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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 μg of each DNA intramuscularly and 5 or 10 μg 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 μg FMSIV DNA intramuscularly and 10 μg 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 $TCID_{50}$ (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 10^8 cell infectious units (CIU) of SeV–Gag three times at weeks 0, 4 and 14 after the initial vaccination and challenged intravenously with 100 $TCID_{50}$ 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 TGTTCCTGTTTCCACCACTA) and probes (GCATTCACGCAGA-AGAGAAAGTGAAACA and ACTGAGGAAGCAAAACAGATAGT-GCAGAGA).

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 $<0{\cdot}05$ %.

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, $<2\times10^3$ 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, $>1\times10^6$ 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 $>1\times10^5$ 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 ($<2\times10^3$ copies ml^{-1}) until week 24 after challenge, but began to increase after that. The animal maintained high viral loads, about 1×10^6 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 1×10^4 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

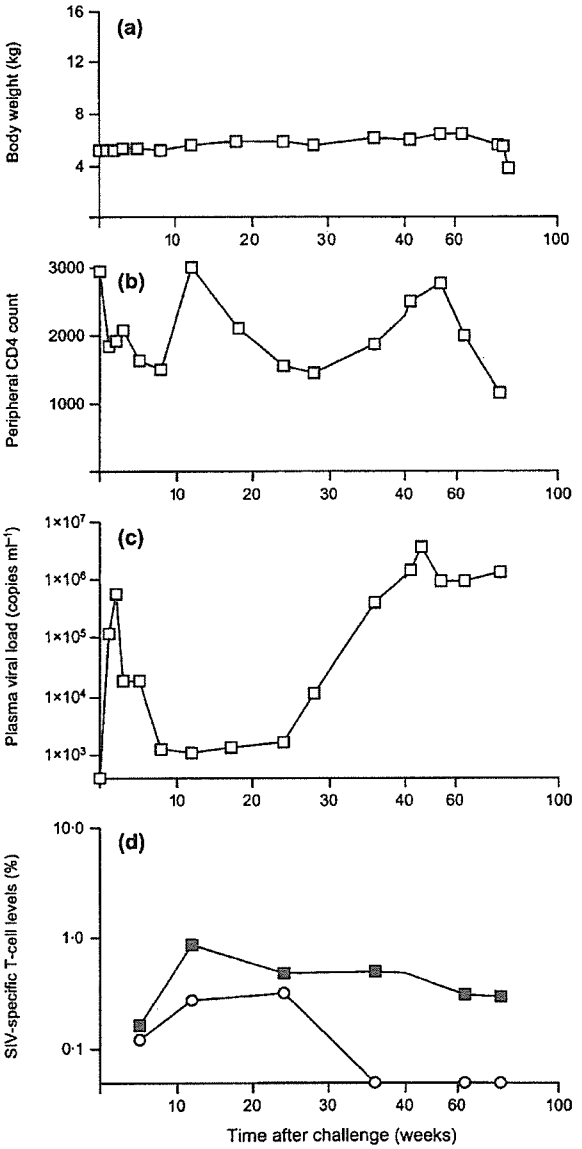

Fig. 1. Follow-up of macaque #18 after SIV challenge. (a) Body weight (kg); (b) peripheral CD4$^+$ T-cell count (μl^{-1}); (c) plasma viral load (SIV RNA copy number ml^{-1}); (d) SIV-specific T-cell level, measured by assessing frequencies of IFN-γ producing cells after SIVGagPol-specific stimulation. 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

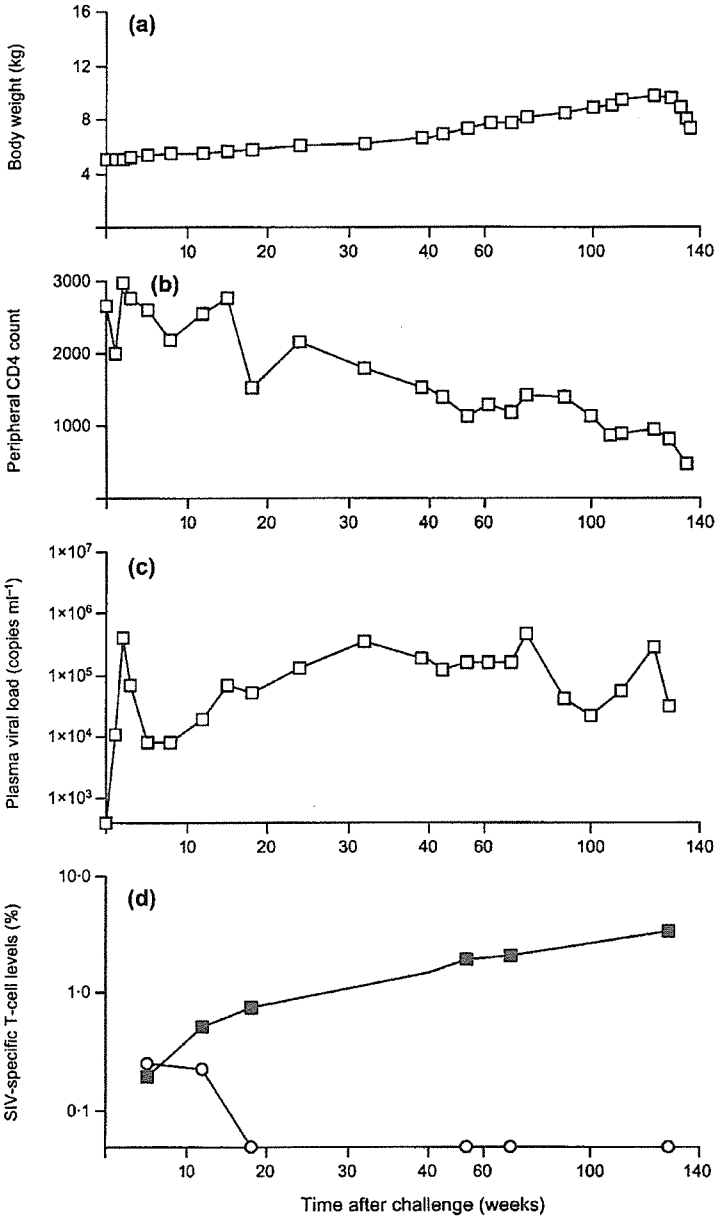

Fig. 2. Follow-up of macaque #20 after SIV challenge. Legend as for Fig. 1.

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.

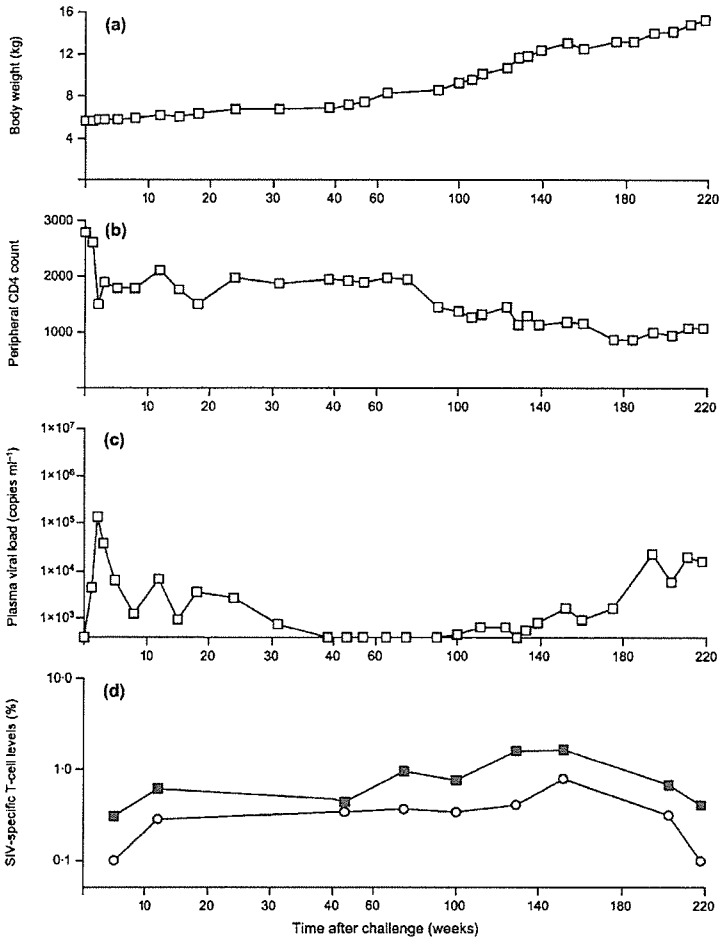

Fig. 3. Follow-up of macaque #21 after SIV challenge. Legend as for Fig. 1.

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 $>1\times10^5$ 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

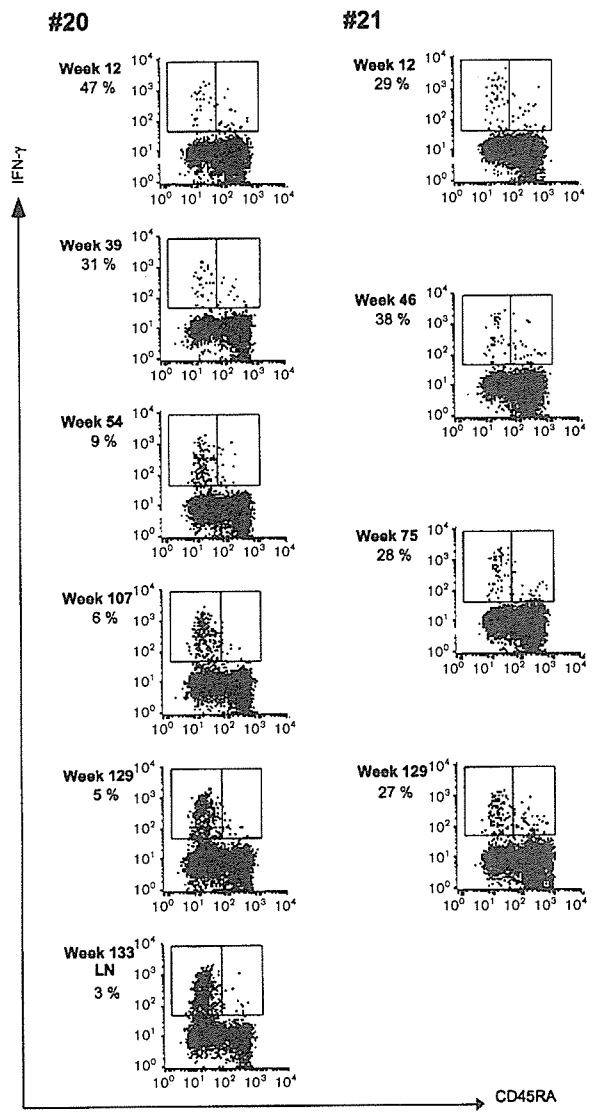

Fig. 4. Dot plots showing CD45RA$^+$ and CD45RA$^-$ subpopulations in SIV-specific CD8$^+$ T cells in macaques #20 and #21. PBMCs at indicated time-points were subjected to intracellular IFN-γ staining after SIVGagPol-specific stimulation. Gating was performed on the CD3$^+$ CD8$^+$ lymphocyte subpopulation. 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 ^{51}Cr-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

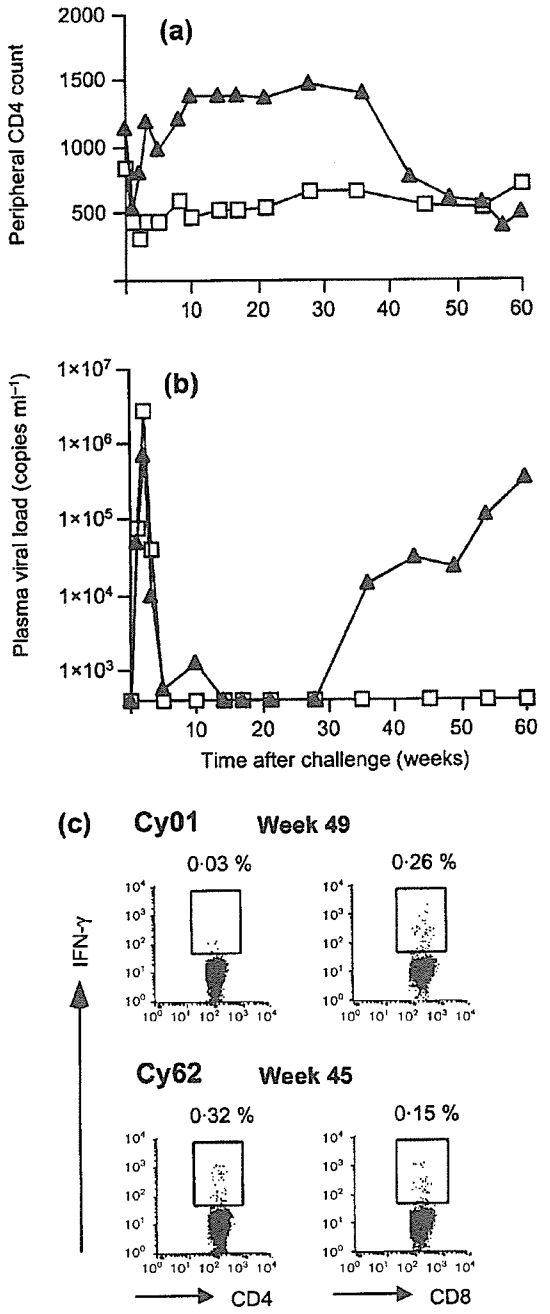

Fig. 5. Follow-up of SeV–Gag-vaccinated macaques, Cy01 (▲) and Cy62 (□), after SIV challenge. (a) Peripheral CD4$^+$ T-cell counts (μl^{-1}); (b) plasma virus loads (SIV RNA copy number ml^{-1}); (c) dot plots showing SIV-specific CD4$^+$ T cells and CD8$^+$ T cells in PBMCs that were subjected to intracellular 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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Short Communication

Stimulation of Virus-Specific T Cell Responses by Dendritic Cell Vaccination in the Chronic Phase of Simian AIDS Models

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SUMMARY: Virus-specific $CD8^+$ cytotoxic T lymphocyte (CTL) responses play an important role in the control of immunodeficiency virus infections. Therapeutic immunization with antigen-pulsed dendritic cells (DC) may be a promising strategy for stimulating CTL. However, decreases in DC number and function have been suggested in the host persistently infected with the virus, and this may constitute an obstacle to DC-based immunotherapy in the chronic phase. In this study, we show that virus-specific CTL responses were augmented by therapeutic immunization with inactivated virus-pulsed autologous DC in rhesus macaques that had maintained prophylactic vaccine-based control of virus replication for more than 3 years after simian or simian-human immunodeficiency virus challenge. Our results indicate the potential of DC in the chronic phase for efficiently stimulating CTL in vivo, suggesting the feasibility of therapeutic DC immunization for replenishing virus-specific CTL responses in the chronic phase after the prophylactic vaccine-based control of primary immunodeficiency virus infection.

Recently, preclinical trials of prophylactic vaccines inducing virus-specific $CD8^+$ cytotoxic T lymphocyte (CTL) responses have been shown to control the primary infections of a pathogenic simian-human immunodeficiency virus (SHIV) that induces acute $CD4^+$ T-cell depletion in macaques (1-4). In the chronic phase after such prophylactic vaccine-based control, the loss of control due to the appearance of a CTL escape mutant has been observed in a macaque (5). In another report (6), macaques maintaining vaccine-based control of SHIV infection have shown, in the chronic phase, a rise in plasma viral loads after anti-CD8 monoclonal antibody treatment inducing $CD8^+$ T-cell depletion. Thus, the maintenance of virus-specific $CD8^+$ T-cell responses has been indicated to be required for the sustained control of virus replication, and therapeutic immunization replenishing virus-specific $CD8^+$ T-cell responses is likely to contribute to this sustained control.

Therapeutic immunization with antigen-pulsed dendritic cells (DC) may be a promising strategy for stimulating virus-specific T-cell responses. However, decreases in DC number and function have been suggested in the host persistently infected with HIV-1 and it may be an obstacle to DC-based immunotherapy in the chronic phase (7,8). Indeed, precise evaluation of DC-based immunotherapy in the chronic phase has not been performed in macaque AIDS models. It has remained unclear if virus-specific $CD8^+$ T-cell responses can be stimulated by therapeutic DC immunization in the chronic phase even in those macaques maintaining low or undetectable viral loads. In the present study, we have administered inactivated virus-pulsed autologous DC into three rhesus macaques that had maintained prophylactic vaccine-based control of virus replication for more than 3 years after challenge.

The three male rhesus macaques (*Macaca mulatta*) used in this study received prophylactic vaccinations and were challenged with simian immunodeficiency virus (SIV) or SHIV as described previously (3,9) (Table 1). They were maintained in accordance with the Guideline for Laboratory Animals of the National Institute of Infectious Diseases. All of these macaques (#21, #19, and R005) showed low viral loads at the setpoint after challenge. In macaque #21 which had been immunized with prophylactic DNA vaccine and challenged with SIVmac239 (10), plasma viral loads were below or just above the detectable level from week 31 to week 139 after challenge. This animal then showed viremia but maintained plasma viral loads at low levels, around 2×10^4 RNA copies/ml, until DC immunization at week 227. In macaque #19 which had been immunized with DNA-prime/Gag-expressing Sendai virus vector (SeV-Gag)-boost and challenged with $SHIV_{DH12R}$ (11), plasma viral loads were undetectable after week 28 until therapeutic DC immunization at week 205. In macaque R005 which had been immunized with DNA-prime/SeV-Gag-boost and challenged with SHIV89.6PD (12), plasma viral loads were undetectable after week 8 until DC immunization at week 176.

Monocyte-derived DC were prepared from blood at weeks 215 and 223 in macaque #21, at weeks 197 and 201 in macaque #19, and at weeks 165 and 169 in macaque R005 (Table 1). For DC preparation, peripheral blood mononuclear cells (PBMC) were prepared from 20 ml of whole blood by using Ficoll-Paque Plus (Amersham Biosciences, Piscataway, N.J., USA). $CD14^+$ monocytes were isolated from PBMC by positive selection using CD14 MicroBeads and magnetic cell separator Mini MACS (Miltenyi Biotec, Gladbach, Germany). These cells were suspended in RPMI 1640 (Life Technologies, Rockville, Md., USA) supplemented

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Table 1. Vaccine and challenge protocol

Macaque	#21	#19	R005
Prophylactic vaccine	DNA (wk 0, 1, 2, 6, 12 pv)	DNA (wk 0, 1, 2, 6, 12 pv)	DNA (wk 0, 1.5, 1, 6 pv)
		SeV-Gag (wk 33 pv)	SeV-Gag (wk 12 pv)
Challenge	SIVmac239 (wk 24 pv)	$SHIV_{DH12R}$ (wk 37 pv)	SHIV89.6PD (wk 26 pv)
DC preparation	6×10^5 (wk 215 pc)	3×10^5 (wk 197 pc)	4×10^5 (wk 165 pc)
	4×10^5 (wk 223 pc)	9×10^5 (wk 201 pc)	8×10^5 (wk 169 pc)
DC vaccine	1.0×10^6 (wk 227 pc)	1.2×10^6 (wk 205 pc)	1.2×10^6 (wk 176 pc)

At prophylactic vaccination, macaque #21 received defective proviral DNA vaccinations, whereas macaques #19 and R005 received the DNA vaccinations and an SeV-Gag-boost. In DC preparation, the number of AT-2-inactivated virus-pulsed DC prepared from 20 ml blood at each time point is shown. These cells were used for therapeutic DC vaccination. pv, post-vaccination; pc, post-challenge.

with 10% fetal bovine serum (Hyclone, Logan, Utah, USA), 2000 U/ml of granulocyte-macrophage colony-stimulating factor (Genzyme-Techne, Cambridge, Mass., USA), and 3000 U/ml of interleukin-4 (Genzyme-Techne), and were divided into 8 wells in 6-well plates (BD Biosciences, San Jose, Calif., USA) for cell culture. After 6 days, the cells were pulsed with inactivated SIVmac239 (for macaque #21-derived DC), $SHIV_{DH12R}$ (for #19), or SHIV89.6PD (for R005) (0.5 μg of p27 [SIV CA]/well) for 2 h. The inactivated viruses were obtained by incubation of SIV or SHIV with 250 μM aldrithiol-2 (AT-2) (Sigma, St. Louis, Mo., USA) (13) at 37°C for 1 h. The cells were cultured in the presence of 50 ng/ml of tumor necrosis factor-α (R&D system, Minneapolis, Minn., USA) for 1 day from day 7. For flow-cytometric analysis, immature DC and mature DC were harvested from 1 well of culture on day 6 and on day 8, respectively. The analysis confirmed CD86 expression in the DC just before the antigen exposure and increases in its expression after that, indicating DC maturation (14) (data not shown). For therapeutic immunization, the inactivated virus-pulsed DC were harvested on day 8 and frozen until use. The numbers of recovered DC from 20 ml of blood were between 3×10^5 and 9×10^5. Upon immunization, the frozen DC were thawed and inoculated subcutaneously into macaques.

Macaques #21, #19, and R005 were inoculated with $1.0 \times 10^6 \sim 1.2 \times 10^6$ autologous DC at weeks 227, 205, and 176, respectively (Table 1). No macaques displayed apparent clinical symptoms after the therapeutic DC immunization. Nor were apparent changes in $CD4^+$ T-cell counts, $CD8^+$ T-cell counts, or plasma viral loads observed.

In order to determine the effect of the therapeutic DC immunization on SIV-specific T-cell responses, we measured SIV-specific T-cell frequencies in PBMC before and after immunization. This was done by detecting SIV-specific interferon-γ (IFN-γ) induction as described previously (3,9). In brief, COS-1 cells were cotransfected with an *env*- and *nef*-deleted SHIV proviral DNA, SIVGP1, and a plasmid pVSV-G that expresses vesicular stomatitis virus G protein (VSV-G) (BD Biosciences) in order to obtain a VSV-G-pseudotyped SIV, SIVGP1(VSV-G). PBMC were cocultured with autologous herpesvirus papio-immortalized B lymphoblastoid cell lines (B-LCL) infected with SIVGP1(VSV-G) for SIV-specific stimulation. Intracellular IFN-γ staining was performed using a Cytofix-Cytoperm kit (BD Biosciences), fluorescein isothiocyanate (FITC)-conjugated anti-human CD4, peridinin chlorophyll protein (PerCP)-conjugated anti-human CD8, allophycocyanin (APC)-conjugated anti-human CD3, and phycoerythrin (PE)-conjugated anti-human IFN-γ antibodies (BD Biosciences). SIV-specific T-cell frequencies were calculated by subtracting the IFN-γ^+ T-cell frequencies after non-specific stimulation with VSV-G-pseudotyped murine leukemia virus-infected B-LCL from those after the SIV-specific stimulation. The SIV-specific T cells detected in this assay did not include Env-specific or Nef-specific T cells. The background IFN-γ^+ T-cell frequencies after non-specific stimulation were less than 200 cells per million PBMC. SIV-specific T-cell frequencies less than 100 cells per million PBMC were considered negative, those between 100 and 200 borderline, and those greater than 200 positive.

All three macaques showed efficient augmentation of SIV-specific $CD8^+$ T-cell responses after DC immunization (Fig. 1). In macaques #21 and 19, SIV-specific $CD8^+$ T cells were detectable even before the immunization, though their frequencies were increased by about eight-fold (#21) or four-fold (#19) 1 week after that. In macaque R005, SIV-specific $CD8^+$ T cells were undetectable before the immunization but were induced efficiently 1 week after that. SIV-specific $CD8^+$ T-cell frequency 2 weeks after DC immunization was still higher than that before the immunization in macaques #21 and #19 but below the detectable level in macaque R005.

SIV-specific $CD4^+$ T-cell responses were also augmented after DC immunization (Fig. 1). It has been indicated that virus-specific $CD4^+$ T-cell as well as $CD8^+$ T-cell responses play an important role in the control of immunodeficiency virus infections (15,16). Recent studies, however, have reported that HIV-1-infected patients with viremia frequently keep HIV-1-specific $CD4^+$ T cells able to produce IFN-γ but do not have those able to produce interleukin-2 in response to HIV-1 antigens, suggesting that the HIV-1-specific $CD4^+$ T-cell subpopulation able to produce IFN-γ may not contribute to the proliferative responses necessary for the $CD4^+$ T-cell helper function (17,18). Therefore, it has remained unclear if SIV-specific $CD4^+$ T-cell responses induced by DC immunization can contribute to the sustained control of virus replication in this study.

In macaques #19 and R005 that had received SeV-Gag booster before challenge, SeV-specific T-cell levels were also examined. In both of them, SeV-specific T-cell responses were undetectable before and after DC immunization (at weeks 205 and 206 in #19, and at weeks 176 and 177 in R005), confirming that the effect of the DC immunization on cellular immune responses was SIV-specific.

The present study is the first trial of DC-based immunotherapy for stimulating virus-specific $CD8^+$ T-cell responses in the chronic phase in the host maintaining prophylactic vaccine-based control of immunodeficiency virus replication. Immunization with inactivated virus-pulsed DC in the chronic phase was seen to augment SIV-specific $CD8^+$ T-cell responses in all three macaques that had controlled SIV or SHIV replica-

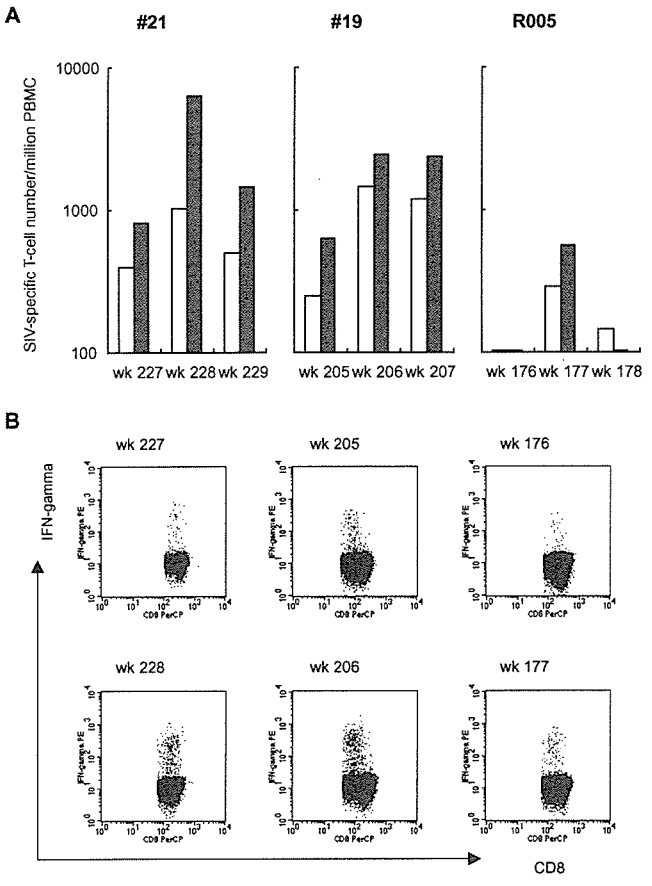

Fig. 1. Augmentation of SIV-specific T-cell responses after DC immunization. Macaques #21, #19, and R005 received DC immunization at weeks 227, 205, and 176, respectively. (A) Frequencies of SIV-specific $CD4^+$ T cells (open bar) and $CD8^+$ T cells (shaded bar) in PBMC before and after DC immunization. (B) Representative dot plots showing IFN-γ induction after SIV-specific stimulation in $CD8^+$ T cells just before and 1 week after DC immunization. In each dot plot, $CD3^+CD8^+$ lymphocytes are gated and shown.

tion for more than 3 years, indicating the potential of their DC for efficiently stimulating virus-specific $CD8^+$ T-cell responses in vivo. Whereas therapeutic immunizations have been studied in HIV-1-infected individuals and SIV-infected macaques during antiretroviral treatment (19,20), our results suggest the feasibility of therapeutic DC immunization for replenishing virus-specific T-cell responses in the chronic phase after the prophylactic vaccine-based control of primary immunodeficiency virus infections.

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IL-15 is superior to IL-2 in the generation of long-lived antigen specific memory CD4 and CD8 T cells in rhesus macaques

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Abstract

Using tetanus toxoid (TT) and influenza (Flu) immunization of rhesus macaques as a model, the effect of IL-2 and IL-15 on the generation and maintenance of antigen specific memory T cells was evaluated following primary and secondary immunization. Daily cytokine administration expanded primarily effector but not memory cells, while spacing cytokine administration to q3–7 days markedly enhanced TT and Flu specific memory responses. Following primary immunization, TT specific CD4 and influenza matrix protein (Flu-MP) specific CD8 effector responses were enhanced by IL-2 administration but CD8 specific memory responses were no different from cytokine non-treated monkeys. In contrast, expansion of Flu specific CD8 cells with IL-15 was only modest but resulted in significantly elevated levels of memory cells at 6 months. IL-15 also significantly enhanced early and late TT specific CD4 responses. The highest levels of primary effector and memory T cells were observed following alternate administration of both IL-2 and IL-15. Following booster immunization, however, only IL-15 appeared able to enhance CD8 T cell responses while IL-2 or IL2/IL-15 administration were less effective.

Keywords: Macaque; T cell; IL-2; IL-15

1. Introduction

The recruitment, expansion, and contraction of the frequencies and absolute numbers of CD4 and CD8 T cells during antigen recognition followed by memory development is a subject of intense investigation [1–3]. Thus, recent data suggest that the magnitude of the peak of T cell expansion correlates with levels of long-term memory T and B cells being present in a given individual [4], supporting the notion that the frequencies of T cells specific for any given epitope result from a preset percentage of regulatory cell death among the expanded T cell clones. However, these findings do not imply that such preset levels of T cell death cannot be influenced by the milieu in which the expansion and/or contraction of such pools occurs nor during the homeostatic maintenance of memory T cells. This is of particular relevance not only in the case of chronic infections but also for immunization and vaccines strategies. Thus, aside from a number of factors and strategies known to increase antigen presentation to enhance the magnitude of T cell expansion [5–8], several other strategies have been tested to boost levels of memory T cells via homeostatic mechanisms, e.g. using defined T cell stimulatory cytokines [9–11] and/or costimulatory signals [12,13]. Among the prime candidate reagents readily available for evaluation are the cytokines IL-2, IL-7, and IL-15 [14–18]. The tandem use of IL-2 and IL-15 in particular has drawn a lot of attention since both cytokines signal via heterotrimeric (α) receptors, whereby receptors for each of the two cytokines use the same β and γ subunits, allowing for significant overlap between biological effects delivered by either factor [19,20]. However, it

Abbreviations: B-LCB, B lymphocytoblast; EIA, enzymatic immunosorbent assay; Flu, influenza live attenuated isolate A/PR8; Flu-MP, influenza matrix protein; HIV, human immunodeficiency virus; IL, interleukin; IV, intravenously; mab, monoclonal antibody; MHC, major histocompatibility complex; PBMC, peripheral blood mononuclear cell; p-CTL, CTL precursor; rMamu, recombinant *Macaca mulatta*; SIV, simian immunodeficiency virus; SubQ, subcutaneously; TT, tetanus toxoid

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soon became apparent that salient differences in the quality of signal delivered by either cytokine were identifiable, in particular their role in the regulation of T cell activation and survival. Thus, while IL-2 induced rapid mobilization, activation, and expansion of T cells expressing the IL-2R, this mobilization was accompanied by a heightened susceptibility to undergo apoptosis as evidenced by the upregulation of Fas [21,22], Fas-ligand [23], and downregulation of Bcl-2, gamma-c [14] and Bcl-xL [24]. Comparable T cell mobilization in the presence of IL-15 appears to generate a less vigorous effector response, however, upregulation of pro-apoptotic factors is also far less prominent and such IL-15 treated cells are more likely to generate higher levels of antigen specific memory cells [14,15]. While most such studies have been pioneered in defined murine models and/or in vitro expansion models, a rigorous evaluation of the effect of IL-2 versus IL-15 on primary and secondary T cell responses has not been formally addressed in humans nor in outbred large animal models. Our laboratory has been focused on the evaluation of select recombinant cytokines as potential adjunct therapeutic agents for the augmentation of virus specific immunity in the simian immunodeficiency virus (SIV) model of human AIDS [25–27]. One of the lessons that we learnt from the studies utilizing recombinant rhesus macaque IL-12 was the use of these cytokines requires careful in vivo evaluation of the doses and the schedules of administration to maximize their potential beneficial effects and to minimize their potential toxic effects and/or negative or blunting effects on the induction and maintenance of antigen specific immune responses. In light of these findings coupled with a growing need for improved vaccines against both new and old pathogens whose effector responses rely primarily on cell mediated immune responses, e.g. *M. tuberculosis*, malaria, and human immunodeficiency virus (HIV), provided the rationale for the studies performed herein. Thus, groups of healthy young rhesus macaques were immunized with prototype antigens such as tetanus toxoid (TT) and live attenuated influenza virus strain A/PR8 (Flu), respectively, in the presence and absence of varying schedules of doses of recombinant rhesus IL-2 alone, IL-15 alone or in a limited study a combination of IL-2 and IL-15.

2. Materials and methods

2.1. Animals

Juvenile to young adult rhesus macaques were immunized and administered cytokines as described below for this study. The monkeys were maintained in accordance with the instructions of the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council and the US Public Health Service Guidelines, Guide for the Care and Use of Laboratory Animals.

2.2. Tetanus toxoid and influenza immunizations

All monkeys received a primary immunization of 100 μg of TT emulsified in incomplete Freund's adjuvant subcutaneously (subQ). The TT booster immunization consisted of 10 μg of TT administered without adjuvant intravenously (IV) as detailed elsewhere [28–30]. Each macaque was also administered 1024 HA units of Influenza A/PR8 grown in embryonated chicken eggs (courtesy of T. Rowe, CDC, Atlanta, GA) in a volume of 1 ml administered dropwise intranasally for either the primary or booster immunization.

2.3. Preparation of rMamu IL-2 and IL-15

The genes of rhesus macaque IL-2 and IL-15 were previously cloned by our lab [31] and expressed in *E. coli* using the pET32a expression vector (Novagen, Madison, WI). Both cytokines were tested for absence of endotoxin content using the LAL test (Biowhitaker, Walkersville, MD), quantified by enzymatic immunosorbent assay (EIA) [31,32] against a human standard and for bioactivity using the IL-2 responsive murine cell line HT-2 [32]. The administration of each cytokine was performed subQ at doses and schedules indicated for each experiment.

2.4. Evaluation of biological activity of Mamu IL-2 and IL-15

Both cytokines signal via receptors that share the β and γ subunits, and thus, the biological activity of each cytokine was quantitated using the same IL-2 dependent HT-2 murine cell line [32].

Briefly, HT-2 cells in logarithmic growth phase are starved overnight in media without IL-2. The next day, standard dilutions of rhu IL-2 or IL-15 are set up in triplicate microcultures as well as three-fold dilutions of each sample to be analyzed. To each culture well is then added 2×10^4 starved HT-2 cells and cultured for 24 h before adding 1 μCi of tritiated thymidine per well. After another 16 h pulse, all microcultures were harvested using a Tomtec harvester and the incorporated radioactivity read with a Wallac Trilux β-scintillation counter (Wallac, Turku, Finland). A unit of bioactivity was then calculated based on the dilution of each sample inducing a half maximal HT-2 proliferative response [32]. The recombinant *Macaca mulatta* (rMamu) IL-2 lot used had a 3.8×10^6 units of bioactivity/mg and rMamu IL-15, 5.6×10^6 units/mg.

2.5. Isolation of PBMCs and preparation of transformed cell lines

Heparinized whole blood was centrifuged at $450 \times g$ for 10 min and the plasma removed. The cells were separated using Ficoll hypaque gradients, washed free of platelets and resuspended to the desired cell concentration for the appropriate assay. Prior to the immunization and cytokine

administration, aliquots of PBMCs from each monkey were incubated in vitro with Herpes papio for the derivation of predominantly B cell lineage lymphocytoblasts (B-LCB). These cultures were routinely monitored and the emerging transformed cells expanded, aliquoted, and cryopreserved.

2.6. ELISPOT enumeration of Flu and TT specific T cells

The ELISPOT analyses were performed essentially as published previously [33]. Briefly, Millipore multiscreen plates were coated with either 5 μg/ml of monoclonal anti-human IFN-γ antibody (clone GZ-4, Mabtech Inc., Nacka, Sweden) or anti-IL-2 (clone 419A7A3, Biosource Int., Camarillo, CA). Then, 1×10^5 PBMCs were plated per well in RPMI 1640 complete medium (RPMI 1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 10% fetal calf serum) in the presence of either 10 μg of a control ovalbumin peptide (SYNFEKL), Flu matrix protein peptide 58–66 (GILGFVFTL), TT-P2 peptide (830–843, QYIKANSKFIGITE) or whole TT protein. Following a 36 h incubation at 37 °C in a 7% CO_2 humidified atmosphere, the cells were removed and IFN-γ or IL-2 "spots" detected using the anti-IFN-γ biotinylated monoclonal antibody (mab) clone 7B6.1 (Chromogenix, West Chester, OH) or the biotinylated anti-IL-2 mab clone 297C16G2 (Biosource), respectively. Detection was performed using streptavidin–horse radish peroxidase conjugate followed by diaminobenzidine substrate addition. Spot counting was performed using an automated ImmunoSpot counter (Becton-Dickinson, Mountain View, CA).

2.7. p-CTL analysis

The CTL precursor (p-CTL) assay was performed using limiting dilution assays (LDA) as standardized by our laboratory [25,28,34–36]. Briefly, aliquots of B-LCBs (2×10^6 cells/ml) from the monkey to be assayed were cultured in vitro for 6–8 h with 10 μM of Flu matrix protein peptide (58–66). Alternatively, the B-LCBs were infected with the recombinant vaccinia containing the influenza matrix protein (Flu-MP) at an MOI of 10 overnight (first in a small volume of 0.5–1.0 ml for 1 h and then overnight in a T-30 flask). The cells were washed in media and irradiated (8000 rad), washed and adjusted to 2×10^5 cell/ml and 0.1 ml of this cell suspension dispensed into each well of a 96-well round bottom plate. Two-fold dilutions of the effector cells to be tested ranging from 10^3 cells to 6.4×10^4 cells were then added to sets of 24 replicate wells. The cultures were incubated at 37 °C in a 7% CO_2 and humidified atmosphere. On days 7 and 11 each culture was supplemented with 20 units/ml of IL-2. On day 14, the expanded cells from each of the 96 wells were split into two. For the analysis of peptide specific effectors, one set of autologous B-LCBs were pulsed with 10 μM Flu-MP peptide, while the other control set was pulsed with the OVA peptide. For the whole MP protein, one B-LCB set was pulsed with FLU-MP expressing vaccinia while the control set was pulsed with wild type vaccinia. All antigen and control antigen pulsed B-LCBs were then labeled with ^{51}Cr, washed and dispensed in the appropriate wells of 96-well round bottom microplates (1×10^4 cells per well). With each set of effector and target cell assay was included six replicate wells that received the ^{51}Cr-labeled target cells incubated in media alone (spontaneous release) and ^{51}Cr-labeled target cells that were incubated with 1% Triton-X 100 (maximal release). The microtiter plates were centrifuged ($150 \times g$), incubated for 6 h at 37 °C in a 7% CO_2 humidified atmosphere, centrifuged once again at $150 \times g$ and 0.1 ml supernatant fluid removed from each microtiter well to determine the amount of radioactivity released. Individual wells that had supernatant fluids with counts three standard deviations above the mean cpm of the spontaneous release were scored as being positive. The Jackknife statistical program [37] was utilized to calculate the precursor frequencies per million effector cells. An assay was only considered valid if the mean cpm of the spontaneous release for a given target cell was less that 20% of the maximal release for the same target cell population. The values obtained against the wild type vaccinia virus varied but were in all cases <120 p-CTL/10^6 effector cells while OVA specific responses were consistently <100 p-CTL/10^6 effectors. Data as presented are net p-CTL which were derived by subtracting the CTL values obtained with the wild type vaccinia infected or OVA pulsed target cells from those obtained with the Flu-MP vaccinia infected or Flu-MP pulsed target cells.

2.8. Tetanus toxoid specific proliferative responses

Quadruplicate microtiter cultures were established with 5×10^5 cells per well pulsed with 10 μg/ml of TT in complete RPMI 1640 media. After 3 days of incubation at 37 °C/7% CO_2, the cultures were pulsed with 1 μCi of tritiated thymidine (Perkin-Elmer, Boston, MA) for an additional 16 h. The incorporated radioactivity in each culture was then collected using a Tomtec plate harvester and read using a Wallac Trilux β-counter. Mean thymidine uptake and standard deviations of the quadruplicate cultures were calculated. Control wells received PBMCs alone. The major histocompatibility complex (MHC) class II restriction was verified by the blocking of the proliferative response (>75%) by anti-MHC class II but not anti-MHC class I mab pretreatment of the target cells (data not shown).

2.9. Statistical analyses

Statistically significant differences between multiple independent normally distributed data collected from ELISPOT and T cell proliferation assays were determined by one way analysis of variance (ANOVA) with contrasts using Dunnet 95% confidence intervals for error protection. Calculation were performed using Analyze-It statistical software for Microsoft Excel (Leeds, England, UK). Statistical estimates of precursor frequencies of p-CTLs were performed using

a software algorithm based on the Jackknife version of the maximum likelihood method [37].

3. Results

3.1. Homeostatic dose response to IL-2 and IL-15 in vivo

Two groups of monkeys were administered increasing single doses of rMamu IL-2 or IL-15 subQ at 2 weeks intervals between each dose. The number of CD4 T cells was evaluated in each animal at 5 days following cytokine administration and just before cytokine administration to verify that baseline values were restored (data not shown). As illustrated in Fig. 1, both IL-2 and IL-15 administration induced dose dependent increase in absolute numbers of CD4 T cells reaching a plateau at 5 μg/kg of IL-2 and 10 μg/kg of IL-15. IL-2 appeared to induce a higher increase in circulating CD4 T cells than IL-15 (687 versus 533), however, this difference was not significant ($P = 0.076$). Based on these initial dose–response findings, all subsequent cytokine administration used 5 μg/kg of rMamu IL-2 and 10 μg/kg of rMamu IL-15, respectively, since doses the respective cytokines above 5 and 10 μg/kg failed to induce further increases in CD4+ T cell numbers ($P > 0.4$).

3.2. In vivo half life of rMamu IL-2 and IL-15

Next we determined the pharmacokinetics of each cytokine after administration of a single optimal dose of either cytokine subQ to groups of six monkeys each, followed by sequential blood collections at 5, 30, 60, 90, 120, 240 min, and at 24 h post administration. As shown in Table 1, the average $T_{1/2}$ values for rMamu IL-2 in vivo were 3.28 and 2.37 h as determined by EIA and bioassay, respectively. The average $T_{1/2}$ values for rMamu IL-15 were even smaller, with values of 1.31 and 0.92 h based on EIA and bioassay, respectively, suggesting that both cytokines are being rapidly consumed and/or metabolized from the circulation.

Table 1
Pharmacokinetics of rMamu IL-2 and rMamu IL-15 ($T_{1/2}$ in h) in rhesus macaques

Monkey	Testing			
	EIA		Bioassay	
	IL-2	IL-15	IL-2	IL-15
1	3.27 ± 0.83	1.22 ± 0.29	2.92 ± 0.24	0.80 ± 0.11
2	3.31 ± 0.66	1.17 ± 0.22	1.92 ± 0.11	0.94 ± 0.15
3	2.98 ± 0.32	1.45 ± 0.32	1.84 ± 0.23	0.89 ± 0.09
4	3.54 ± 0.61	1.41 ± 0.15	1.79 ± 0.17	0.76 ± 0.21
5	3.39 ± 0.52	1.29 ± 0.14	2.56 ± 1.3	1.14 ± 0.14
6	3.17 ± 0.21	1.32 ± 0.17	3.21 ± 0.6	0.96 ± 0.13
Mean	3.28 ± 0.53	1.31 ± 0.22	2.37 ± 0.44	0.92 ± 0.14

3.3. Influence of the administration schedule of cytokines on the expansion of antigen specific effector and memory T cells

Preliminary data using rMamu IL-12 administration to rhesus macaques showed a marked cytokine refractory period when the administration was repeated at close intervals (data not shown). It was reasoned that even though human clinical studies generally follow a daily dosing schedule, this may not be the most optimal administration schedule to expand and maintain antigen specific T cells. Thus, a total of 18 monkeys were each immunized with TT and live attenuated influenza, followed by a booster immunization with the same antigens 2 weeks later (Fig. 2A). Starting 3 days after the booster immunization groups of three animals each were subjected to 6 weeks of administration of 5 μg/kg of IL-2 or 10 μg/kg of IL-15 either daily, every 2, 3, 5 or 7 days. Evaluation of frequencies of antigen specific T cells at 0, 6, and 12 weeks (IL-2 treatment) or 0, 9, and 15 weeks (IL-15 treatment) post boost suggested different

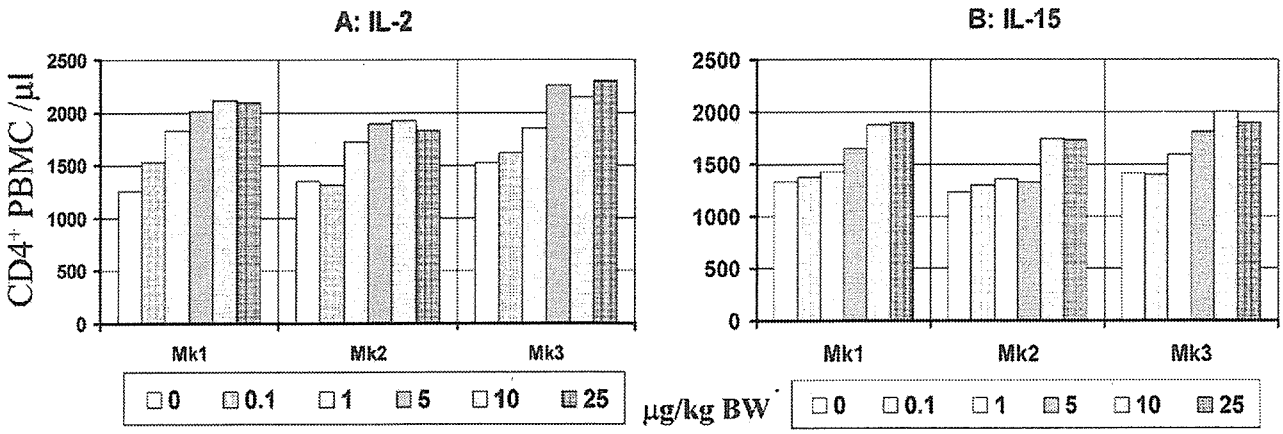

Fig. 1. Dose–response of IL-2 (A) vs. IL-15 (B) on absolute numbers of peripheral CD4 T cells in sets of three rhesus macaques administered increasing doses of cytokines at 2 weeks intervals. CD4 T cell counts were determined 5 days following each administration and prior to the next administration to verify for returns to baseline values.

levels of enhancement and maintenance of such frequencies following IL-2 or IL-15 treatment over untreated control monkeys according to the cytokine administration schedule (Fig. 2B–E). First, administration of either cytokine according to any schedule led to markedly enhanced antigen specific effectors early post boost over those seen with non cytokine treated controls. Thus, IL-2 treated monkeys showed most marked increases in Flu-MP and TT specific effector T cells (at 6 weeks post boost, $P < 0.001$) when the cytokine was administered daily or q2 days for Flu-MP (Fig. 2B) or daily, q2 or q3 days for TT (Fig. 2C) as compared with the other dose schedules. Evaluation of memory T cells at 12 weeks post boost in IL-2 treated monkeys showed a different pattern, whereby spacing of IL-2 administration to q2, 3, and 5 days for Flu-MP and q3, 5 or 7 days for TT provided better enhancement of such memory responses in PBMCs as compared to other dose schedules. In the case of IL-15, as noted above, a marked enhancement of both early Flu-MP specific CD8 and TT specific CD4 T cell responses was noted for all administration schedules (Fig. 2C and E) with optimal enhancement noted for q3 and q5 days IL-15 administration for Flu-MP (Fig. 2B) and q3 days for TT effectors

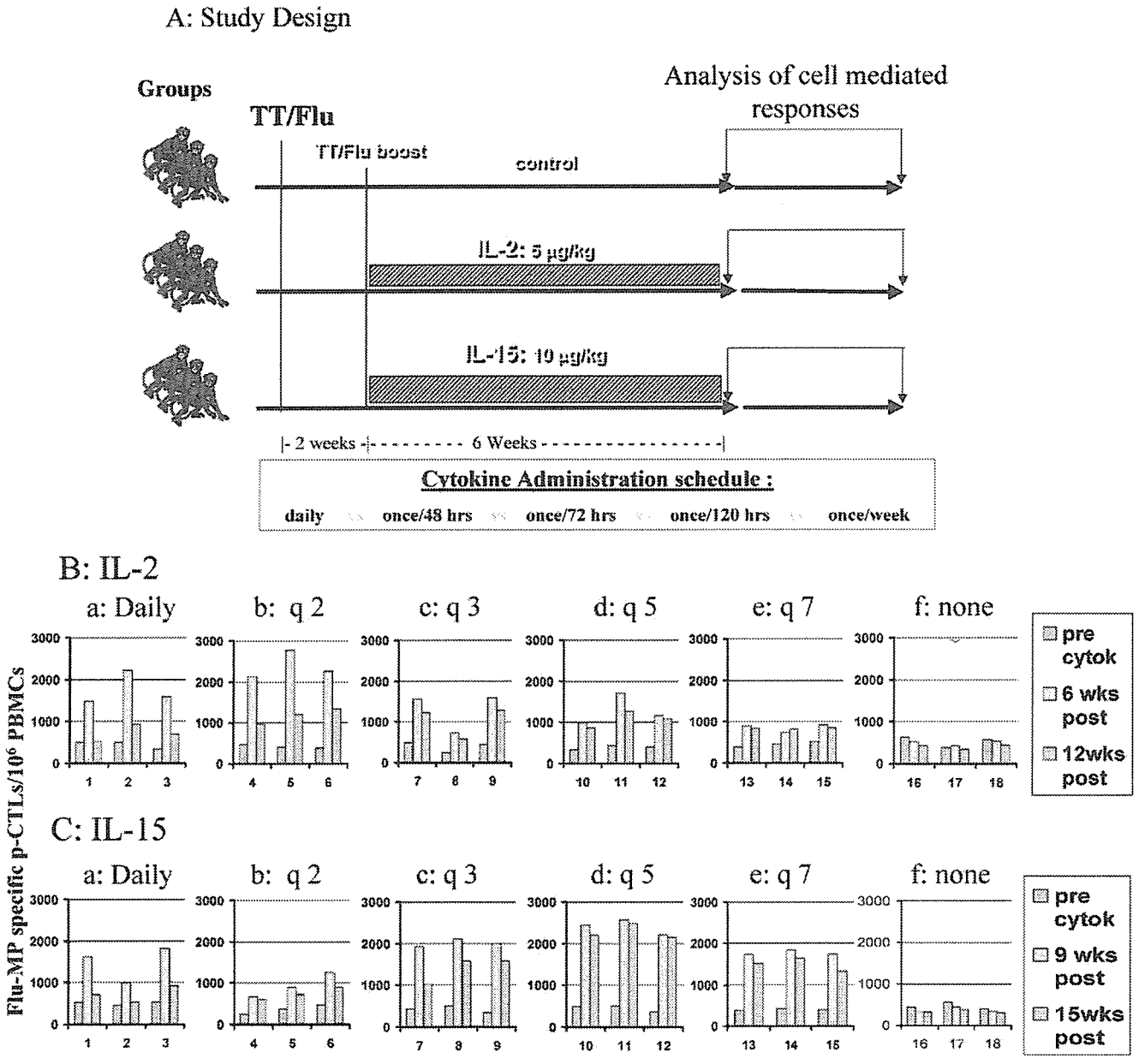

Fig. 2. Experimental design (A) of the evaluation of IL-2 and IL-15 administration frequency on the expansion and maintenance of Flu-MP specific p-CTL responses (B and C) and TT specific IL-2 ELISPOT responses (D and E). Groups of three rhesus macaques each were immunized with TT and live attenuated Flu A/PR8 followed by repeated administration of IL-2 (B and D) or 10 µg/kg IL-15 (C and E) for 6 weeks at the indicated daily intervals. Antigen specific responses were assayed before cytokine administration and at 6 and 12 weeks following booster immunization (IL-2 treated monkeys) or at 9 and 15 weeks following booster immunization.

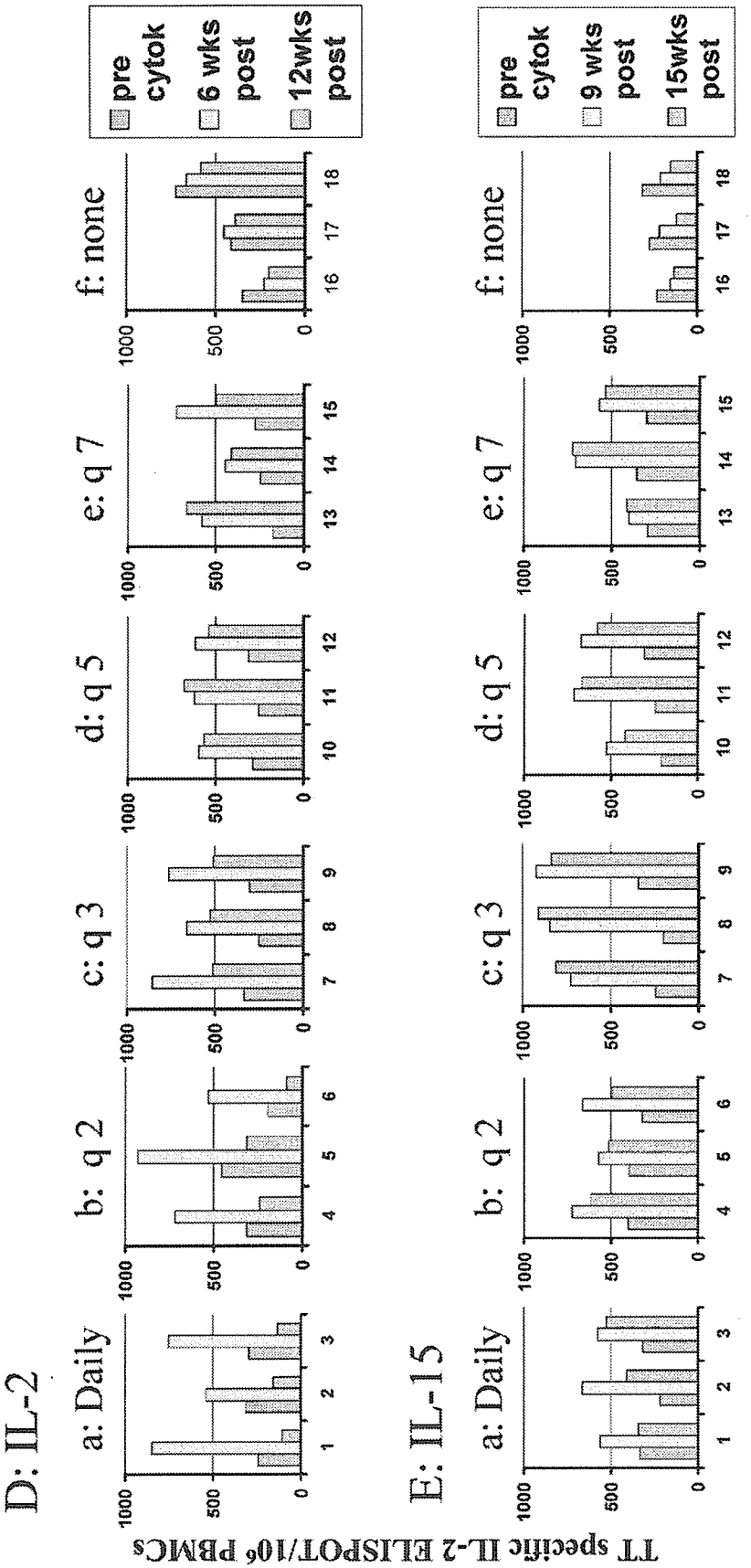

Fig. 2. (*Continued*).

(Fig. 2E). Evaluation of levels of antigen specific memory cells at 15 weeks showed a maximum retention of Flu-MP specific CD8 T cells when IL-15 was administered q5 days and TT specific CD4 T cells when IL-15 was administered q3 days (Fig. 2E). Based on these results, we submit that spacing of IL-2 and IL-15 therapeutic dosage provides distinct benefits in terms of enhancement and maintenance of antigen specific responses. In fact daily or q2 days administration of IL-2 resulted even in diminished levels of TT specific CD4 memory T cells (Fig. 2D), although the number of animals per group precludes definitive conclusions. In contrast, TT specific memory T cells and to a lower extent Flu-MP specific memory cells appeared to be better maintained following IL-15 treatment as exemplified by the week 15 levels of TT specific T cell levels which were not statistically different from the levels measured at 9 weeks for all administration schedules. These data appear to suggest that there are differences between retention of antigen specific memory responses that are augmented by IL-2 as compared with IL-15, the latter being more effective.

3.4. IL-2/IL-15 mediated effects on primary immune response in monkeys

Based on the findings presented above, it was reasoned that administration of IL-2 (5 μg/kg) and IL-15 (10 μg/kg) according to a twice weekly dosing schedule would represent the most optimal compromise for enhancement and maintenance of antigen specific CD4 and CD8 T cells. Hence such a schedule was utilized for all subsequent experiments. The findings above also prompted us to examine the potential of combining IL-2 with IL-15 cytokine therapy as an additional parameter. Thus, the modulatory effect of IL-2 and IL-15 was evaluated on groups of four monkeys each after a primary Flu/TT immunization followed by 4 weeks of IL-2, IL-15 or a combination of alternating IL-2 and IL-15 twice weekly administration (Fig. 3). A group of immunized but otherwise untreated monkeys served as a control. The effect of the cytokine administration was then evaluated on Flu-MP specific CD8 T cell responses and TT specific CD4 T cell responses both at 1 month post immunization (following the discontinuation of cytokine administration) or at 6 months as a measure of memory levels. As illustrated in Fig. 4A, IL-2 administration induced significantly higher levels of Flu-MP specific effector T cells when compared to primed monkeys that did not receive cytokines (Fig. 4A, $P < 0.0022$), while IL-15 only marginally enhanced such levels ($P > 0.05$). The combination of IL-2 and IL-15 appeared to induce a synergistic enhancement of such antigen specific T cell expansion over primed but untreated control monkeys ($P < 0.0001$) or over IL-2 treated monkeys ($P < 0.0001$). At 6 months post prime, however, the monkeys administered IL-2 exhibited Flu specific T cell levels no different from the control primed/non cytokine treated monkeys (Fig. 4B). In contrast, animals given IL-15 following the primary immunization, in spite of a modest initial expansion of Flu-MP specific CD8 effectors showed significantly elevated levels of Flu-MP specific memory T cells when compared to IL-2 treated and untreated primed monkeys (Fig. 4B, $P < 0.0001$), suggesting better retention of the expanded T cells by the action of IL-15. IL-2/IL-15 treated monkeys again showed the highest level of Flu-MP specific memory T cells with a significant enhancement even over IL-15 only treated monkeys (Fig. 4B, $P < 0.0001$).

Similar analyses were performed in the same monkeys for levels of CD4 mediated TT specific responses (Fig. 4C and D). In contrast to Flu specific CD8 mediated responses, while IL-2 significantly expanded the TT specific CD4 T

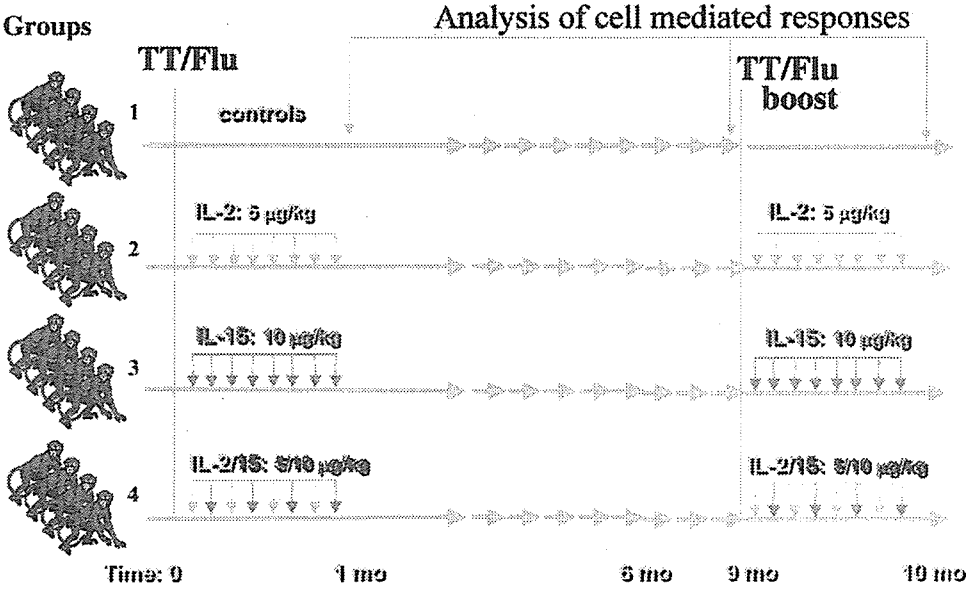

Fig. 3. Experimental design of the IL-2, IL-15, and IL-2/15 mediated enhancement of primary and secondary immune responses to Flu and TT immunization.