

Loss of virus-specific CD4⁺ T cells with increases in viral loads in the chronic phase after vaccine-based partial control of primary simian immunodeficiency virus replication in macaques

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Virus-specific cellular immune responses play an important role in the control of immunodeficiency virus replication. However, preclinical trials of vaccines that induce virus-specific cellular immune responses have failed to contain simian immunodeficiency virus (SIV) replication in macaques. A defective provirus DNA vaccine system that efficiently induces virus-specific CD8⁺ T-cell responses has previously been developed. The vaccinated macaques showed reduced viral loads, but failed to contain SIVmac239 replication. In this study, macaques that showed partial control of SIV replication were followed up to see if or how they lost this control in the chronic phase. Two of them showed increased viral loads about 4 or 8 months after challenge and finally developed AIDS. Analysis of SIV-specific T-cell levels by detection of SIV-specific gamma interferon (IFN- γ) production revealed that these two macaques maintained SIV-specific CD8⁺ T cells, even after loss of control, but lost SIV-specific CD4⁺ T cells when plasma viral loads increased. The remaining macaque kept viral loads at low levels and maintained SIV-specific CD4⁺ T cells, as well as CD8⁺ T cells, for more than 3 years. Additional analysis using macaques vaccinated with a Gag-expressing Sendai virus vector also found loss of viraemia control, with loss of SIV-specific CD4⁺ T cells in the chronic phase of SIV infection. Thus, SIV-specific CD4⁺ T cells that were able to produce IFN- γ in response to SIV antigens were preserved by the vaccine-based partial control of primary SIV replication, but were lost with abrogation of control in the chronic phase.

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INTRODUCTION

Cellular immune responses play a critical role in the control of immunodeficiency virus infections (Brander & Walker, 1999; Seder & Hill, 2000). The importance of virus-specific CD8⁺ T cells, especially cytotoxic T lymphocytes (CTLs), in this control has been indicated in human immunodeficiency virus type 1 (HIV-1)-infected individuals (Borrow *et al.*, 1994; Koup *et al.*, 1994; Ogg *et al.*, 1998) and in macaque AIDS models (Matano *et al.*, 1998; Jin *et al.*, 1999; Schmitz *et al.*, 1999). Therefore, CTL-based vaccine strategies may be promising for the development of AIDS vaccine candidates.

AIDS vaccine strategies have been evaluated in macaque models by using simian immunodeficiency viruses (SIV) or simian–human immunodeficiency viruses (SHIV)

(Nathanson *et al.*, 1999). Macaques infected with a pathogenic SIV strain, SIVmac239 (Kestler *et al.*, 1990), generally show chronic clinical courses in the development of AIDS, whereas infections with a pathogenic SHIV strain, SHIV89.6P (Karlsson *et al.*, 1997), induce acute CD4⁺ T-cell depletion in a few weeks, leading to the acute onset of AIDS in macaques. Recently, in the latter model, several groups have developed vaccine strategies that induced high levels of virus-specific CTLs, leading to containment of SHIV89.6P replication (Barouch *et al.*, 2000; Amara *et al.*, 2001; Matano *et al.*, 2001; Rose *et al.*, 2001; Shiver *et al.*, 2002). However, it has been suggested that SIV infection models may reflect HIV-1 infections in humans more closely, whereas no preclinical vaccine trials successfully contained SIV replication in rhesus macaques (Feinberg & Moore, 2002; Horton *et al.*, 2002).

We previously developed a DNA vaccine system by using FMSIV (Matano *et al.*, 2000), which is a chimeric SHIV with ecotropic Friend murine leukaemia virus (FMLV) *env* in place of SHIV *env*, in combination with the FMLV receptor, mCAT1 (Albritton *et al.*, 1989), which is not normally expressed in primate cells. Vaccination of macaques with both FMSIV proviral DNA and mCAT1 expression plasmid DNA allowed mCAT1-dependent FMSIV replication and efficiently induced SIV-specific CD8⁺ T-cell responses (Matano *et al.*, 2000; Takeda *et al.*, 2000). After intravenous SIVmac239 challenge, the vaccinated macaques showed low plasma viral loads during the early phase of infection, although they failed to contain SIV replication.

Further, we established a Sendai virus (SeV) vector-based vaccine system that efficiently induced virus-specific CD8⁺ T-cell responses (Kano *et al.*, 2002). Intranasal immunization of macaques with a recombinant SeV vector expressing SIV Gag (SeV-Gag) elicited Gag-specific CD8⁺ T-cell responses, leading to marked reduction in set point plasma viral loads after intravenous SIVmac239 challenge (Kano *et al.*, 2000).

This study is a longitudinal analysis of those vaccinated macaques that showed partial control of primary SIVmac239 replication after challenge. Analysis revealed that some of them failed to keep plasma viral loads at low levels. Analysis of SIV-specific T-cell levels by detection of SIV-specific gamma interferon (IFN- γ) production revealed that the increases in viral loads in the chronic phase were accompanied by loss of SIV-specific CD4⁺ T cells, but occurred in the presence of SIV-specific CD8⁺ T cells.

METHODS

DNA and SeV vectors. DNA of an infectious SHIV clone, SHIV_{MD14YE} (Shibata *et al.*, 1997), was provided by M. A. Martin. The gene fragment encoding the Env surface protein of SHIV_{MD14YE} was removed and replaced with an FMLV *env* fragment (Koch *et al.*, 1983) to obtain infectious FMSIV clone DNA, as described previously (Matano *et al.*, 2000). The 3' portion of the *env* gene and the 5' quarter of the *nef* gene were deleted in the FMSIV DNA. Therefore, the FMSIV DNA has SIV-derived long terminal repeat, *gag*, *pol*, *vif*, *vpx* and partial *vpr* sequences, HIV-1-derived partial *vpr*, *tat*, *rev* and partial *env* (containing the second exon of *tat*, the second exon of *rev*, and RRE) sequences and FMLV-derived *env* sequences. A plasmid expressing mCAT1, pJET (Albritton *et al.*, 1989), was provided by J. M. Cunningham. An *env*- and *nef*-deleted SHIV proviral DNA, SIVGP1 DNA, was obtained by removing the whole FMLV *env* region from the FMSIV DNA (Matano *et al.*, 2001). An infectious SIVmac239 clone DNA, pBRmac239, was provided by T. Kodama and R. C. Desrosiers (Kestler *et al.*, 1990). The plasmid pVSV-G, which expresses vesicular stomatitis virus G protein (VSV-G), was purchased from Clontech. A recombinant SeV vector expressing SIV Gag (SeV-Gag) was prepared as described previously (Kato *et al.*, 1996; Kano *et al.*, 2002).

Animal experiments. All Indian rhesus macaques (*Macaca mulatta*) and cynomolgus macaques (*Macaca fascicularis*) used in this study were male and were maintained in accordance with the Guidelines for Laboratory Animals of the National Institute of Infectious Diseases. These macaques tested negative for SeV and SIV

before use. Blood collection, lymph node (LN) biopsy, vaccination and virus challenge were performed under ketamine anaesthesia. The DNA vaccine experiment using rhesus macaques was performed as described previously (Matano *et al.*, 2000). Rhesus macaques #20, #21 and #18 received FMSIV DNA and pJET (100 or 200 μ g of each DNA intramuscularly and 5 or 10 μ g of each DNA by gene gun) five times at weeks 0, 1, 2, 6 and 12 after the initial vaccination. Rhesus macaque #17 received 200 μ g FMSIV DNA intramuscularly and 10 μ g FMSIV DNA by gene gun five times at weeks 0, 1, 2, 6 and 12 as a control. Rhesus macaque #22 was a naive control. These macaques were challenged intravenously with 100 TCID₅₀ (50 percent tissue culture infective dose) of SIVmac239 12 weeks after the last vaccination. Macaque #21 was observed until week 218 after challenge and other rhesus macaques were observed until their death. The SeV-Gag vaccine experiment using cynomolgus macaques was performed as described previously (Kano *et al.*, 2000). Cynomolgus macaques Cy01 and Cy62 were immunized intranasally with 10⁸ cell infectious units (CIU) of SeV-Gag three times at weeks 0, 4 and 14 after the initial vaccination and challenged intravenously with 100 TCID₅₀ of SIVmac239 8 weeks after the last vaccination. These macaques were observed until week 60. Diagnosis of AIDS was based on clinical signs, such as diarrhoea and loss of body weight, and histological signs, such as lymphocyte depletion and lymphoma.

Quantification of plasma viral loads. Plasma RNA was extracted by using a High Pure Viral RNA kit (Roche). For quantification of plasma SIV RNA levels, serial five-fold dilutions of RNA samples were amplified in quadruplicate by reverse transcription and nested PCR using SIV *gag*-specific primers to determine the end point as described previously (Shibata *et al.*, 1997). For preparing the RNA standard, we first set up the method for quantification of SHIV RNA copy number by using HIV-1 *vpu*-specific primers and an HIV-1 standard, which was quantified by an Amplicor HIV-1 monitor (Roche). By using this method, we prepared an SHIV standard for the present assay. At several time-points, RNA copy number was reassessed by real-time PCR using the LightCycler system (Roche) with SIV *gag*-specific primers (GTAGTATGGGCAGCAAATGA and TGTTCCTGTTCCACCACTA) and probes (GCATTCACGCAGAGAGAAAGTGAAACA and ACTGAGGAAGCAAAACAGATAGTGCAGAGA).

Flow cytometric analysis of SIV-specific IFN- γ induction. We measured frequencies of SIV-specific T cells by flow cytometric analysis of intracellular IFN- γ induction after SIV-specific stimulation, as described previously (Matano *et al.*, 2001). In brief, COS-1 cells were cotransfected with SIVGP1 and pVSV-G to obtain a pseudotyped SIV bearing VSV-G, SIVGP1(VSV-G). Alternatively, another pseudotyped SIV, SIV239(VSV-G), was obtained by cotransfection of COS-1 cells with pBRmac239 and pVSV-G. Peripheral blood mononuclear cells (PBMCs) were prepared by density-gradient centrifugation using Ficoll-Paque PLUS (Amersham Biosciences) and frozen until use. For SIV-specific stimulation, PBMCs were co-cultured with autologous herpesvirus papio-immortalized B lymphoblastoid cells (BLCs) (Voss *et al.*, 1992) that were infected with SIVGP1(VSV-G) or SIV239(VSV-G). Stimulation with SIVGP1(VSV-G)-infected BLCs was expected to stimulate SIV Gag-, Pol-, Vif- and Vpx-specific T cells and is referred to as Env-independent SIV-specific or SIVGagPol-specific stimulation. On the other hand, stimulation with SIV239(VSV-G) was expected to stimulate all T cells that were reactive to SIV antigens and is referred to as SIVGagPolEnv-specific stimulation. For non-specific stimulation, a VSV-G-pseudotyped murine leukaemia virus (MLV), MLVGP(VSV-G), was used instead of SIVGP1(VSV-G) or SIV239(VSV-G). After co-culture in the presence of GolgiStop (monensin) (Becton Dickinson) for 6 h, intracellular IFN- γ staining was performed by using a Cytofix-Cytoperm kit (Becton Dickinson).

FITC-conjugated anti-human CD4, peridinin chlorophyll protein-conjugated anti-human CD8, allophycocyanin-conjugated anti-human CD3, FITC-conjugated anti-human CD45RA and phycoerythrin-conjugated anti-human IFN- γ antibodies (Becton Dickinson) were used. Stained samples were collected by FACSCalibur and analysed by using CellQuest software (Becton Dickinson). SIV-specific T-cell levels were calculated by subtracting the IFN- γ ⁺ T-cell frequencies after non-specific stimulation from those after SIV-specific stimulation. The frequencies of CD4⁺ IFN- γ ⁺ T cells in CD4⁺ T cells or those of CD8⁺ IFN- γ ⁺ T cells in CD8⁺ T cells after non-specific stimulation were <0.05%.

RESULTS

Follow-up of DNA-vaccinated macaques after SIV challenge

In our previous study (Matano *et al.*, 2000), two rhesus macaques (#20 and #21) that had been immunized with our DNA vaccine system using FMSIV and mCAT1 DNA were challenged intravenously with SIVmac239; both of them showed reduced plasma viral loads, compared to the control rhesus macaques (#22 and #17) in the early phase of infection. In addition, rhesus macaque #18 was subjected to the same FMSIV and mCAT1 DNA vaccine and SIVmac239 challenge protocol and also showed partial control of primary SIV replication with low plasma viral loads, <2 × 10³ copies ml⁻¹, at the set point. We followed up these macaques in the chronic phase of SIV infection in this study.

The unvaccinated macaque, #22, failed to control viraemia after challenge and showed acute onset of AIDS, as described previously (Matano *et al.*, 2000). The animal lost its body weight with severe diarrhoea and maintained high plasma viral loads, >1 × 10⁶ RNA copies ml⁻¹, until its death at week 17. Macaque #17, which was vaccinated with FMSIV DNA alone, also failed to control viraemia, with high set point plasma viral loads of >1 × 10⁵ RNA copies ml⁻¹, as described previously (Matano *et al.*, 2000), and showed disease progression with loss of body weight until euthanasia at week 45 (data not shown). The autopsy revealed malignant lymphoma and *Pneumocystis carinii* pneumonia.

Three macaques that were vaccinated with FMSIV and mCAT1 DNA showed partial control of primary SIV replication, but two of them lost this control and developed AIDS 2 or 3 years after challenge. In macaque #18 (Fig. 1), plasma viral loads were kept at low levels (<2 × 10³ copies ml⁻¹) until week 24 after challenge, but began to increase after that. The animal maintained high viral loads, about 1 × 10⁶ RNA copies ml⁻¹, after week 36, began to lose body weight after week 54 and died at week 81. In macaque #20 (Fig. 2), plasma viral loads were kept at low levels, about 1 × 10⁴ RNA copies ml⁻¹, until week 12, but began to increase after that. Peripheral CD4⁺ T-cell counts decreased gradually after week 15. The animal remained alive with a high viral load for more than

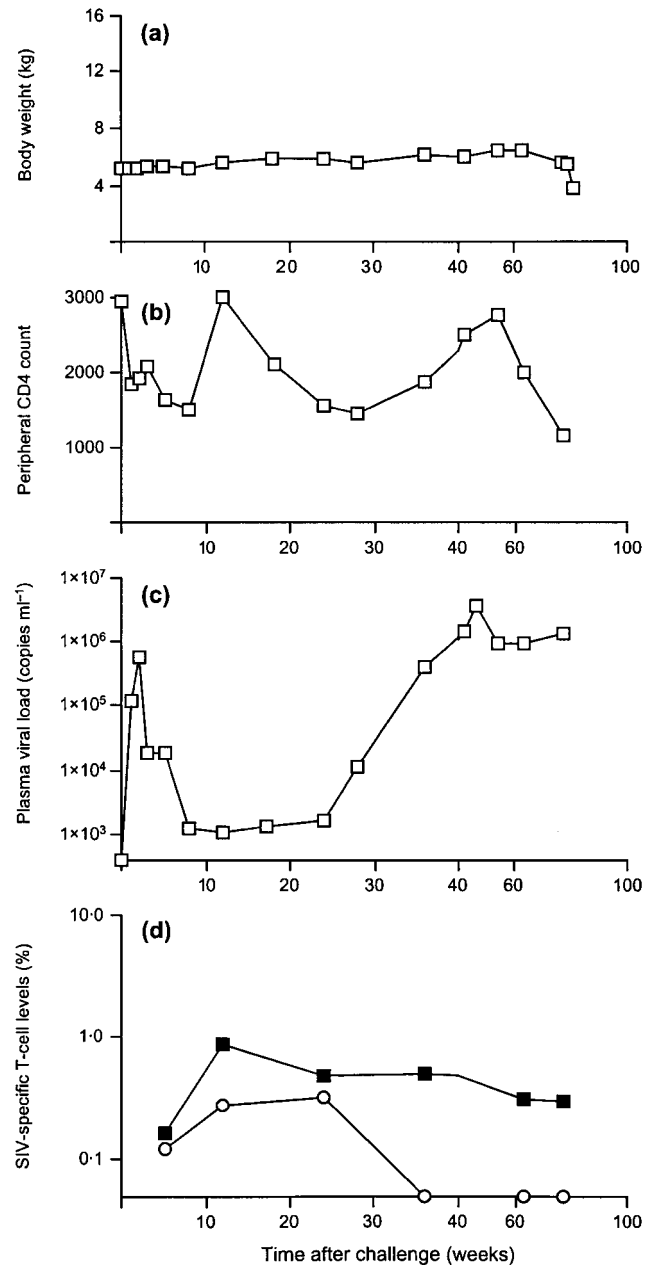


Fig. 1. Follow-up of macaque #18 after SIV challenge. (a) Body weight (kg); (b) peripheral CD4⁺ T-cell count (μl⁻¹); (c) plasma viral load (SIV RNA copy number ml⁻¹); (d) SIV-specific T-cell level, measured by assessing frequencies of IFN- γ producing cells after SIVGagPol-specific stimulation. Proportions of SIVGagPol-specific CD4⁺ T-cell number in the total CD4⁺ T-cell number (○) and of SIVGagPol-specific CD8⁺ T-cell number in the total CD8⁺ T-cell number (■) are shown.

2 years, but finally developed AIDS and was euthanized at week 136. On the other hand, macaque #21 showed sustained control of SIV replication without disease for more than 3 years (Fig. 3). Plasma viral loads were below or just above the detectable level from week 31 to week

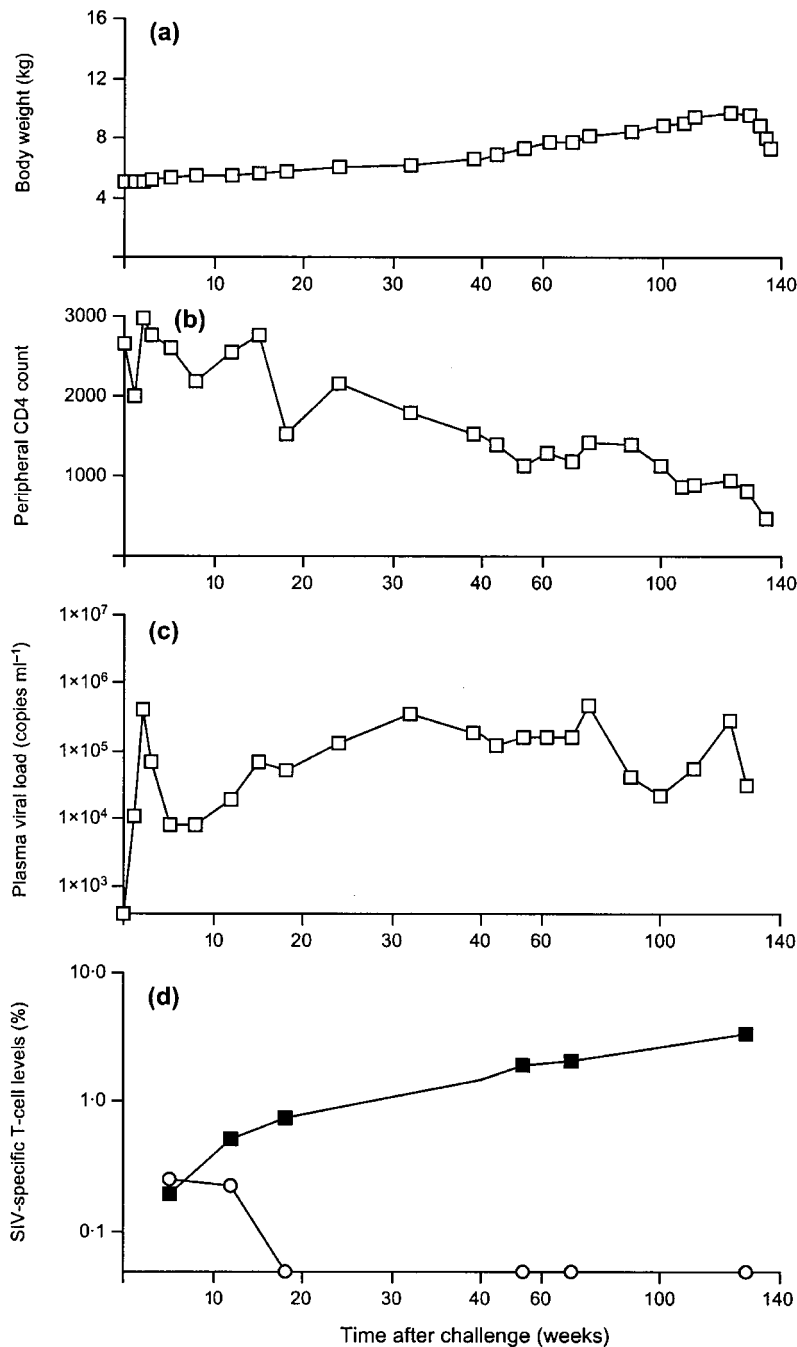


Fig. 2. Follow-up of macaque #20 after SIV challenge. Legend as for Fig. 1.

139 and then began to increase gradually, but were still $< 2 \times 10^4$ RNA copies ml^{-1} , even at week 218.

SIV-specific T-cell levels in DNA-vaccinated macaques after SIV challenge

The FMSIV DNA used in the vaccine has SIVmac239 Gag-, Pol-, Vif- and Vpx-coding regions. To detect T cells that were specific for the FMSIV-derived SIV antigens, we previously developed the SIVGagPol-specific stimulation method (see Methods). We measured SIV-specific T-cell

levels by using this method in the present study. In addition, we examined IFN- γ induction after SIVGagPolEnv-specific stimulation (see Methods) at several time-points to detect all T cells that were reactive to SIV antigens, including Env.

We first examined SIV-specific T-cell levels at week 12 after challenge in both control macaques that failed to control viraemia (data not shown). SIV-specific CD8⁺ T cells were undetectable in macaque #22, but were detected in macaque #17. However, SIV-specific CD4⁺ T cells were undetectable in both macaques.

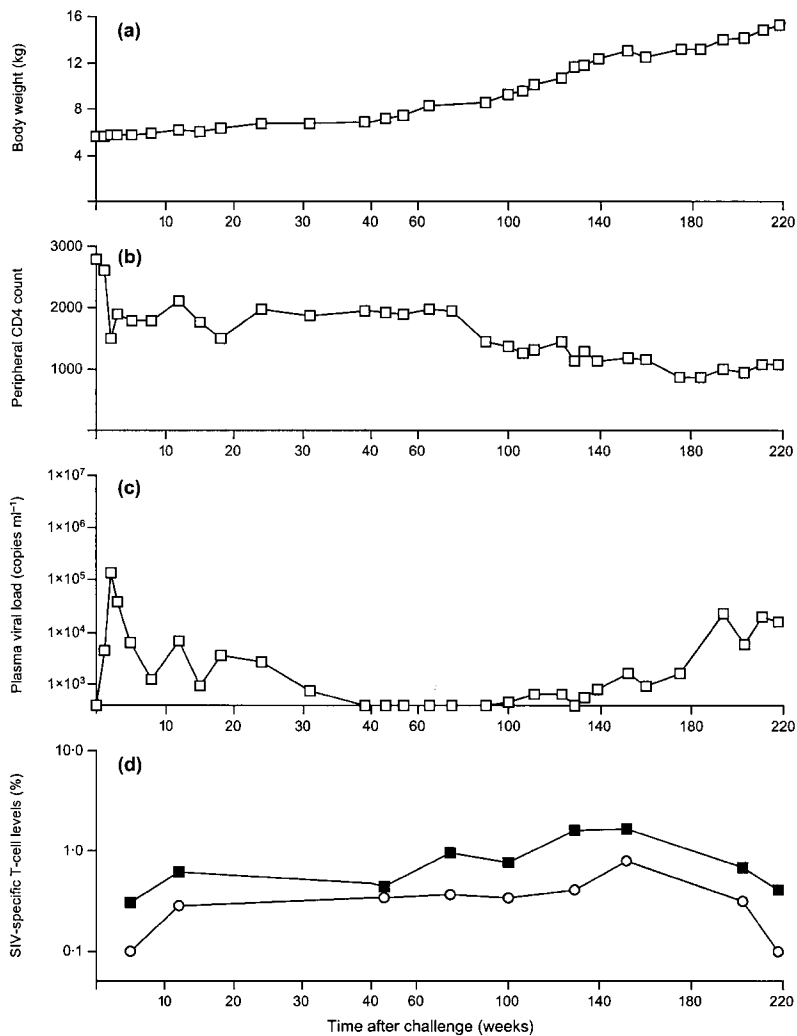


Fig. 3. Follow-up of macaque #21 after SIV challenge. Legend as for Fig. 1.

In contrast, SIV-specific CD4⁺ T cells, as well as CD8⁺ T cells, were detected in the early phase in all three vaccinated macaques, which showed low set point viral loads. In macaque #18, SIV-specific CD4⁺ T cells and CD8⁺ T cells were detected at week 5 and their levels increased at week 12. Levels of both were maintained at week 24, when the animal still kept viral loads at low levels, but SIV-specific CD4⁺ T cells were lost suddenly at week 36, when plasma viral loads increased (Fig. 1). In contrast, SIV-specific CD8⁺ T cells were maintained after that until death.

In macaque #20, SIV-specific CD4⁺ T cells and CD8⁺ T cells were both maintained until week 12, but SIV-specific CD4⁺ T cells were lost at week 18, when plasma viral loads increased to $> 1 \times 10^5$ RNA copies ml⁻¹ (Fig. 2). In contrast, SIV-specific CD8⁺ T cells were maintained after that and their levels increased until death. Not only SIVGagPol-specific CD4⁺ T cells, but also SIVGagPolEnv-specific CD4⁺ T cells were undetectable after loss of the partial control in macaques #18 and #20 (#18, at weeks 42 and 77; #20, at weeks 32, 62 and 90).

In macaque #21, which showed sustained control, SIV-specific CD4⁺ T cells, as well as CD8⁺ T cells, were detected in the early phase and were maintained for more than 3 years (Fig. 3). After this time, the animal showed gradual increases in plasma viral loads and decreases in SIV-specific CD4⁺ T-cell levels. However, these cells were still detectable at week 218.

Specific CD8⁺ T cells with IFN- γ -producing function can be divided into CD45RA⁻ and CD45RA⁺ subpopulations; the latter has been suggested to be more differentiated (Hamann *et al.*, 1997; Sallusto *et al.*, 1999). We examined whether the CD45RA⁺ subpopulation in SIV-specific CD8⁺ T cells was maintained in macaques #20 and #21 (Fig. 4). In macaque #21, about 30% of SIV-specific CD8⁺ T cells were CD45RA⁺ at week 12 and this proportion was almost constant, even in the late phase, indicating maintenance of SIV-specific CD8⁺ CD45RA⁺ T cells. Macaque #20 showed a higher percentage of the CD45RA⁺ subpopulation in SIV-specific CD8⁺ T cells, compared to macaque #21, at week 12. The fraction decreased, but was still $> 30\%$ at week 39. Although levels of SIV-specific

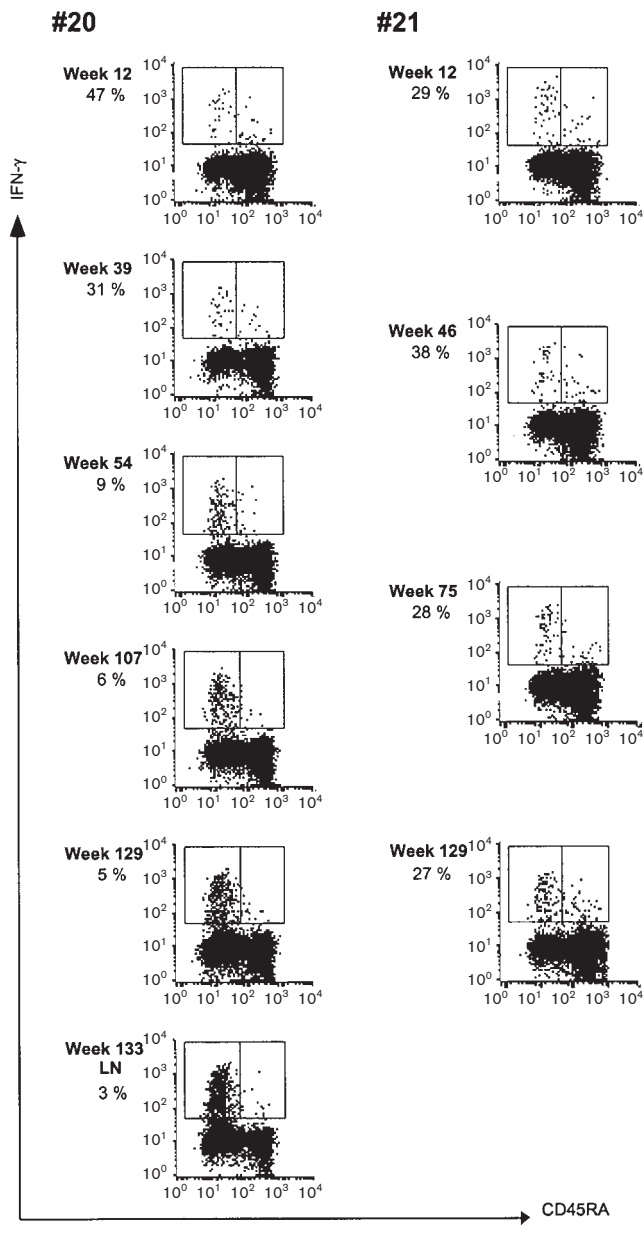


Fig. 4. Dot plots showing CD45RA⁺ and CD45RA⁻ subpopulations in SIV-specific CD8⁺ T cells in macaques #20 and #21. PBMCs at indicated time-points were subjected to intracellular IFN- γ staining after SIVGagPol-specific stimulation. Gating was performed on the CD3⁺ CD8⁺ lymphocyte subpopulation. The proportion of the CD45RA⁺ IFN- γ ⁺ cell number in the IFN- γ ⁺ cell number in each dot plot is shown.

CD8⁺ T cells in this animal were increasing, most of them were CD45RA⁻ after that. We further examined levels in the inguinal LN at week 133, but found no significant difference between PBMCs and the LN. These results show loss of the CD45RA⁺ subpopulation in SIV-specific CD8⁺ T cells after increases in viral loads in macaque #20.

SIV-specific T-cell levels in SeV-Gag-vaccinated macaques in the chronic phase of SIV infection

We also examined SIV-specific T-cell levels in the chronic phase of SIVmac239 infection in SeV-Gag-vaccinated cynomolgus macaques. Two, Cy01 and Cy62, received intranasal SeV-Gag vaccinations and then were challenged intravenously with SIVmac239. These two macaques showed greatly reduced set point plasma viral loads (Fig. 5). However, one of them (Cy01) lost control of viraemia at week 30 post-challenge and showed decreases in peripheral CD4⁺ T cells after that. In this animal, SIV-specific CD4⁺ T cells were undetectable, whereas high levels of SIV-specific CD8⁺ T cells were observed at week 49. In contrast, we found significant levels of SIV-specific CD4⁺ T cells, as well as CD8⁺ T cells, at week 45 in macaque Cy62. Again, SIV-specific CD4⁺ T cells were maintained in the macaque that kept control of viraemia, but were undetectable in the one that lost this control.

DISCUSSION

In our previous studies (Kano *et al.*, 2000; Matano *et al.*, 2000), macaques immunized with FMSIV plus mCAT1 DNA or SeV-Gag showed high levels of virus-specific CD8⁺ T cells, leading to reductions in plasma viral loads during the early phase of SIVmac239 infections. In the present follow-up study of these macaques, some of them failed to keep control of SIV replication and showed increased viral loads in the presence of SIV-specific CD8⁺ T cells. These results indicate that, in macaques with high viral loads, these cells were not able to contain SIV replication.

Recent reports suggested functional impairment of virus-specific CD8⁺ T cells during the chronic phase of immunodeficiency virus infections (Appay *et al.*, 2000; Champagne *et al.*, 2001; Kostense *et al.*, 2001; Vogel *et al.*, 2001; Migueles *et al.*, 2002). The SIV-specific CD8⁺ T cells observed in this study were able to produce IFN- γ in response to SIV antigens. We then examined a differentiation marker, CD45RA, in virus-specific CD8⁺ T cells. The macaque that showed sustained control (#21) maintained the CD45RA⁺ subpopulation of SIV-specific CD8⁺ T cells, even in the late phase. In contrast, macaque #20 lost SIV-specific CD8⁺ CD45RA⁺ T cells 1 year after infection. This may be a consequence of increases in viral loads, but could promote the increases if it is related to functional impairment of SIV-specific CD8⁺ T cells. Regarding killing function, we performed a ⁵¹Cr-release assay as described previously (Matano *et al.*, 2000) and Gag-specific killing activities in PBMCs that were subjected to 1 week Gag-specific stimulation were detected in macaque #20, as well as #21, at week 129 (data not shown), indicating that these SIV-specific CD8⁺ T cells were able to kill the target cells, at least after *ex vivo* stimulation.

It has been indicated that virus-specific CD4⁺ T cells, as well as CD8⁺ T cells, play an important role in the control

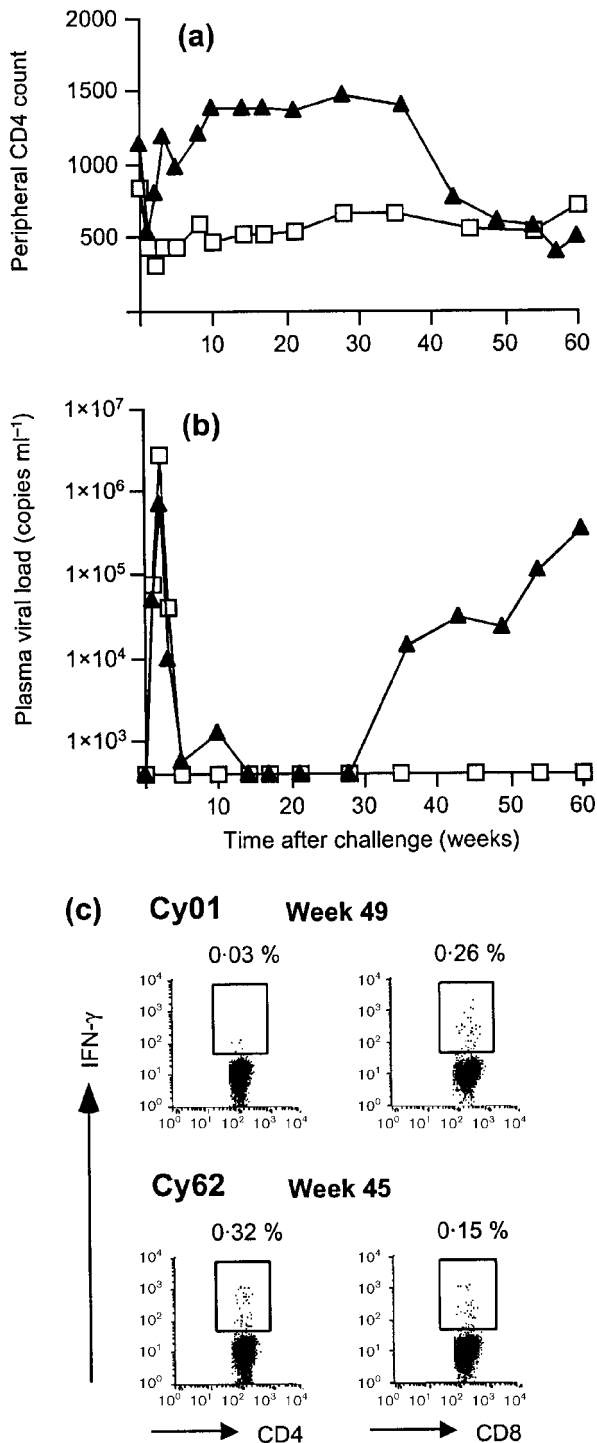


Fig. 5. Follow-up of SeV-Gag-vaccinated macaques, Cy01 (▲) and Cy62 (□), after SIV challenge. (a) Peripheral CD4⁺ T-cell counts (μl⁻¹); (b) plasma virus loads (SIV RNA copy number ml⁻¹); (c) dot plots showing SIV-specific CD4⁺ T cells and CD8⁺ T cells in PBMCs that were subjected to intracellular IFN-γ staining after SIVGagPol-specific stimulation. Gating was performed on the CD3⁺ CD4⁺ (left panels) or CD3⁺ CD8⁺ (right panels) lymphocyte subpopulations. The proportion of the IFN-γ⁺ cell number in the gated cell number in each dot plot is shown.

of immunodeficiency virus infections (Matloubian *et al.*, 1994; Rosenberg *et al.*, 1997; Altfeld & Rosenberg, 2000). Recent studies have reported that HIV-1-infected patients with viraemia frequently retain HIV-1-specific CD4⁺ T cells that are able to produce IFN-γ, but do not have those that are able to proliferate and produce interleukin-2 (IL2) in response to HIV-1 antigens (Iyasere *et al.*, 2003; Harari *et al.*, 2004). The studies suggested that the HIV-1-specific CD4⁺ T-cell subpopulation that is able to produce IFN-γ in HIV-1-infected patients with viraemia may not contribute to proliferative responses for CD4⁺ T-cell helper function. On the other hand, macaques that failed to keep control of SIV replication in this study lost SIV-specific CD4⁺ T cells that were able to produce IFN-γ in response to SIV antigens when they lost this control. It is possible that the SIV-specific CD4⁺ T-cell subpopulation that is able to produce IFN-γ in vaccinated macaques may have had some function in supporting the control of SIV replication. It remains to be determined how it was lost in this study, but loss of the SIV-specific CD4⁺ T-cell subpopulation that is able to produce IFN-γ and loss of that that is able to proliferate and produce IL2 would have different effects on disease progression. Understanding of the function of each SIV-specific CD4⁺ T-cell subpopulation and the effect of its loss may provide insights into the pathogenesis of immunodeficiency virus infections.

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