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Rapid Appearance of Secondary Immune Responses and Protection from Acute CD4 Depletion after a Highly Pathogenic Immunodeficiency Virus Challenge in Macaques Vaccinated with a DNA Prime/Sendai Virus Vector Boost Regimen

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Heterologous prime/boost regimens are AIDS vaccine candidates because of their potential for inducing cellular immune responses. Here, we have developed a prime/boost regimen leading to rapid control of highly pathogenic immunodeficiency virus infection in macaques. The strategy, priming by an *env* and *nef* deletion-containing simian-human immunodeficiency virus (SHIV) proviral DNA followed by a single booster with a Gag-expressing Sendai virus (SeV-Gag), efficiently induced virus-specific T cells, which were maintained for more than 3 months until challenge. While all naive control macaques showed acute $CD4^+$ T-cell depletion at week 2 after an intravenous SHIV89.6PD challenge, all the macaques vaccinated with the prime/boost regimen were protected from depletion and showed greatly reduced peak viral loads compared with controls. Vaccination with the DNA alone or SeV-Gag alone was not enough to confer the consistent protection from the depletion, although it led to efficient secondary $CD8^+$ T-cell responses at week 2 after challenge. At week 1, a difference in the secondary responses between the protected and the unprotected macaques was clear; rapid augmentation of virus-specific $CD8^+$ T cells was detected in the former but not in the latter. Thus, our results indicate the importance of rapid secondary responses for reduction in the peak viral loads and protection from acute $CD4^+$ T-cell depletion.

Cellular immune responses play a critical role in the control of immunodeficiency virus infections (8, 25). The importance of $CD8^+$ T cells in this control has been indicated in human immunodeficiency virus type 1-infected individuals (7, 15, 22) and in macaque AIDS models (11, 20, 24). Also, virus-specific $CD4^+$ T-cell responses have been shown to be essential for effective cytotoxic-T-lymphocyte responses and for controlling virus infections (3, 18, 23). Therefore, a strategy inducing virus-specific T-cell responses efficiently can be a promising AIDS vaccine candidate.

For efficient induction of the responses, we previously developed a DNA vaccine system (19) using FMSIV, which is a chimeric simian-human immunodeficiency virus (SHIV) with ecotropic Friend murine leukemia virus (FMLV) *env* in place of SHIV *env*, in combination with the FMLV receptor, mCAT1 (1), which is not normally expressed in primate cells. Vaccination of macaques with both the FMSIV proviral DNA and mCAT1 expression plasmid DNA allowed mCAT1-dependent FMSIV replication and induced resistance against intravenous challenge with a pathogenic strain of simian immunodeficiency virus, SIVmac239; the macaques vaccinated with FMSIV DNA plus mCAT1 DNA showed reduced viral loads at both the peak and the set point compared with controls (19). Further, we established an efficient antigen expression system using Sendai virus (SeV), which is a nonsegmented negative-strand RNA virus considered nonpathogenic for humans and nonhuman primates (13, 14, 21). Intranasal immunization of macaques with a recombinant SeV vector expressing SIV Gag (SeV-Gag) elicited resistance against intravenous SIVmac239 challenge, leading to marked reduction in the set-point plasma viral loads to below or just above the detectable level, although the primary acute viremia was not controlled (12). In this study, we combined these two systems to develop a prime/boost vaccine strategy and evaluated its protective efficacy in a macaque AIDS model using a highly pathogenic immunodeficiency virus. We used 14 rhesus macaques (*Macaca mulatta*) divided into four groups for the evaluation (Table 1).

The frequency of specific T cells measured by flow-cytometric analysis of intracellular interferon-γ (IFN-γ) induction is regarded as an index of antigen-specific cellular immune responses, although it does not always correlate with antigen-specific cytolytic activity (5, 9, 10, 16). Then, we examined the frequencies of the T cells reactive to the SHIV antigens other than Env and Nef, which are expected to be induced by FMSIV DNA–SeV-Gag vaccinations. In brief, an SHIV proviral DNA with *env* and *nef* deleted, SIVGP1 DNA, was obtained by removing the whole FMLV *env* region from the FMSIV DNA. COS-1 cells were cotransfected with SIVGP1 DNA and a plasmid DNA expressing vesicular stomatitis virus G protein

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TABLE 1. Vaccination and challenge protocol in macaques

Group (vaccination) and animal[a]	DNA vaccination[b]	SeV vaccination (wk)[c]	SHIV challenge (wk)
I (Naive)			
R009			SHIV89.6PD
R004	pCMVmCAT1	SeV control (12)	SHIV89.6PD (26)
R010	pCMVmCAT1	SeV control (12)	SHIV89.6PD (26)
R014		SeV control (0)	SHIV89.6PD (14)
II (DNA alone)			
R002	FMSIV + pCMVmCAT1	SeV control (12)	SHIV89.6PD (26)
R021	FMSIV + pCMVmCAT1		SHIV89.6PD (14)
R022	FMSIV + pCMVmCAT1		SHIV89.6PD (14)
III (SeV-Gag alone)			
R013		SeV-Gag (0)	SHIV89.6PD (14)
R015		SeV-Gag (0)	SHIV89.6PD (14)
R017		SeV-Gag (0)	SHIV89.6PD (14)
IV (DNA + SeV-Gag)			
R007	FMSIV + pCMVN	SeV-Gag (12)	SHIV89.6PD (26)
R011	FMSIV + pCMVN	SeV-Gag (12)	SHIV89.6PD (26)
R005	FMSIV + pCMVmCAT1	SeV-Gag (12)	SHIV89.6PD (26)
R012	FMSIV + pCMVmCAT1	SeV-Gag (12)	SHIV89.6PD (26)

[a] All the macaques were male and negative for SIV and simian retrovirus type D before use. They were maintained in accordance with the institutional guidelines for laboratory animals.

[b] The FMSIV proviral DNA, a plasmid expressing the FMLV receptor (pCMVmCAT1), and a control plasmid (pCMVN) were constructed as described previously (19). At each DNA vaccination, animals received 800 μg of individual DNA intramuscularly and 10 μg of individual DNA by gene gun as described previously (19). DNA vaccinations were performed at weeks 0, 0.5, 1, and 6 after the initial vaccination.

[c] SeV-Gag and a control SeV were recovered as described previously (12,13,14). At the SeV vaccination, animals received 10^8 cell infective units of SeV-Gag or SeV control intranasally.

(pVSV-G; Clontech, Palo Alto, Calif.) to obtain a pseudotyped SHIV bearing VSV-G, SIVGP1(VSV-G). For the SHIV-specific stimulation, 10^6 peripheral blood mononuclear cells (PBMC) were cocultured with 10^5 herpesvirus papio-immortalized B-lymphoblastoid cells (26) infected with SIVGP1 (VSV-G) for 6 h (in the presence of GolgiStop [monensin; Pharmingen, San Diego, Calif.] for the last 5 h). For nonspecific stimulation, a VSV-G-pseudotyped MLV, MLVGP(VSV-G), was used instead of SIVGP1(VSV-G). Then, intracellular IFN-γ staining was performed with a Cytofix-Cytoperm kit (Pharmingen) according to the manufacturer's protocol. Fluorescein isothiocyanate-conjugated anti-human CD4 (Pharmingen), peridinin chlorophyll protein-conjugated anti-human CD8 (Becton Dickinson, San Jose, Calif.), allophycocyanin-conjugated anti-human CD3 (Pharmingen), and phycoerythrin-conjugated anti-human IFN-γ (Pharmingen) antibodies were used. Stained samples were collected by FACScalibur and analyzed using CellQuest software (Becton Dickinson). Gating was performed on mononuclear cells and then on $CD3^+$ $CD4^+$ or $CD3^+CD8^+$ subpopulations. From the ratio of $CD3^+$ $CD4^+$ IFN-γ^+ or $CD3^+$ $CD8^+$ IFN-γ^+ cells to mononuclear cells, the frequency of $CD4^+$ IFN-γ^+ or $CD8^+$ IFN-γ^+ T cells per 10^6 cells was calculated. Then, the frequency of SHIV-specific IFN-γ^+ cells was calculated by subtracting the frequency after the nonspecific stimulation from that after the SHIV-specific stimulation.

The frequencies of specific T cells in macaque PBMC after vaccination were examined by flow-cytometric analysis (Fig. 1A). None of the group I macaques showed SHIV-specific IFN-γ induction before SHIV challenge (data not shown). In all the group II macaques vaccinated with the DNA alone, SHIV-specific T cells were clearly induced and remained detectable until challenge. In all the group III macaques vaccinated with SeV-Gag alone, significant levels of specific T cells were seen at week 2 following vaccination, but their numbers declined to marginal levels before challenge. Four animals in group IV were given SeV-Gag boosters at week 12. Before the SeV-Gag booster, two (R007 and R011) received only FMSIV DNA (with control DNA), while the other two (R005 and R012) were vaccinated with FMSIV DNA plus mCAT1 DNA. In the former two, SHIV-specific T cells were detected faintly before the SeV-Gag booster, but only a single SeV-Gag vaccination resulted in efficient induction of SHIV-specific T cells. In the latter two, the DNA vaccination induced SHIV-specific T cells efficiently and the SeV-Gag booster led to more vigorous responses. Thus, SHIV-specific T cells expanded efficiently after a single SeV-Gag booster and were kept at high levels for more than 3 months until challenge in all the group IV macaques (Fig. 1B).

For the challenge, we used a highly pathogenic SHIV89.6PD virus stock provided by Y. Lu (17). Before the present vaccine study, we had confirmed that its intravenous inoculation at the dose of 10 50% tissue culture infective doses caused almost complete depletion of peripheral $CD4^+$ T cells within 2 weeks in rhesus macaques. Then, all 14 macaques (Table 1) were challenged intravenously with 10 50% tissue culture infective doses of SHIV89.6PD. We challenged macaques in group IV with SHIV89.6PD more than 3 months after the SeV-Gag booster to examine its long-term efficacy, although intravenous challenge was performed no more than 6 weeks after the last vaccination in many previous studies.

All the macaques in groups I and III showed almost complete depletion of peripheral $CD4^+$ T cells at week 2 after challenge (Fig. 2). In group II, one animal (R022) was protected completely from depletion and a second (R002) was partially protected, but a third (R021) showed depletion. In marked contrast to these, all four macaques in group IV were

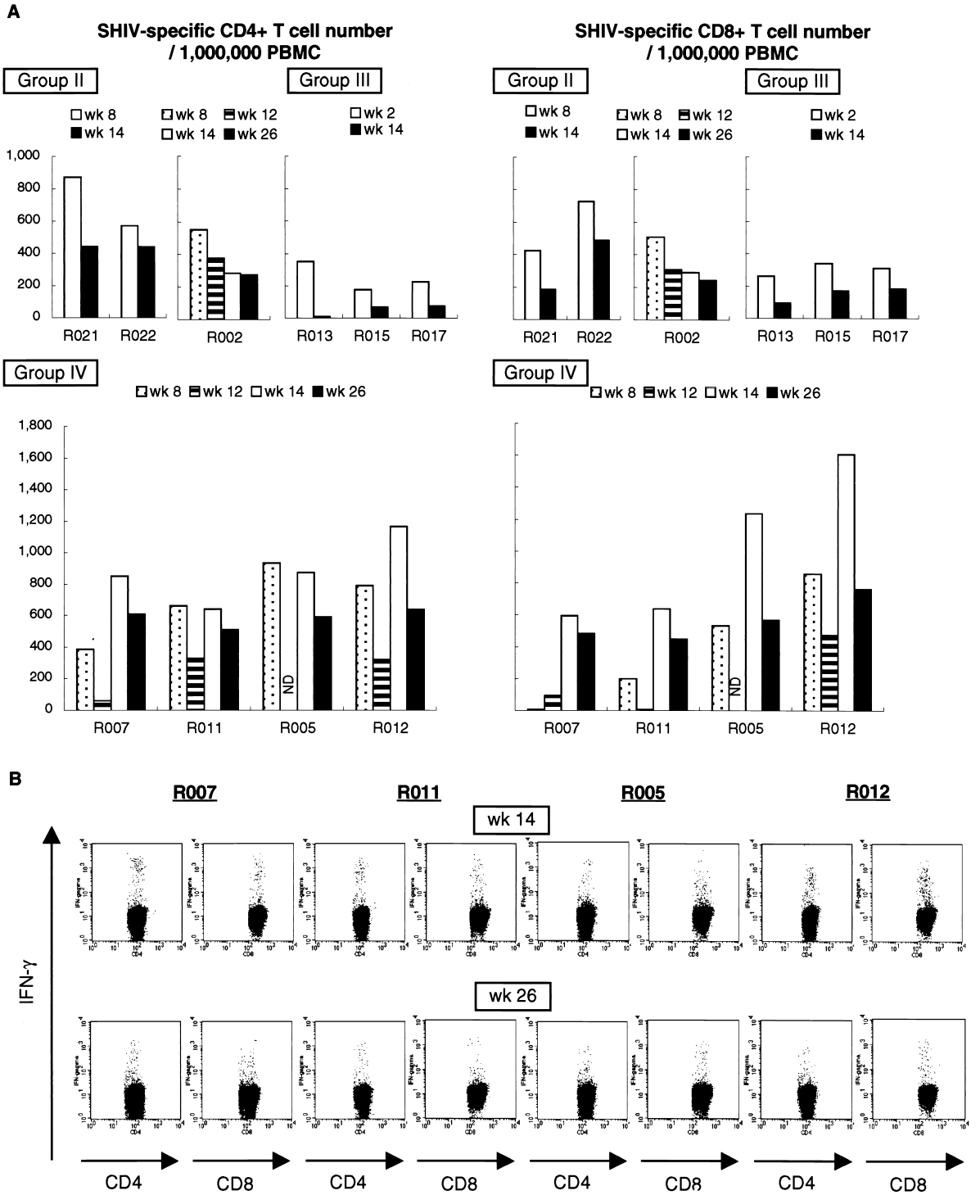

FIG. 1. SHIV-specific T-cell frequencies after vaccination. (A) SHIV-specific $CD4^+$ and $CD8^+$ T-cell frequencies in PBMC obtained at the indicated time points after the initial vaccination in group II, III, and IV macaques. In all macaques, SHIV-specific IFN-γ induction was undetectable before the initial vaccination. ND, not determined. (B) Dot plots showing SHIV-specific IFN-γ induction in group IV macaques after a SeV-Gag booster (2 weeks after the booster [wk 14] and just before challenge [wk 26]). CD4, SHIV-specific $CD4^+$ T cells, gated on $CD3^+$ $CD4^+$; CD8, SHIV-specific $CD8^+$ T cells, gated on $CD3^+$ $CD8^+$.

completely protected from $CD4^+$ T-cell depletion (Fig. 2 and Table 2).

All the group IV macaques showed greatly reduced plasma SHIV loads compared with those in group I (Fig. 2). The reduction at the peak (week 2) was striking (Table 2), and viremia was undetectable at the set point in group IV. In group II, the peak viral load was greatly reduced in R022 protected from the CD4 depletion, while no reduction was observed in

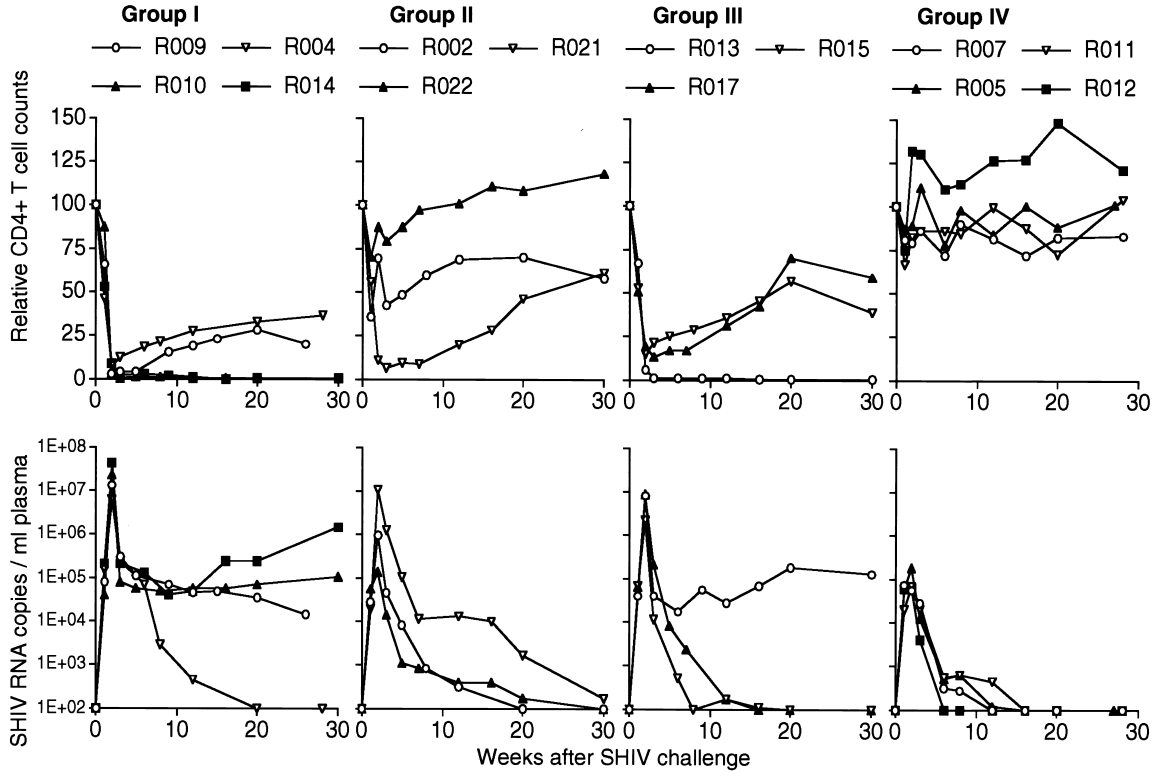

FIG. 2. Protection after SHIV89.6PD challenge. (Top) For each animal, the CD4 counts relative to that at challenge (set at 100) are shown. (Bottom) Changes in plasma SHIV RNA copy number were quantified as described previously (19).

the unprotected R021. The peak level in the partially protected R002 was between the other two. In group III, no reduction in the peak viral loads was observed, but the set-point viral loads declined to undetectable levels in two animals.

We examined frequencies of SHIV-specific T cells in PBMC at weeks 1 and 2 after challenge (Fig. 3). Augmented SHIV-specific $CD4^+$ and $CD8^+$ T cells appeared at week 1 in all the group IV macaques, while no significant secondary responses were observed at week 1 or 2 in the group I macaques. In group II, the protected macaque R022 showed augmented SHIV-specific $CD4^+$ and $CD8^+$ T cells at week 1. In contrast, the unprotected macaque R021 showed significant secondary responses of SHIV-specific $CD4^+$ T cells at week 1, but SHIV-specific $CD8^+$ T cell responses were delayed. Thus, poor protection in R021 can be explained by insufficient augmentation of SHIV-specific $CD8^+$ T cells at week 1. The group III macaques showed no significant secondary responses at week 1, but efficient augmentation of SHIV-specific $CD8^+$ T cells appeared at week 2.

AIDS vaccine strategies have been evaluated in macaque models using pathogenic SIV or SHIV (2). While most macaques develop AIDS a few years after SIV challenge, the pathogenic SHIV model shows acute CD4 depletion a few weeks after challenge. In the latter model, this study showed excellent protection by our *env*-independent prime/boost vaccination against acute CD4 depletion. Interestingly, a single protein, Gag, was sufficient as the booster antigen for protection. The macaques vaccinated with the prime/boost regimen showed rapid secondary responses of virus-specific T cells after challenge.

At week 1 after challenge, augmented SHIV-specific $CD8^+$ T cells were observed in all the protected macaques but not in any of the unprotected macaques. The group III macaques without SHIV-specific T-cell augmentation at week 1 were not protected from the $CD4^+$ T-cell depletion at week 2, although they showed efficient secondary $CD8^+$ T-cell responses at week 2. The secondary responses may lead to reduction in the set-point viral loads, as has been indicated (4, 6), but the appearance of SHIV-specific $CD8^+$ T cells at week 2 would be too late for a reduction in peak viral loads and protection from acute CD4 depletion. Thus, the turning point determining whether macaques can be protected from acute CD4 depletion is within a week after challenge. This is consistent with a previous study showing that the effects of CD8 depletion on viral loads are greater in macaques treated with anti-CD8

TABLE 2. Mean CD4 counts and viral loads at week 2 after challenge

Group	Relative mean CD4 count[a]	Plasma viral load[b] (geometric mean)
I	5.1	1.67×10^7
II	56.1	1.10×10^6
III	13.0	5.33×10^6
IV	95.4	8.30×10^4

[a] See Fig. 2, top. The CD4 counts in group IV were significantly higher than those in group I and those in group III ($P = 0.0003$ and 0.0026 by t test using StatView software, respectively).

[b] See Fig. 2, bottom. The plasma SHIV RNA copy numbers in group IV were significantly lower than those in group I and those in group III ($P < 0.0001$ and $P = 0.0004$, respectively).

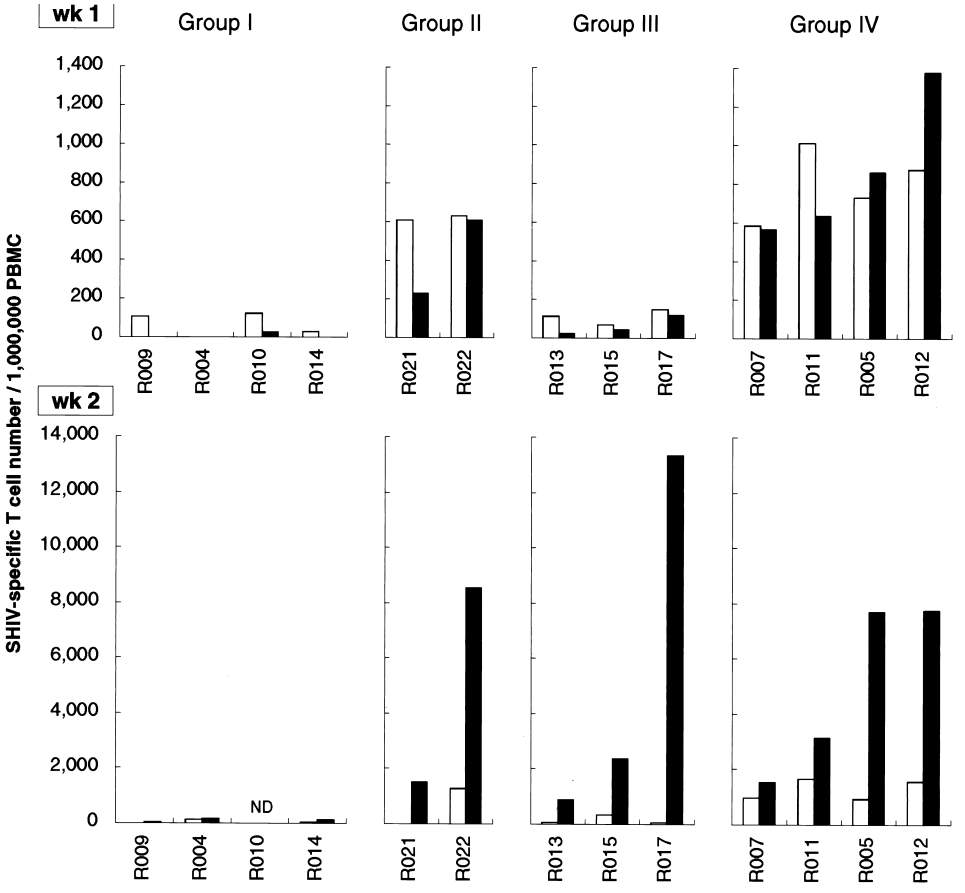

FIG. 3. SHIV-specific T-cell frequencies after SHIV89.6PD challenge. SHIV-specific $CD4^+$ (open bars) and $CD8^+$ (closed bars) T-cell frequencies in macaque PBMC at 1 and 2 weeks after challenge are shown. The frequencies in R002 were not determined because we failed to obtain enough samples from the animal. ND, not determined.

antibody just before SHIV challenge than in those treated at week 1 after challenge (20). Taken together, our results indicate that rapid secondary responses of SHIV-specific T cells, particularly $CD8^+$ T cells, were essential for the marked reduction in peak viral loads and the protection from acute $CD4^+$ T-cell depletion after challenge.

In group IV, SHIV-specific T-cell induction was not efficient before SeV-Gag booster in two macaques (R007 and R011) primed with FMSIV DNA only, but they showed levels of protection similar to those in the other two (R005 and R012) primed with FMSIV plus mCAT1 DNA. In contrast, the group III macaques vaccinated with SeV-Gag alone were not protected from acute $CD4^+$ T-cell depletion and showed delayed secondary responses. Thus, vaccination with FMSIV DNA (without mCAT1 DNA) may be required and sufficient as priming in our prime/boost regimen for the rapid secondary responses and the protection against acute CD4 depletion.

Recently, a DNA vaccine with cytokine augmentation succeeded in preventing SHIV89.6P-induced AIDS in macaques (6). Further, a DNA priming followed by a booster with a recombinant modified vaccinia virus Ankara expressing multiple proteins has been reported to control mucosal SHIV89.6P challenge (4). In vaccinated animals, however, the virus-specific T cells were undetectable in peripheral blood at week 1 after challenge, potentially reflecting the recruitment of specific T cells to the site of infection. The virus-specific secondary responses became detectable later, and the late responses correlated with set-point viral loads but not with peak viral loads. Although there were some differences between experimental conditions in those studies and in ours, the protected macaques in our study showed the fastest detectable secondary responses at week 1 after challenge. The rapid responses may explain the greater reduction in peak viral loads in our study. Thus, our prime/boost regimen showed rapid control of immunodeficiency virus infection, which could contribute to host immune function and help slow the virus epidemic.

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