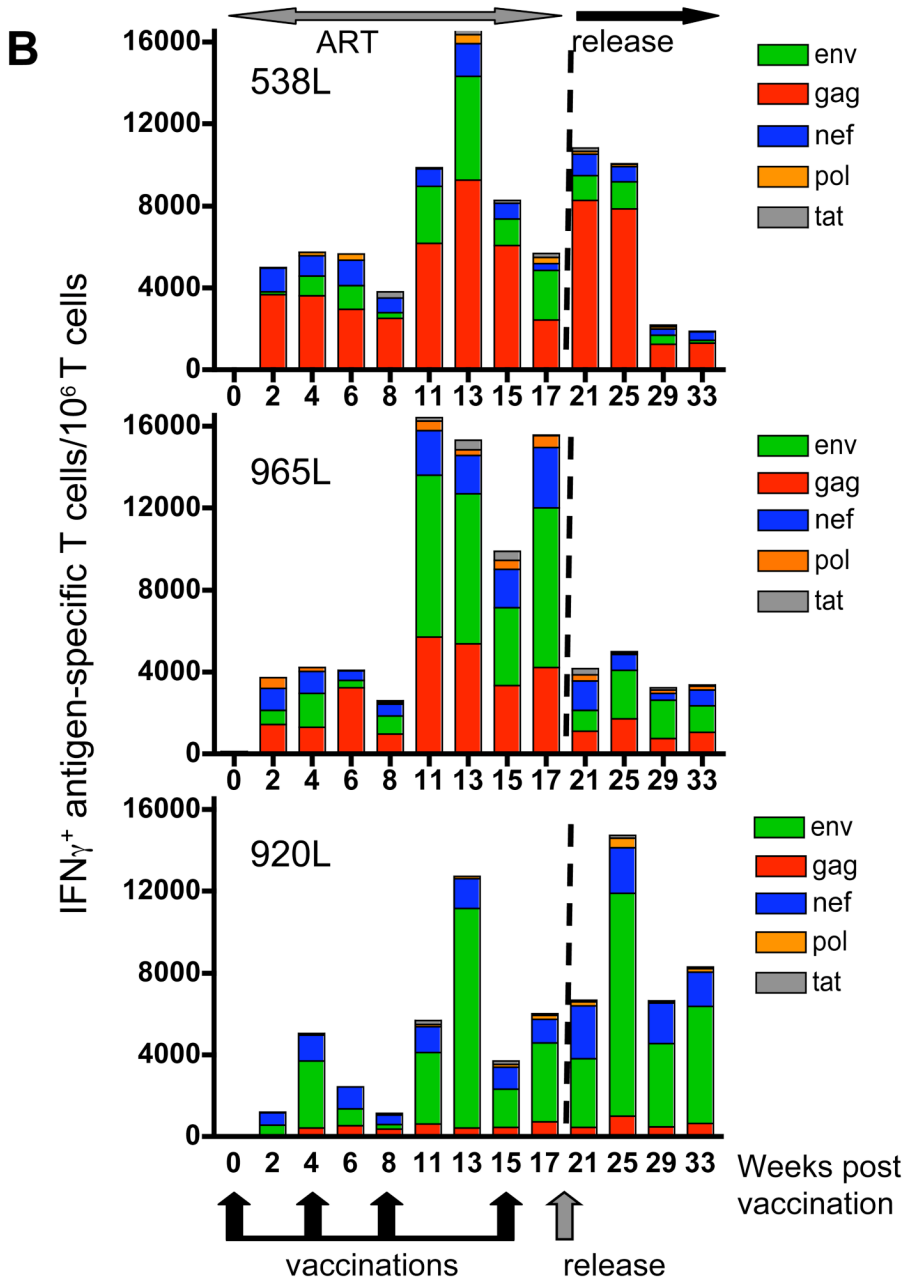
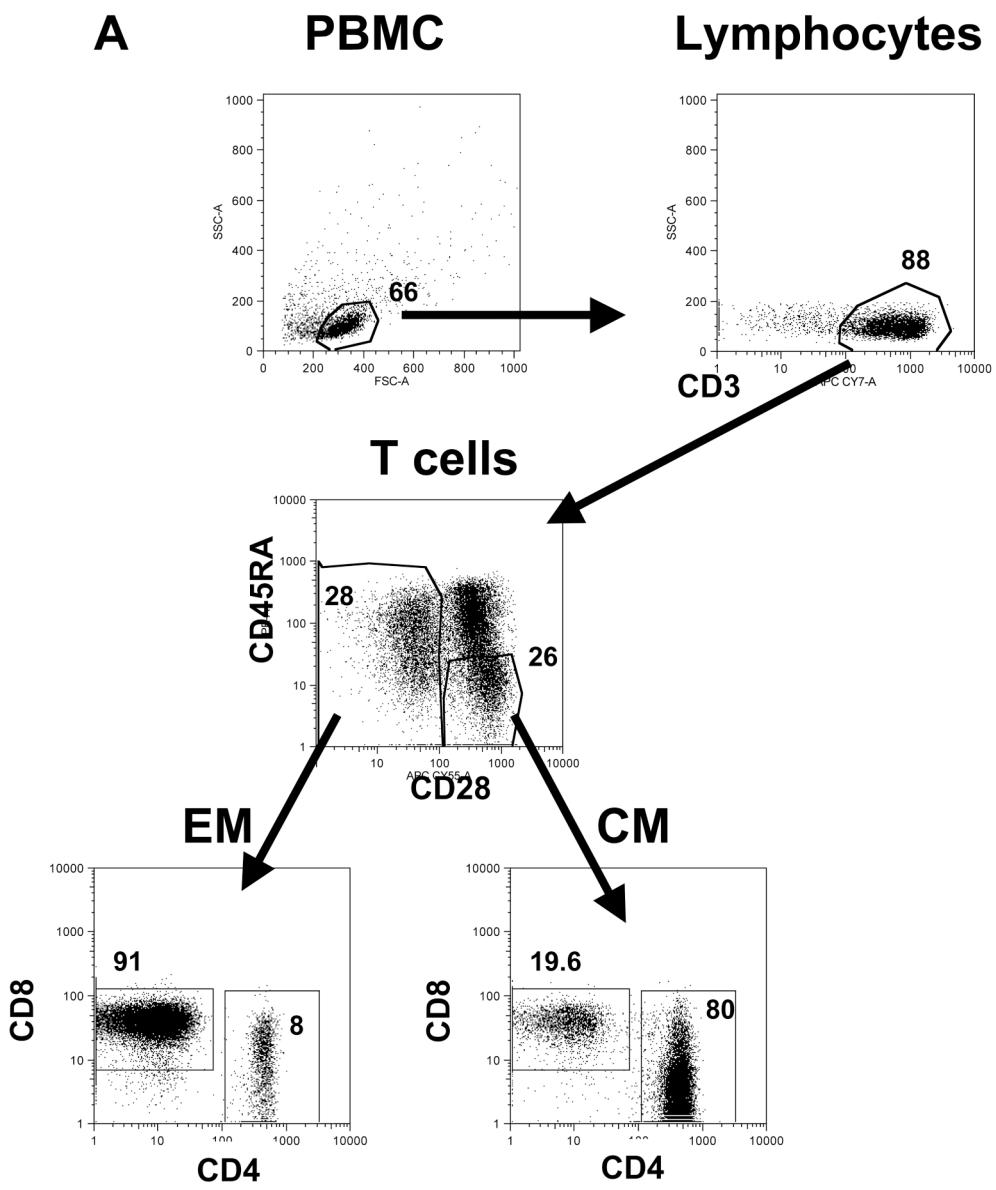


FIG. 2.

Increased SIV-specific IFN-γ producing T-cells in DNA immunized macaques during ART. (**A**) Identification of IFN-γ producing antigen-specific T cells by flow cytometry. PBMC stimulated with different peptide pools of SIV antigens were stained with a cocktail of cell surface antibodies as described in Material and Methods. The main lymphocyte population was gated based on forward and side scatter, and T cells were identified according to CD3 staining. Dot plots show the frequency of IFN-γ^+ T cells upon stimulation with Env, Gag or Nef peptide pools. The background of the assay in the presence of medium alone is shown (data is from macaque 965L at week 3 post EP3). (**B**) The numbers of IFN-γ producing T cells of the three animals after stimulation with Env, Gag, Nef, Pol and Tat peptide pools,

respectively, expressed per million circulating T lymphocytes are shown during ART/DNA and after release from ART.



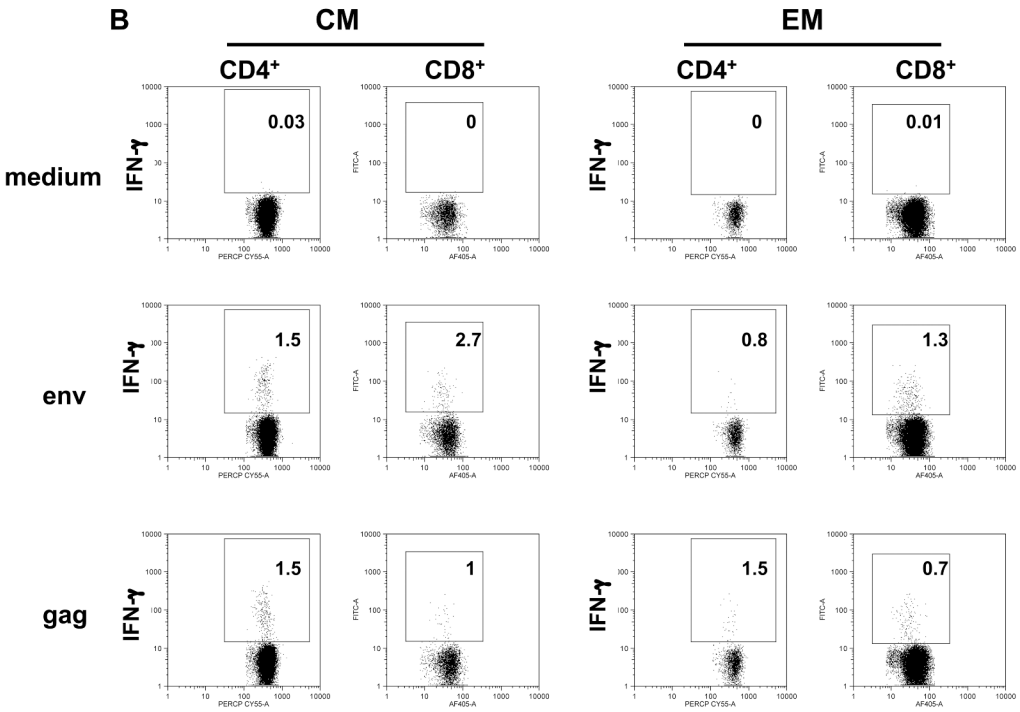
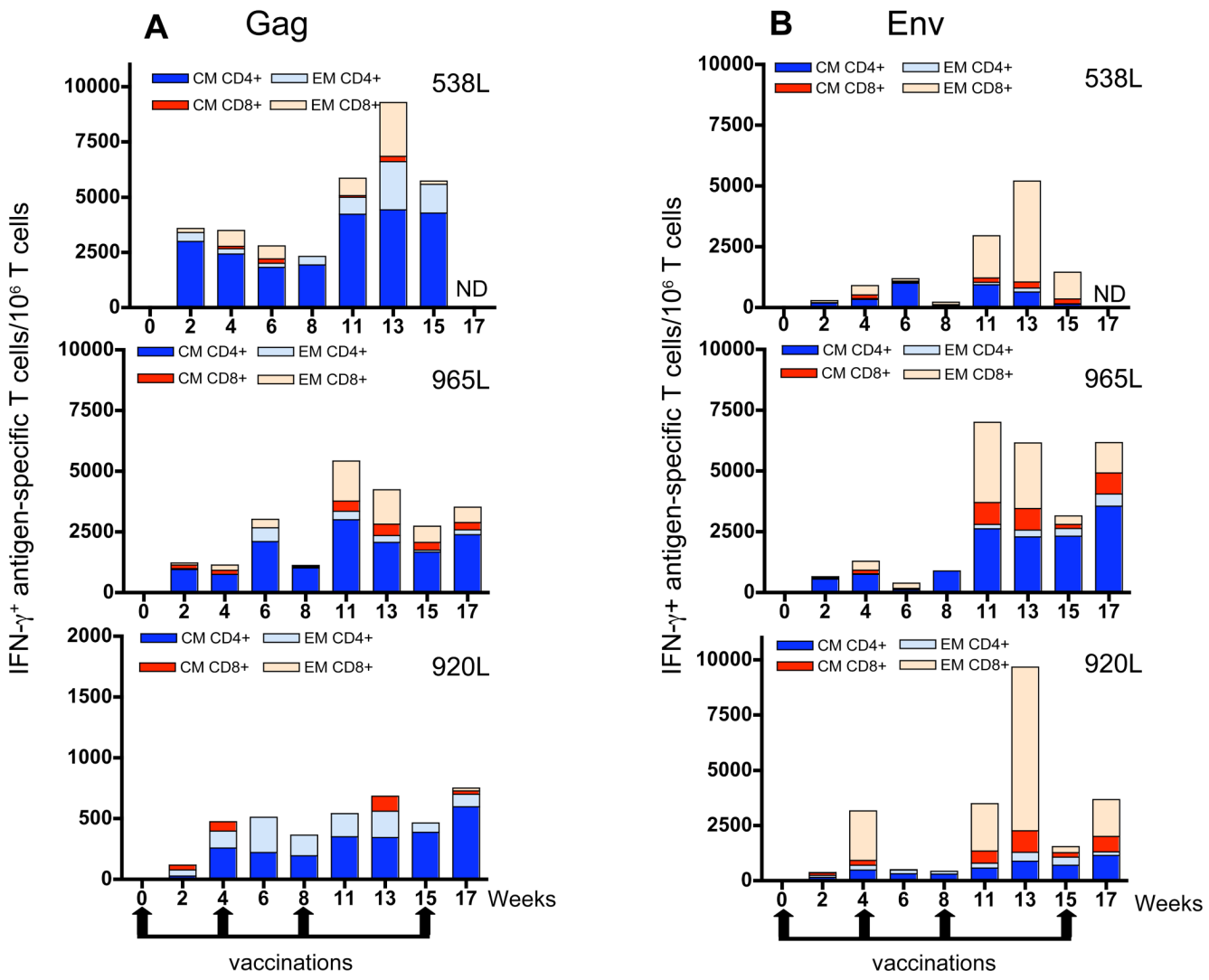


FIG. 3.

Flow cytometric analysis of T cell subsets. (**A**) Identification of the subsets of $CD4^+$ and $CD8^+$ T cells. The T cells were divided in central memory (CM) and effector memory (EM) cells based on the pattern of CD28 and CD45RA expression: $CD3^+CD28^+CD45RA^-$ for CM T cells and $CD3^+CD28^-$ for EM T cells. The two subsets of antigen experienced T cells were further divided in $CD4^+$ and $CD8^+$ populations. Cells with an EM phenotype were mainly $CD8^+$ T cells (91% in macaque 965L), while the majority of CM cells were $CD4^+$ T cells. (**B**) Identification of SIV-specific IFN-γ^+ CM and EM T cells by flow cytometry. Dot plots show the presence of $CD4^+$ and $CD8^+$ T cells with CM and EM markers producing IFN-γ in the presence of Gag and Env SIV peptide pools or medium alone. Numbers inside the gates represent the percentage of IFN-γ^+ T cells within the respective parent population. The data shown were obtained from the same animal 965L (3 weeks post EP3) as used in Fig. 3A.



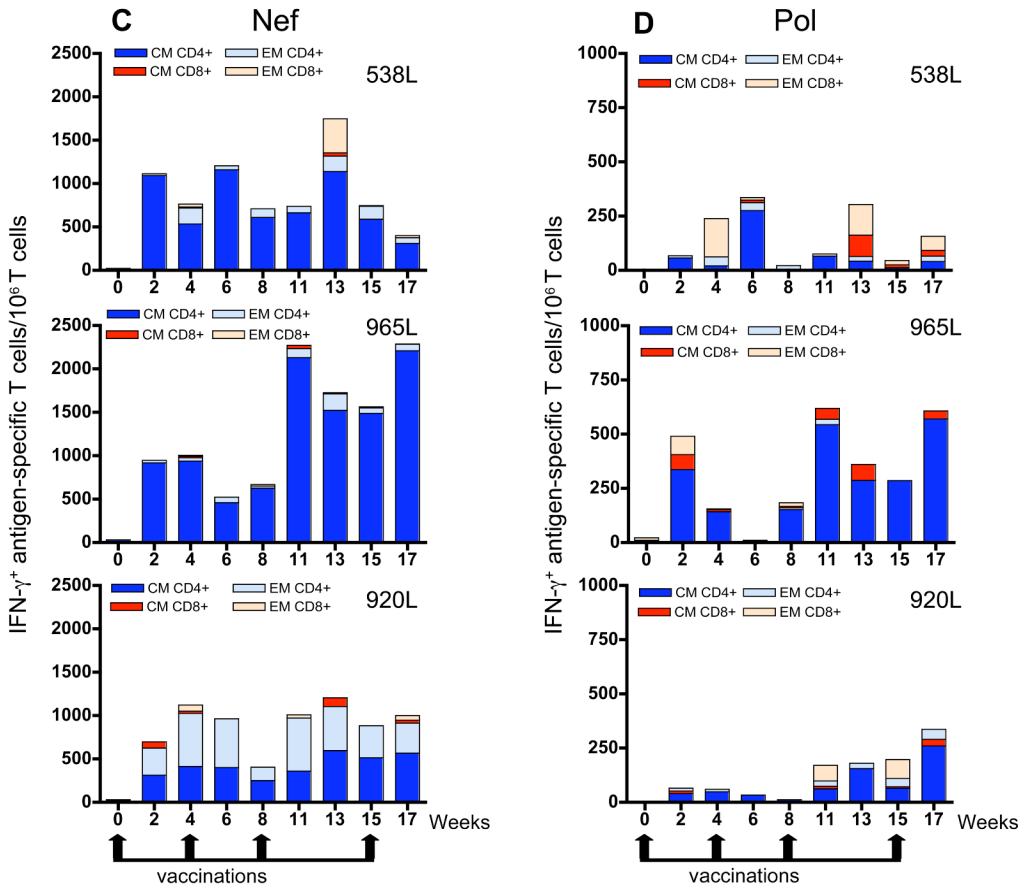


FIG. 4.

Comparison of SIV-specific IFN-γ producing T cell subsets induced by immunization during ART. Frequency of SIV-specific $CD4^+$ and $CD8^+$ T cells with central memory (CM) or effector memory (EM) markers were determined as outlined in Fig. 4. Numbers indicate IFN-γ producing T cells after stimulation with Gag (**A**), Env (**B**), Nef (**C**), and Pol (**D**) peptide pools, expressed per million circulating T lymphocytes.

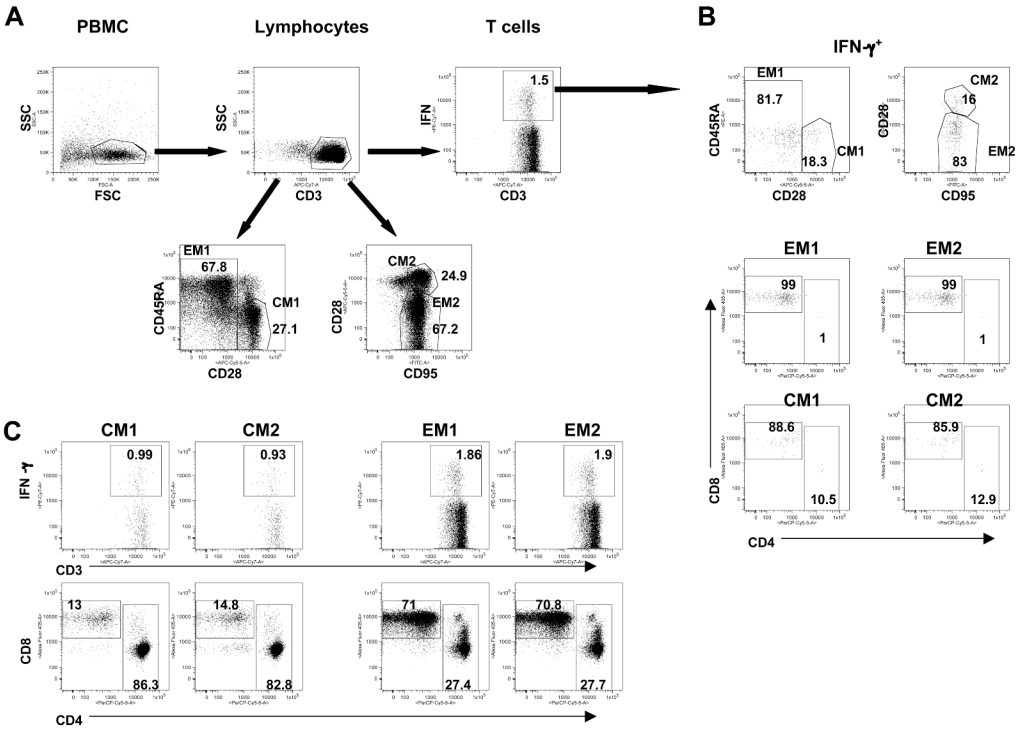


FIG. 5.

Flow cytometric analysis of SIV-specific memory T cell responses using different surface markers. This analysis shows that the frequency and phenotype of the antigen specific cells and of the memory T cell subsets are similar irrespective of the use of the combination of CD28 and CD45RA or CD28 and CD95 markers to define these T cell populations. Briefly, PBMC of macaque M538 (at 7 weeks post EP3) were analyzed by flow cytometry after peptide (env) stimulation and staining with monoclonal antibodies against different sets of markers as described in Materials and Methods. (**A**) The main lymphocyte population was gated based on forward and side scatter, and T cells were identified according to CD3 staining. These T cells were classified as effector memory (EM) and central memory (CM) cells based in the pattern of staining with CD28 and CD45RA (EM1 and CM1) or CD28 and CD95 (EM2 and CM2) (lower plots). (**B**) Phenotypic analysis of the antigen-specific (IFN-γ^+) T cells. The frequency of Env-specific T cells was determined based on IFN-γ production. The cells were classified as EM1 and EM2 and CM1 and CM2 T cells according to the expression of either CD28 and CD45RA or CD28 and CD95, respectively, and the frequency of $CD4^+$ and $CD8^+$ T cells within each of these populations was determined. (**C**) Analysis of the frequency of IFN-γ^+ Env-specific T cells, as well as the $CD4^+$ and $CD8^+$ distribution within the memory subsets as defined in panel A.

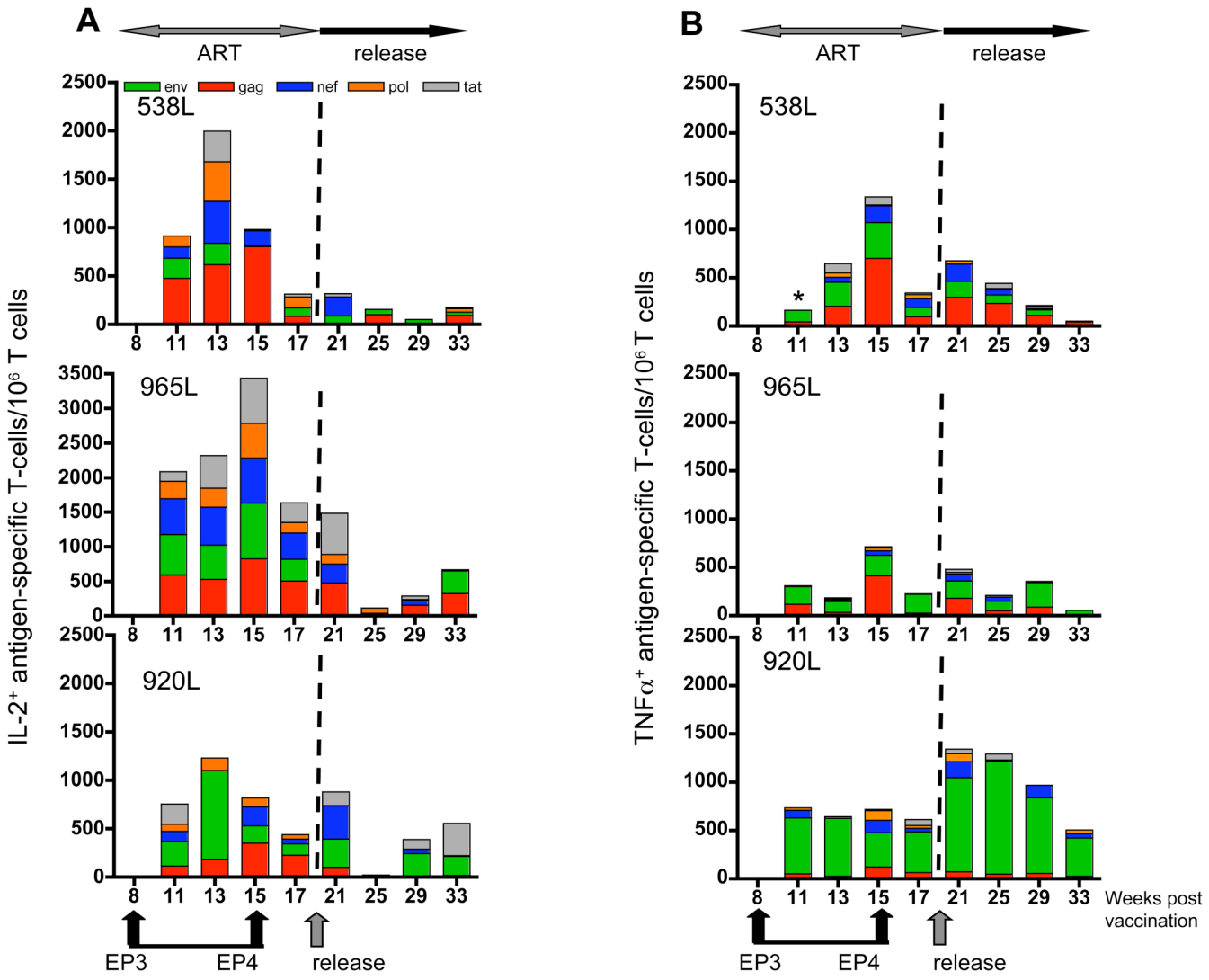


FIG. 6.

Induction of SIV-specific IL-2- and TNFα-producing T cells induced by DNA vaccination during ART. The analysis was performed using similar strategy as described for Fig. 3. IL-2 (**A**) and TNFα (**B**) producing T cells after stimulation with Env, Gag, Nef, Pol, and Tat peptide pools, respectively, were expressed per million circulating T lymphocytes. The ART and release periods are indicated. *, Tat-specific immune response was not determined.

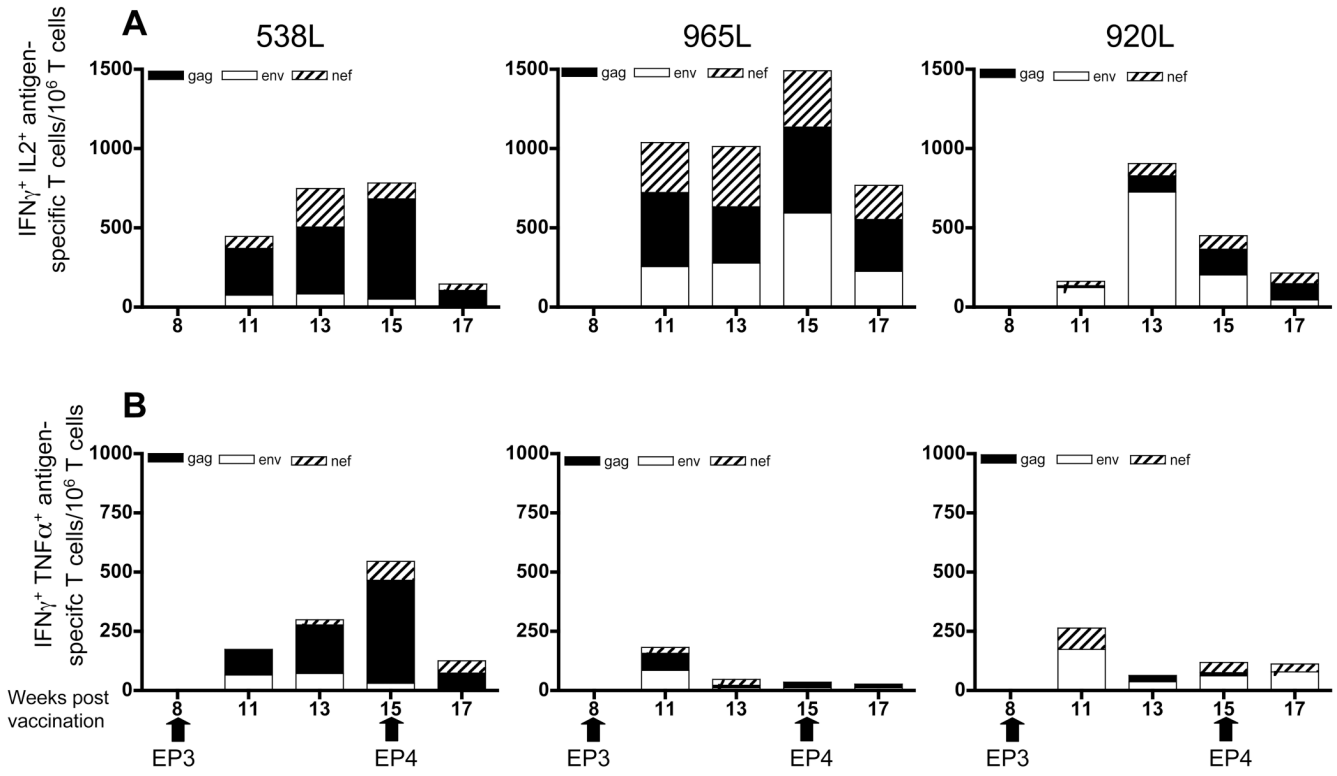


FIG. 7.

Induction of dual cytokine positive SIV-specific T cells in vaccinated animals. The dual IFN-γ plus IL-2 (**A**) and IFN-γ plus TNFα (**B**) producing SIV-specific (Gag, Env, Nef) T cells are indicated.

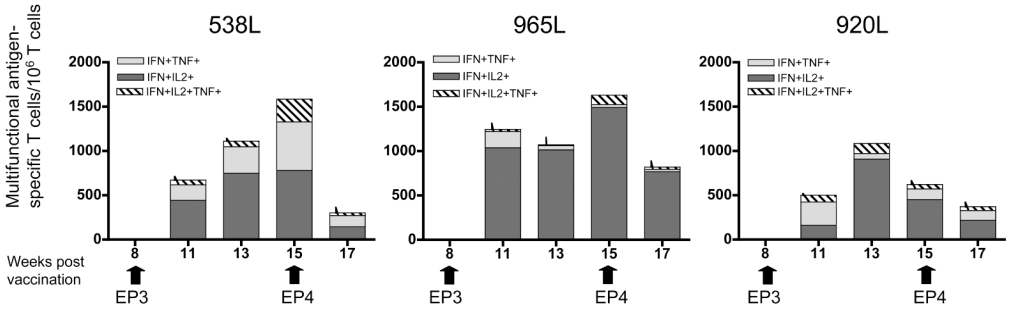


FIG. 8.

Induction of multifunctional SIV-specific T cells. A comparison of the levels of double positive (from Fig. 7; light grey IFNγ$^+$IL-2$^+$; dark grey and IFNγ$^+$ TNFα$^+$) and triple positive (IFNγ$^+$ IL-2$^+$ TNFα$^+$; striped bar) total SIV specific T cells is shown.

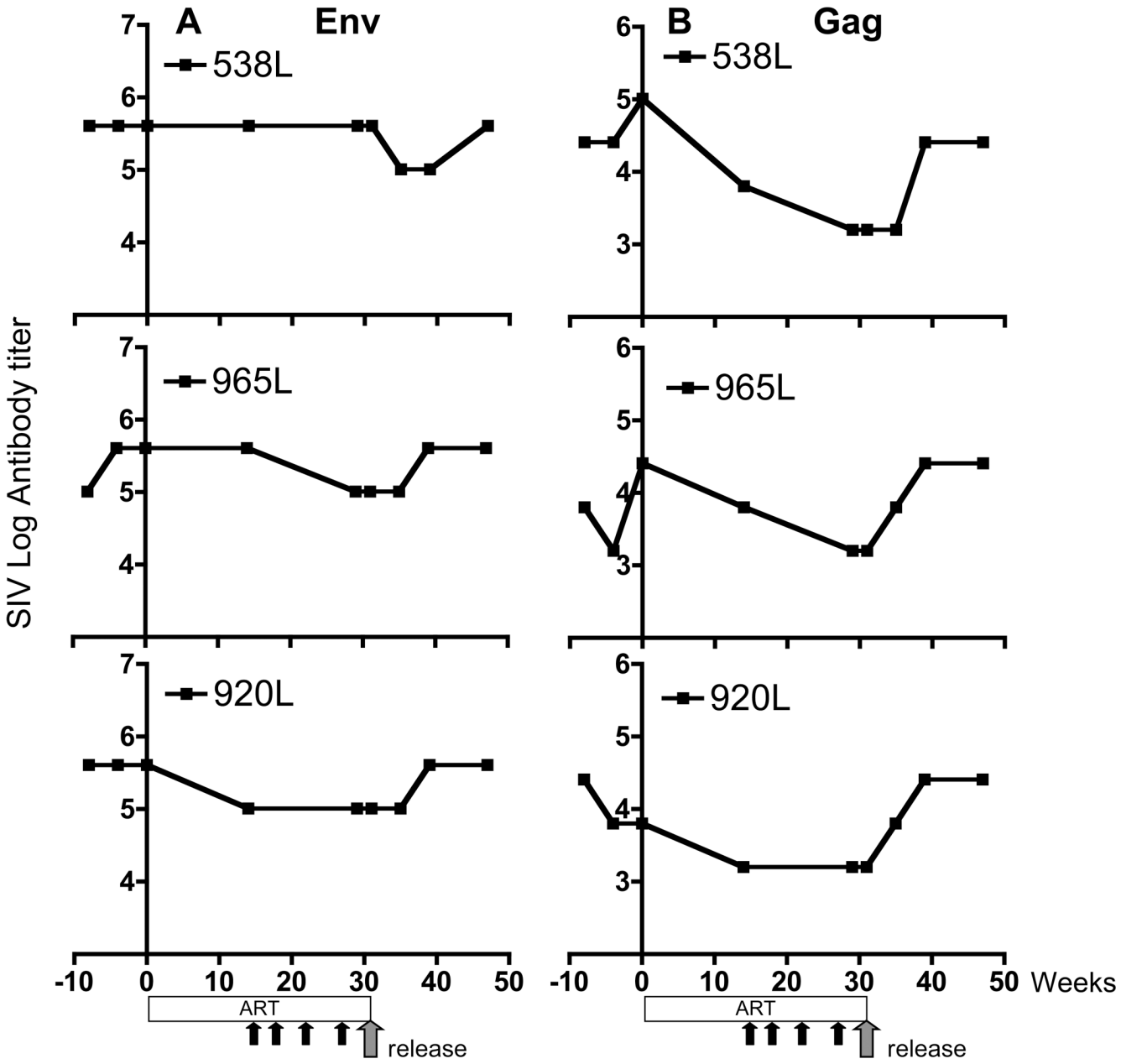


FIG. 9.

Humoral immune responses during ART and DNA vaccination and the period after release from ART. The presence of binding antibodies to Env (gp120) (**A**) and to Gag p27 (**B**) was measured in plasma prior, during and after therapy.