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Augmentation of antigen-specific cytokine responses in the early phase of vaccination with a live-attenuated simian/human immunodeficiency chimeric virus expressing IFN-γ

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Summary. A nef-deleted SHIV-NM-3rN (SHIV-NI) was previously shown to be nonpathogenic and to induce protective immunity. In the present study, a SHIV-NI expressing human interferon-γ (SHIV-IFN-γ) was constructed and the effect of co-expression of IFN-γ on virus replication and immunopotentiation was investigated in macaques that were vaccinated with both viruses, by comparing cytokine responses during the first 4 weeks after vaccination. Peripheral blood mononuclear cells (PBMC) isolated from vaccinated macaques were stimulated with inactivated viral particles for 24 h, and the production of IL-2, IL-4, IL-6, IL-10, IL-12, TNF-α and IFN-γ was determined by ELISA and flow cytometry. All of the vaccinated macaques showed increases in cytokine production. However, the production of IFN-γ (Th1-type cytokine) was more rapidly induced by SHIV-IFN-γ vaccination, and IFN-γ-producing cells appeared to be still increasing at 4 weeks after vaccination, although the difference of virus replication during the time was not significant in contrast to *in vitro* replication in cultured PBMC. These results suggest that co-expression of IFN-γ with SHIV can modulate the antiviral immune responses into the Th1 type response, which would probably provide more protective immunity.

Introduction

Anti-HIV immunity is one of the most important subjects in AIDS research. Induction of adequate immune responses against HIV may prevent viral infection

or disease progression. Primate models of AIDS, such as *Simian immunodeficiency virus* (SIV)-infected monkeys, have demonstrated that live attenuated viruses induce preventive immunity and can be used to identify immune responses that are effective in combating HIV [1, 5, 35]. Especially infection to macaque monkeys with SHIV, the chimera virus of HIV and SIV, may help to identify these responses because SHIVs express the HIV envelope glycoprotein, which is recognized as the main immunogen of HIV in humans. Recently, Ui et al. constructed a new SHIV, NM-3rN-delta-nef (SHIV-NI), which is nonpathogenic and gave macaques complete protection from infection with parental NM-3rN [42].

The mechanism of protective immunity in SIV/SHIV infection has not been elucidated: the mechanism has been proposed to include cellular (Th1-type) responses [13, 15, 17, 23, 25–27, 33, 34, 37, 46], humoral (Th2-type) responses [11, 12, 45] or both Th1- and Th2-type responses [22, 32, 36]. Since cytokines play a crucial role in determining the type of immune responses, the pattern of antigen-specific cytokine production will reflect which type of immunity is induced in virus-infected individuals. Moreover, early events in SIV/SHIV infection have been shown to predict disease outcomes in the later phase [4, 7, 8, 31, 40, 44, 47]. Therefore, analysis of cytokine production just after attenuated SHIV infection will provide clues to the mechanism of protective immunity. Although many studies have investigated cytokine production in SIV/SHIV infection [4, 8, 10, 17, 20, 39, 47], the very early phase of SHIV infection has not been observed in detail. Therefore, we investigated antigen-specific cytokine responses in the initial 4 weeks of very early phase of infection with SHIV-NI.

IFN-γ is a cytokine that possess antiviral activity. IFN-γ, in addition to having a direct effect on virus replication, might also effect the course of infection and induction of protective immunity *in vivo* by modulating the antiviral immune response. Co-delivery of cytokines with HIV/SIV/SHIV has been suggested as a means of modulating the immune response pattern [43]. IFN-γ is a Th1-type cytokine that induces cellular immunity. Several studies have shown that IFN-γ can act as an adjuvant in AIDS vaccines [14, 18, 19, 28]. In the present study, therefore, we constructed SHIV-IFN-γ which has the IFN-γ gene inserted to the nef-deleted part of SHIV-NI, and compared vaccinations with SHIV-IFN-γ and parental SHIV-NI to determine whether co-expression of IFN-γ could alter the antiviral immune response. Although the degree of virus replication was not significantly different between the SHIV-NI- and SHIV-IFN-γ-vaccinated monkeys, our results suggest that co-expression of IFN-γ contributes to the rapid induction of cellular immunity.

Materials and methods

Construction of SHIVs

The construction of SHIV-NM-3rN infectious virus was reported previously [29]. Briefly, this chimeric virus contains the vpr, tat, rev, vpu and env genes of HIV-1 (NL432 isolate) in an SIVmac239 (nef-open) background. Infectious SHIV-NI was constructed by replacing the nef gene (for SHIV-NI) of SHIV-NM-3rN by short polylinkers containing unique endonuclease cleavage sites. SHIV-IFN-γ was constructed by cloning a human IFN-γ-coding sequence into the nef-replaced region. IFN-γ DNA was amplified from a recombinant IFN-γ

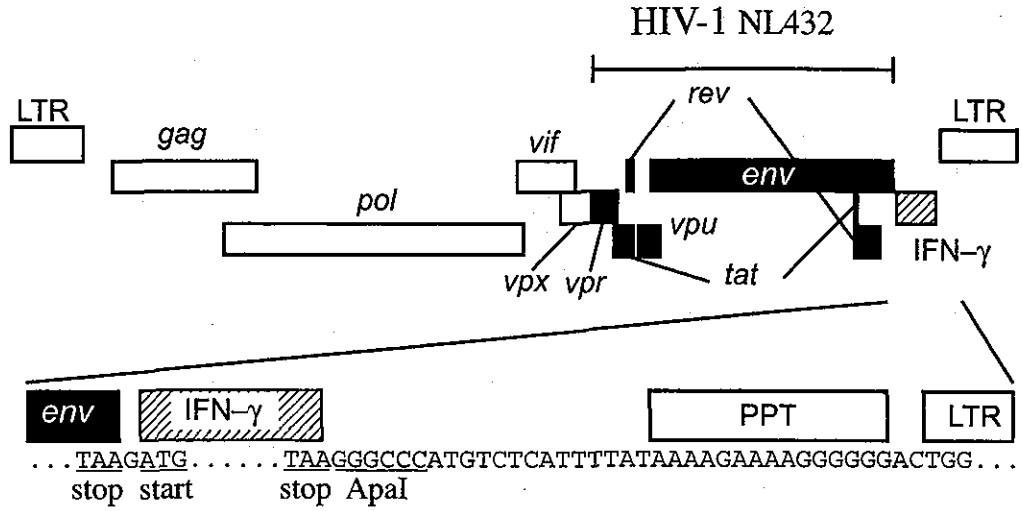

Fig. 1. Genetic structure of SHIV-IFN-γ. The black-colored regions represent sequences derived from HIV-1 (NL432 isolate), the white-colored regions represent SIVmac 239-derived sequences. Human IFN-γ gene DNA (hatched regions) was inserted between the EcoRV and ApaI endonuclease sites of SHIV-NI

DNA-containing plasmid by PCR using IFN-γ-nefF (5′-gAAATATACAAgTTATATCTTggC-3′) and IFN-γ-nefR (5′-gAgggCCCTTACTgggATgCTCTTCg-3′) primers, and ligated to the blunt end of an EcoRV site and sticky end of an ApaI site of SHIV-NI (Fig. 1). The obtained IFN-γ-containing plasmids were multiplied in E. coli DH5a cells. To make sure that no unintended changes had been introduced, all nucleotide sequences derived from PCR were sequenced.

Virus stock production and in vitro *infection*

Plasmids containing full-length retroviral genomes were introduced into a human T-cell line, M8166, by the DEAE-dextran method as described previously [29]. Transfected cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS) and monitored daily to score cytopathic effects. The collected supernatant was used as a viral stock after $TCID_{50}$ measurement. To investigate the growth kinetics of newly constructed clones *in vitro*, macaque PBMC were infected with 10^4 $TCID_{50}$ of filtered cell-free virus stock. The supernatants were harvested with subsequent addition of new medium approximately every 3 days for 25 days after infection and the viral replication was measured by a reverse transcriptase activity assay (RT-assay) as described. As to SHIV-IFN-γ infection, IFN-γ levels in the supernatant were determined by enzyme-linked immunosorbent assay (ELISA) using a commercially available human IFN-γ ELISA Kit (TOYOBO, Osaka, Japan). Biological activity of IFN-γ was confirmed by measuring the inhibition of the cytopathic effect caused by VSV infection in WISH cells, a human amniotic epithelial cell line.

Vaccinations of rhesus macaques

Four rhesus macaques were intravenously inoculated with 10^4 $TCID_{50}$ of SHIV-NI (MM248 and MM252) or SHIV-IFN-γ (MM246 and MM253) and sacrificed at 4 weeks postinfection (p.i.). All animals were housed in a P3 level monkeys storage facility, and were treated in accordance with regulations approved by the Institutional Animal Care and Use Committee of the Institute for Virus Research, Kyoto University. Before infection and at 1, 2 and 4 weeks postinfection, blood samples were collected, treated with sodium citrate to prevent

coagulation, and used to isolate peripheral blood mononuclear cells (PBMC) and plasma samples. To obtain lymph node (LN) cells, axillary or inguinal LNs were surgically removed from each macaque before infection and at 2 and 4 weeks p.i., and mesenteric LNs were removed at 4 weeks p.i. *In vivo* plasma SHIV-IFN-γ viral replication was determined by viral RNA quantitative assays, which were performed by reverse transcriptase PCR (RT-PCR) for the SIV gag region as previously described [29]. The detection limit of the assay was 250 copies/ml. Antibody production was assayed by using a particle agglutination test kit, Serodia HIV (Fujirebio, Tokyo, Japan), which was confirmed to be crossreactive with SHIV.

Virus inactivation procedure

For viral antigen stimulation of PBMC, whole inactivated viral particles were prepared using the method of Rossio et al. [38] with some modifications. 2,2′-dithiodipyridine (aldrithiol-2; AT-2) was added to 150 ml of SHIV-NI stock to a final concentration of 1 mM and the suspension was incubated for 1 h at 37 °C. After inactivation, viral particles were collected by centrifugation for 90 min at 15,000 × g (4 °C). The pellet was washed with RPMI + 10% FBS and then resuspended in 1 ml of RPMI + 10% FBS. The virus concentration in the suspension was determined by antigen capture assay for SIV p27 antigen (Coulter, Miami, FL), according to the manufacturer's instructions.

Stimulation of PBMC and LN cells

PBMC and LN cells were isolated from peripheral blood and LN of vaccinated macaques by centrifugation on Percoll (Amersham Pharmacia Biotech, Buckinghamshire, England) and resuspended in RPMI + 10% FBS. PBMC were cultured in 24-well plates at a cell density of 2×10^6 cells/ml with inactivated viral particles (2.5 μg p27/ml) for 24 h, or with mitogen, Phorbol 12-Myristate 13-Acetate (PMA; 50 ng/ml) and Ionomycin (IM; 500 ng/ml), for 6 h. During the last 2 h of culture, cells were treated with 10 μg/ml of Brefeldin-A (Sigma, St. Louis, Missouri), a transport inhibitor, for an intracellular cytokine staining assay.

Assay of cytokine production

Mitogen-stimulated PBMC and LN cells were examined by flow cytometry to determine intracellular cytokines levels. Briefly, cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD4 (Nichirei, Tokyo, Japan) and PerCP-conjugated anti-CD8 (Becton Dickinson, San Jose, CA) in PBS containing 2% FBS, fixed with 4% paraformaldehyde (IC Fix; BioSource International, Camarillo, CA) and permeabilized with detergent-containing buffer (IC Perm; BioSource International). The permeabilized PBMC were stained with phycoerythrin (PE)-conjugated anti-cytokines (human IFN-γ, IL-4 and TNF-α, Pharmingen, San Diego, CA; human IL-2, BioSource International) in detergent-containing buffer and analyzed on a flow cytometer. In antigen-stimulated PBMC, production of IFN-γ, IL-2, IL-4, IL-6, IL-12 and TNF-α were determined by ELISA of the culture supernatant using commercially available kits (macaque IFN-γ, IL-2 and IL-12, BioSource International; human IL-4 and TNF-α, Pharmingen; human-IL-6, TFB/TORAY, Tokyo, Japan). In addition, intracellular staining was performed on IFN-γ and IL-2.

Results

Replication of SHIV-NI and SHIV-IFN-γ in vitro

The constructed SHIV-NI and SHIV-IFN-γ were tested for infectivity and IFN-γ expression *in vitro* in cultured macaque PBMC. Representative kinetics of

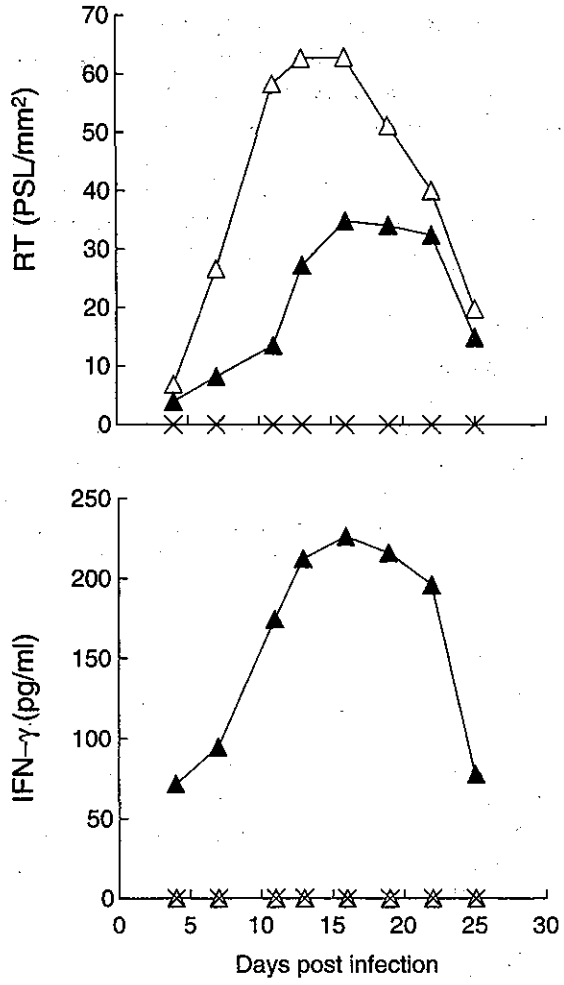

Fig. 2. *In vitro* SHIV infection. Kinetics of viral replication (top) and IFN-γ production (bottom) in macaque PBMC infected with SHIV-NI (△), SHIV-IFN-γ (▲) or mock (×). *PSL*: relative photon-stimulated luminescence

replication and IFN-γ production were shown in Fig. 2. SHIV-NI seemed to replicate slightly faster than SHIV-IFN-γ. The peak of virus replication was observed at around 16 days p.i. Only SHIV-IFN-γ infection resulted in IFN-γ production. The biological activity of the produced IFN-γ was confirmed by using an antiviral assay (data not shown).

To study the stability of the constructs, SHIV-IFN-γ was passaged several times. SHIV-IFN-γ maintained the full-length IFN-γ gene and expressed bioactive IFN-γ for the entire duration of the passage experiment (data not shown).

Change in viral load and induction of antiviral antibodies after in vivo *SHIV vaccination*

Four macaques were intravenously inoculated with 10^4 $TCID_{50}$ of SHIV-NI (MM248, MM252) or SHIV-IFN-γ (MM246, MM253). All animals were viremic within the first week p.i. and the viral load peaked at 2 weeks p.i. (Fig. 3). This is a consistent feature of previous SHIV-NI infections [42]. There was no significant difference in the extent of viremia between SHIV-NI- and SHIV-IFN-γ-vaccinated

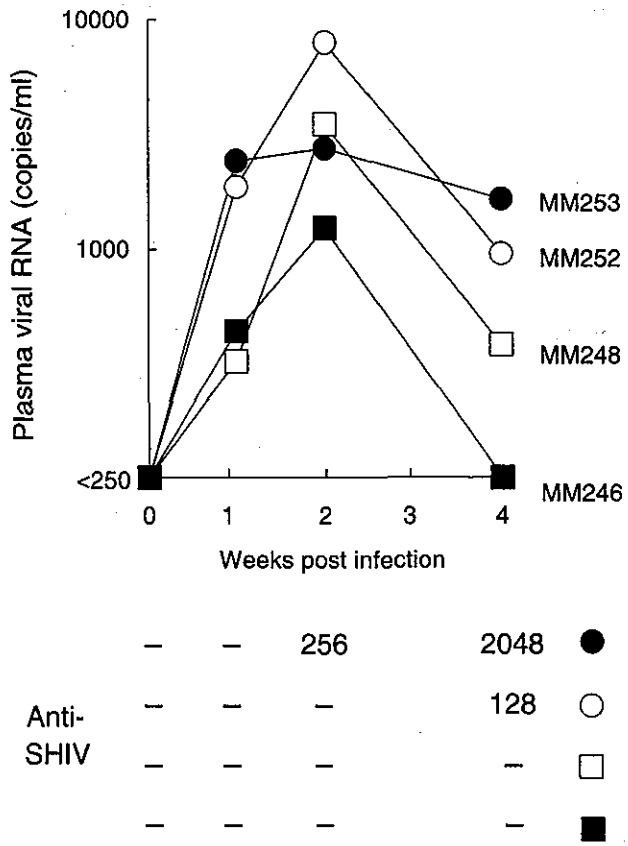

Fig. 3. *In vivo* SHIV vaccination. Changes in viral load (top) and antibody titer (bottom) in MM248 and MM252 after SHIV-NI vaccination, and in MM246 and MM253 after SHIV-IFN-γ vaccination. Antibody titers are expressed as the reciprocal to the maximum dilution in the particle agglutination test. –, under the detection limit

macaques. In MM252 and MM253, anti-SHIV antibody production was observed from 4 weeks and 2 weeks p.i., respectively (Fig. 3).

In all the vaccinated macaques, the number of peripheral CD4+ and CD8+ lymphocytes did not change significantly (data not shown). Similar results were obtained with previous SHIV-NI infections [42].

Cytokine production in antigen-stimulated PBMC

PBMC from the vaccinated macaques were stimulated with inactivated SHIV in 24-well plates. After 24 h, the culture supernatant wa collected and analyzed for the presence of IFN-γ, IL-2, IL-4, IL-6, IL-10, IL-12 and TNF-α by specific ELISAs.

In PBMC from unvaccinated macaques, IFN-γ was below the limit of detection (less than 20 pg/ml). However, after vaccination with SHIVs, IFN-γ production increased in all the macaques (Fig. 4A). Although there appeared to be no significant difference in the increment in IFN-γ production between SHIV-NI and

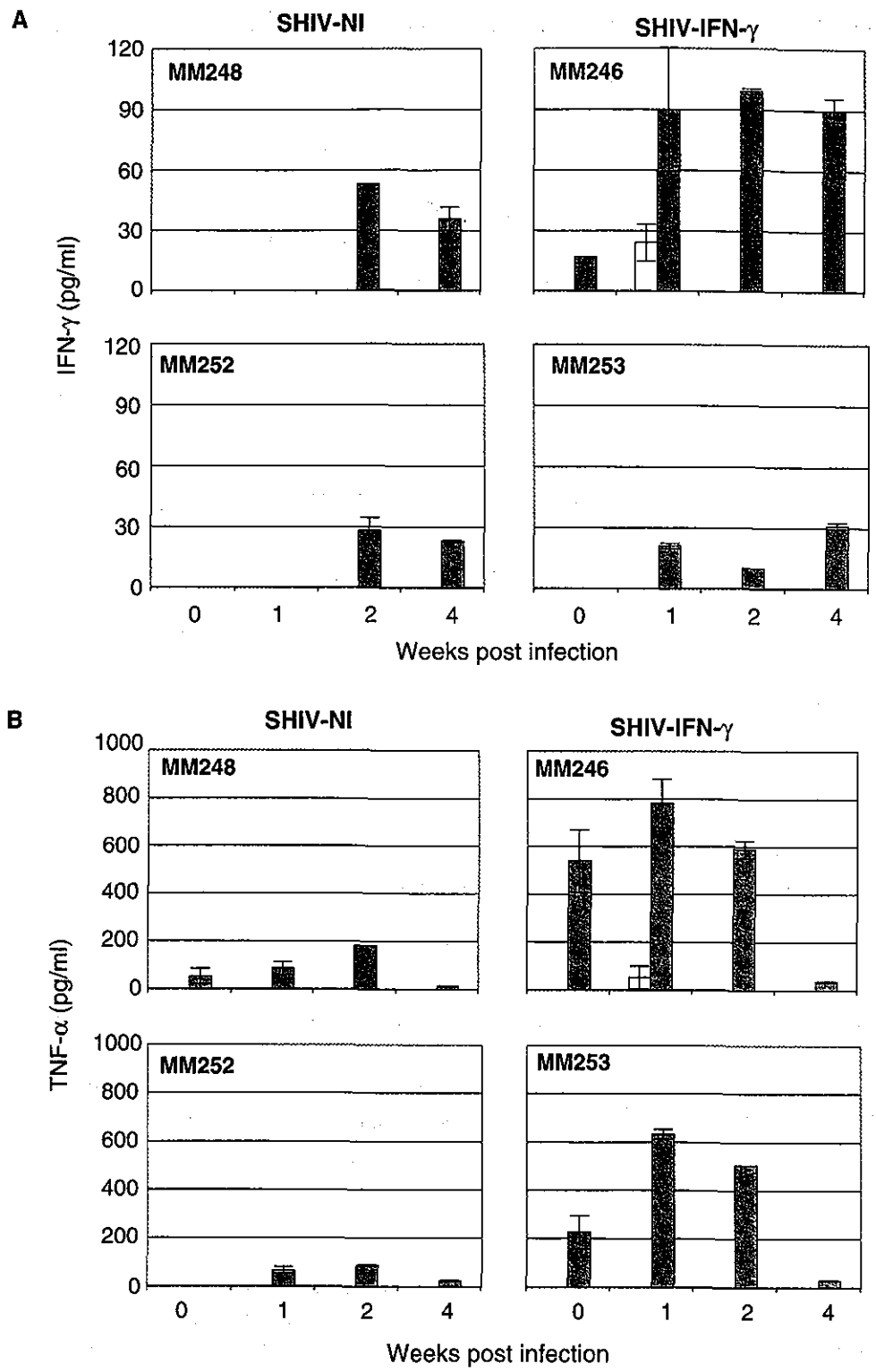

Fig. 4. Change in cytokine response of PBMC to viral antigen stimulation after SHIV vaccination. White columns indicate concentrations of IFN-γ (**A**) and TNF-α (**B**) in the culture supernatant of unstimulated cells. Shadowed columns indicate the corresponding concentrations in the supernatant of inactivated-SHIV-stimulated cells. Standard error bars are shown for the samples that were assayed in duplicate

SHIV-IFN-γ vaccination, the increase occurred earlier in SHIV-IFN-γ vaccination (at 1 week p.i.) than in SHIV-NI vaccination (at 2 weeks p.i.). The plasma IFN-γ concentration was under the detection limit in all the samples (data not shown).

TNF-α production, which was strong in MM246 and MM253 before the vaccination, increased to 100–800 pg/ml in all the macaques after vaccination

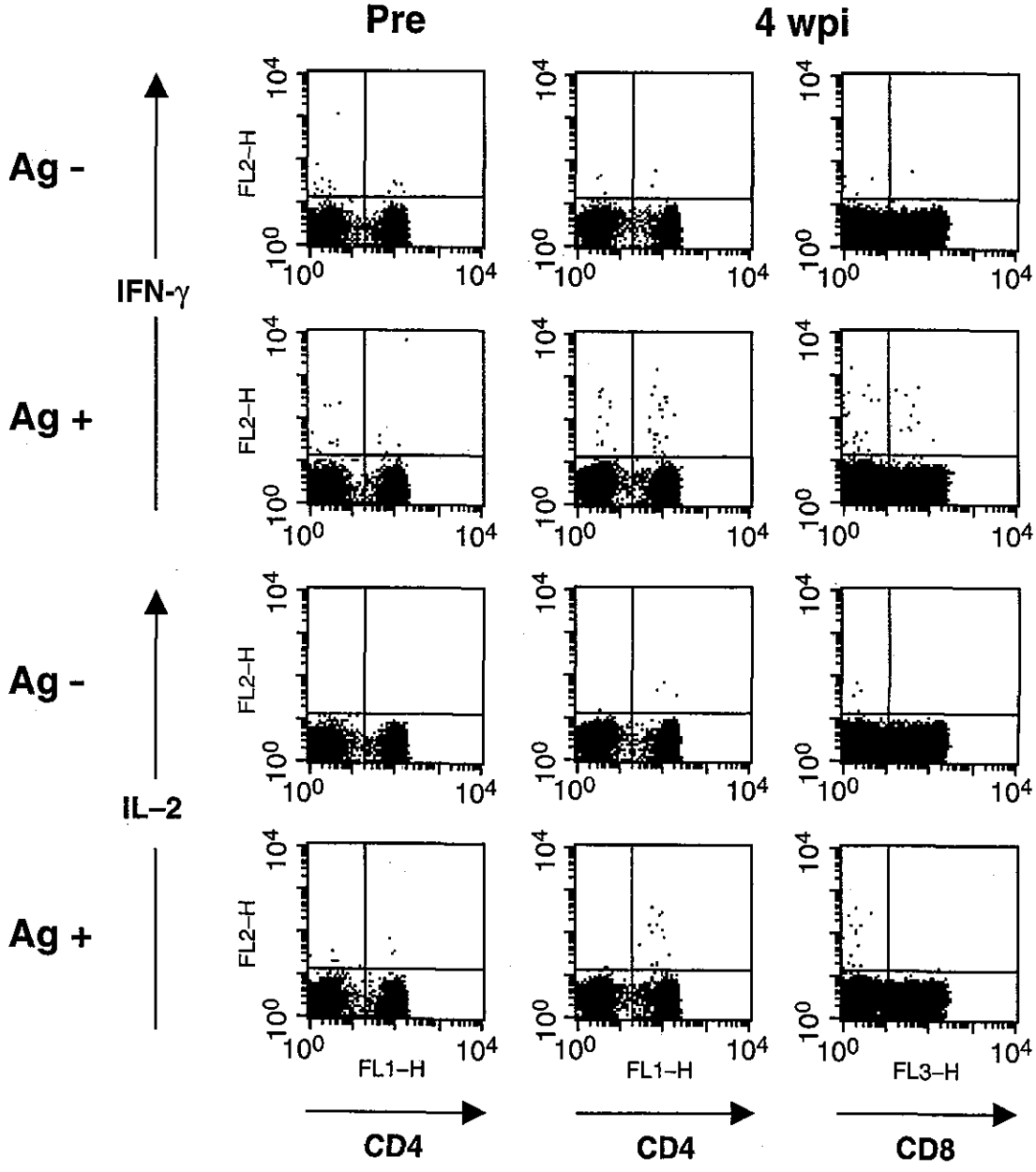

Fig. 5. Flow cytometry results showing intracellular cytokines in viral antigen-stimulated PBMC. Data show representative plots longitudinally obtained from MM246, which was infected with SHIV-IFN-γ. FL-1 represents CD4 or CD8 expression and FL-2 represents IFN-γ expression (top) or IL-2 expression (bottom). Each plot contains about 1×10^4 lymphocytes

(Fig. 4B). The TNF-α production peaked at 1–2 weeks p.i., and increased more in SHIV-IFN-γ-vaccinated macaques than in SHIV-NI-vaccinated macaques.

Although the productions of IL-2, IL-4, IL-6, IL-10 and IL-12 were augmented after vaccination, there were no significant differences in the increments of production of these cytokines between SHIV-NI and SHIV-IFN-γ vaccination (data not shown).

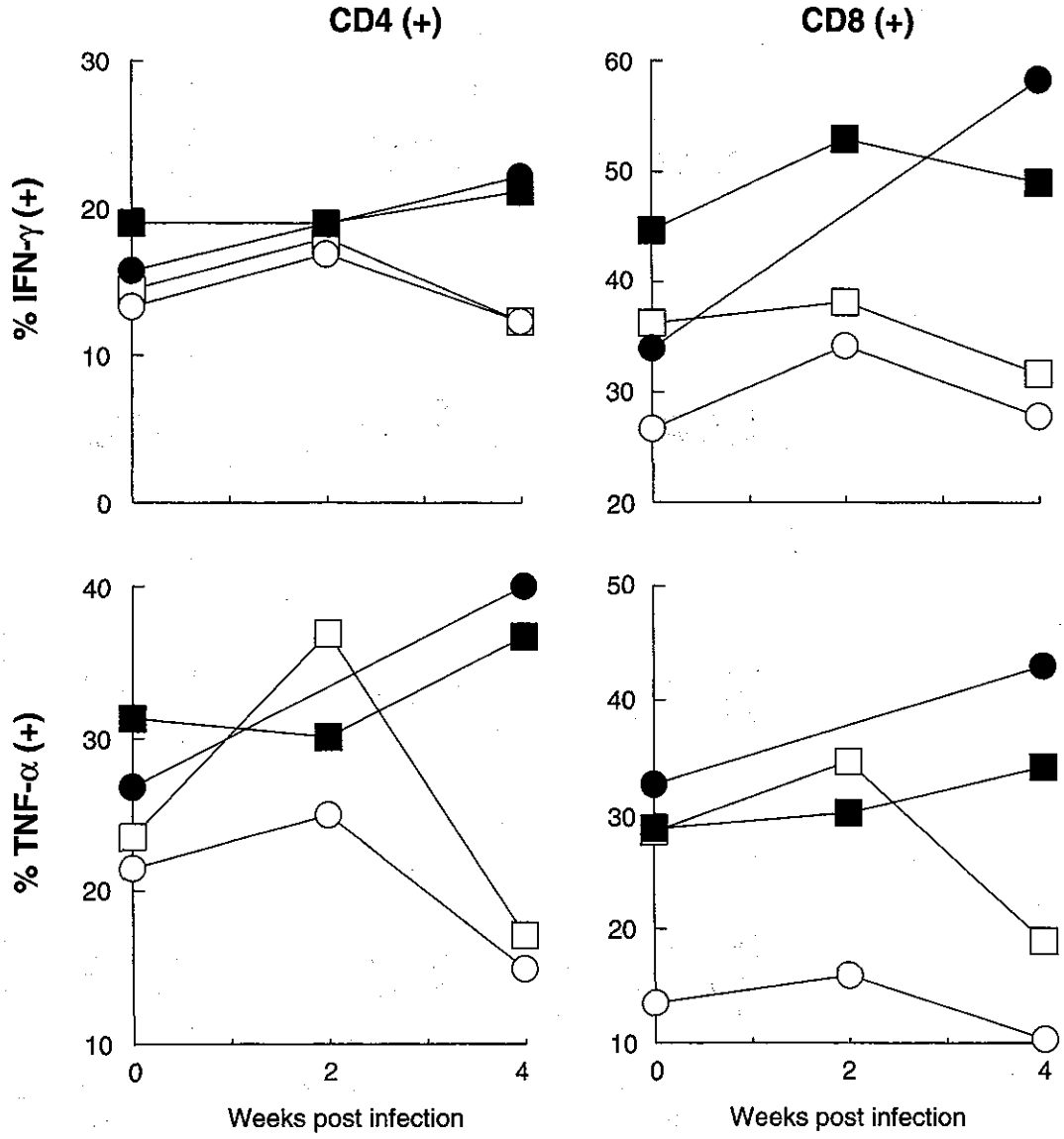

Fig. 6. Change in cytokine production in PMA/IM-stimulated PBMC after SHIV vaccination. Data show percentages of IFN-γ, IL-2, IL-4 and TNF-α producing cells in CD4+ (left) and CD8+ (right) population in PBMC from MM248 (□) and MM252 (○) infected with SHIV-NI, and MM246 (■) and MM253 (●) infected with SHIV-IFN-γ

Detection of intracellular IFN-γ and IL-2 in antigen-stimulated PBMC

The antigen-stimulated PBMC from macaques were analyzed for IFN-γ and IL-2 production by an intracellular cytokine staining assay to determine more precisely. Figure 5 shows representative dot plots obtained in the flow cytometric analysis. At 4 weeks p.i., IFN-γ production and IL-2 production were apparent. IFN-γ was detected in both CD4+ and CD8+ cells, while IL-2 was detected mainly in CD4+ cells.

Cytokine production in antigen-stimulated lymph nodes cells

Mononuclear cells from peripheral and mesenteric lymph nodes were also stimulated with SHIV antigen for 24 h and the culture supernatant was analyzed for the presence of IFN-γ, IL-2, IL-4, IL-12 and TNF-α by specific ELISAs. The cytokine response of lymph nodes cells also increased after SHIV vaccination, although the extent of augmentation was lower than in PBMC in general (data not shown).

Cytokine production in mitogen-stimulated PBMC

Cytokine producing CD4+ and CD8+ cells in PBMC stimulated with PMA and IM were quantified by a multiparametric flow cytometric analysis using antibodies that recognize intracellular cytokines. Figure 6 summarizes changes in the production of IFN-γ and TNF-α after SHIV vaccination. No significant changes were observed in the production of IL-2, IL-4, IL-5, IL-6 or IL-10 (data not shown). In PBMC from the SHIV-NI-vaccinated macaques, the percentage of cytokine-producing CD4+ and CD8+ cells peaked at 2 weeks p.i. All cytokines in CD4+ cells from MM252 exceeded the levels (average + SD of three time-analysis) before vaccination. At 4 weeks p.i., the percentages decreased to levels equal to or less than the preinfection levels. On the other hand, in PBMC from the SHIV-IFN-γ-vaccinated macaques, cytokine-producing cells appeared to be still increasing at 4 weeks p.i. (all values exceeded the levels before vaccination). On the other hand, cytokine production in lymph nodes cells stimulated with PMA and IM did not change significantly (data not shown).

Discussion

In this study we compared the cytokine responses of macaques vaccinated with attenuated SHIV-IFN-γ and SHIV-NI during the early phase following vaccination. As SHIV-IFN-γ infection was analyzed for the first time, *in vitro* production of biologically active IFN-γ from SHIV-IFN-γ was confirmed by ELISA and antiviral assay. SHIV-IFN-γ replicated less rapidly than parental SHIV-NI. This might be due to the antiviral activity of IFN-γ expressed by the virus itself. On the contrary, the *in vivo* kinetics of viral loads in SHIV-IFN-γ-vaccinated macaques was almost the same as that in SHIV-NI-vaccinated ones. This result is inconsistent with that obtained in macaques vaccinated with IFN-γ-containing SIV, in which

the viral loads were lower than they were in the macaques infected with parental SIV-delta-nef [18]. Since infectious SHIV was not isolated from PBMC in either SHIV-NI- or SHIV-IFN-γ-vaccinated macaques at the peak point of plasma viral loads (data not shown), replication levels of these SHIVs *in vivo* were thought to be very low. Therefore, in SHIV-IFN-γ vaccination *in vivo*, it appears that IFN-γ is not produced in sufficient quantities to have any significant effects on viral replication during the observation period.

To characterize antigen-specific cytokine production that has not been investigated in the early phase of SHIV vaccination, PBMC from the vaccinated macaques were stimulated with whole inactivated SHIV particles. Various response patterns were observed among the cytokines quantified by ELISA after the antigen stimulation.

There was almost no antigen-specific IFN-γ and IL-2 production in any of the macaques before vaccination. The production of these cytokines increased after SHIV vaccination as shown by both ELISA and intracellular cytokine staining assay. Especially, the production of IFN-γ was more rapidly induced in monkeys vaccinated with SHIV-IFN-γ, and IFN-γ-producing cells appeared to be still increasing at 4 weeks after vaccination. These results suggest that co-expression of IFN-γ with SHIV can direct the antigen-specific cytokine production into a Th1-type response. IFN-γ is a Th1-type cytokine that activates natural killer (NK) cells and cytotoxic T lymphocytes (CTL). IL-2 is also a Th1-type cytokine and has been suggested to participate in induction of anti-HIV immunity [2, 3, 17, 21, 28]. Although CTL activity was under the detection limit at 4 weeks p.i. (data not shown), PBMC from SHIV-IFN-γ vaccinated macaques produced TNF-α as well as IFN-γ in response to the antigen stimulation. Since production of IFN-γ and/or TNF-α has been shown to be associated with CTL activity [6, 16, 24, 30], these macaques might have started to develop cellular immunity against SHIV. Because CTL responses against HIV/SIV/SHIV play key roles in suppression of virus [13, 15, 17, 23, 25–27, 33, 34, 37, 46], the SHIV-IFN-γ vaccine might be expected to induce better protection than SHIV-NI.

Recently, Stahl-Hennig et al. described live-attenuated SIV expressing IL-4 or IFN-γ [41]. They suggested that SIV-IFN-γ induces better protection in the absence of neutralizing antibodies than SIV-IL-4. Although the cytokine responses during SIV-IFN-γ vaccination were not analyzed in their report, the augmentation of the Th1 cytokine response after vaccination may contribute to the protection against SIV challenge.

On the other hand, strong IL-6 and IL-12 responses to antigen stimulation had already existed before vaccination in all the macaques (data not shown). These non-specific responses were considered to have relevance with innate immunity. Innate immunity is the primary immune response induced immediately after virus infection in which macrophage and NK cells have major roles. Various cytokines, including IL-6 and IL-12, are produced from these cells upon antigen stimulation and augment the innate response. A recent study suggested that innate response is involved in control of viremia in the acute phase of SIV infection [20]. Since the extent of viremia in the acute phase of SIV/SHIV infection has been

shown to correlate with the disease outcomes in the later phase [4, 7, 8, 31, 40, 44, 47], induction of innate response would be important to prevent disease progression. The present result suggested that SHIV-NI and SHIV-IFN-γ replication *in vivo* stimulated macrophages and NK cells to produce IL-6 and IL-12, leading to the efficient induction of innate immunity. In addition, IL-12, a strong inducer of Th1-type immunity, could also contribute to the CTL development.

The present study compared the profiles of cytokine responses in the initial 4 weeks of vaccination with a live-attenuated SHIV expressing IFN-γ and SHIV-NI. Although cytokine production was augmented in all the vaccinated macaques, induction of Th1-type cytokine response was stronger in the SHIV-IFN-γ-vaccinated macaques. However, a greater number of macaques is needed to determine whether SHIV-IFN-γ is better suited as a live-attenuated vaccine because the response of macaques to the virus was highly variable and only the small number of animals was available in this study. Moreover, future studies on the later phase of vaccination and on the response to challenge experiments will be needed to clarify the roles of cytokine production in SHIV vaccination.

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Protective effects of nef-deleted SHIV or that having IFN-γ against disease induced with a pathogenic virus early after vaccination

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Summary. To clarify the involvement of primitive non-specific immune responses in the protective effects of a live, attenuated virus, each two rhesus macaques were intravenously immunized with an attenuated chimeric simian and human immunodeficiency virus (SHIV) in which the nef gene was deleted (SHIV-NI) or a SHIV having human IFN-γ inserted into the deleted nef region (SHIV IFN-γ). These immunized monkeys were intravenously challenged with a heterologous pathogenic SHIV (SHIV-C2/1) at four weeks post immunization (wpi). After vaccination, one of each SHIV-NI- or SHIV IFN-γ-immunized monkeys showed a low level of SIV Gag-specific lymphocyte proliferative response but did not have neutralizing antibodies to both the parental and challenge viruses. After the challenge, the plasma viral RNA loads of the challenge virus were suppressed in all the immunized monkeys and the severe $CD4^+$ T cell loss observed in the unimmunized monkeys was not found. Thus, both SHIV IFN-γ and SHIV-NI infections could prevent from disease progression by a pathogenic virus early after immunization, suggesting that primitive non-specific immune response elicited by attenuated virus infection, in addition to highly acquired virus-specific immunity, contributes to the protective effect against a pathogenic virus.

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Introduction

Various vaccine candidates against human immunodeficiency virus (HIV) have been developed worldwide and their protective effects have been examined using the available animal models. However, it is difficult to induce both neutralizing antibodies and cytotoxic T lymphocytes (CTL) specific to HIV in vaccinated animals. Even though these highly acquired immune responses specific to HIV were effectively established by various vaccine strategies, vaccination did not always result in effective protection against a pathogenic viral challenge. Moreover, HIV-specific memory $CD4^+$ T cells in infected individuals were recently found to contain more HIV viral DNA than other memory $CD4^+$ T cells [6], suggesting that increasing antigen-specific immune responses might not always lead to the better vaccine strategy against HIV. To investigate protective immune system against HIV infection furthermore, it is necessary to understand primitive non-specific immune responses, which is the first line to prevent against pathogens and also triggers highly acquired immunity. The functions of various cells such as NK cell, macrophage and gamma-delta T cell are associated with the immunity [21]. Thus, both virus-specific acquired immunity and non-specific immunity will be required for immunological therapy and vaccination against HIV.

The macaque model using nonhuman primate lentiviruses is an important tool for mimicking HIV infection in humans and for evaluating protective effects of vaccine candidates against viral infection. Live attenuated immunodeficiency viruses, in which pathogenicity-associated genes such as nef and vpr were deleted, have been shown to elicit strong immune responses in macaque monkeys and prevent infection from a pathogenic virus challenge [4, 5, 17, 35]. These studies showed that not only virus-specific immune responses but also non-specific immune responses would contribute to protective effect against challenge virus [1, 22, 30]. We also previously reported that macaque monkeys immunized with a chimeric simian and human immunodeficiency virus (SHIV) with the deleted nef gene were protected against a heterologous pathogenic SHIV that is not cross-reactive in virus neutralizing as well as against a parental virus [7, 15, 34]. The resistance to virus infection did not always correlate with the presence of antigen-specific immune responses including CTL and neutralizing antibodies.

Further attenuation of live attenuated virus and immune-potentiation has been attempted in several laboratories by constructing the nef deletion mutant expressing the immunostimulatory cytokine [9, 10, 12, 13, 16, 19, 20]. Especially, a nef-deleted SIV or SHIV expressing IFN-γ could modulate the antiviral immune response to a T-helper cell type-1 (Th1) during the acute infection [9, 10, 16]. IFN-γ has been reported to play an important role in antiviral immunity, such as the lysis of infected cells by CTL and direct inhibition of viral replication [18, 33]. In the present study, we attempted to examine whether the primitive non-specific immune responses induced by an attenuated virus can contribute to the protective immunity against a pathogenic virus challenge early after vaccination. For this purpose, rhesus macaque monkeys were intravenously immunized with SHIV having the deleted nef gene (SHIV-NI) or SHIV having human IFN-γ at the deleted nef region (SHIV IFN-γ). After four weeks immunization, when immune

responses against the virus are supposed to be immature or low, all the immunized monkeys were challenged with a heterologous pathogenic SHIV which is not cross-reactive in neutralization to the vaccine viruses, that is to avoid involvement of highly acquired virus specific immunity.

Materials and methods

Viruses

In this study, SHIV-NI and SHIV IFN-γ were used as attenuated viruses. SHIV-NM-3rN is a chimeric simian and human immunodeficiency virus, having the envelope gene of HIV-1 pNL432, and can infect macaque monkeys [15]. SHIV-NI was constructed by deleting the nef gene from SHIV-NM-3rN and SHIV IFN-γ was constructed by insertion of a human IFN-γ gene into the nef-deleted site of SHIV-NI [16]. These virus stocks were prepared from supernatants of a human T cell line, M8166, transfected with pSHIV-NI or pSHIV IFN-γ.

For a challenge virus, we used a heterologous pathogenic virus, SHIV-C2/1, which is not cross-reactive in virus neutralization with SHIV-NM-3rN, a parent virus of SHIV-NI and SHIV IFN-γ. SHIV-C2/1 was previously generated by in vivo passage of SHIV-89.6 (containing env, tat, rev, and vpu derived from primary isolates of HIV-1) through cynomolgus monkeys [28]. SHIV-C2/1 infection causes rapid $CD4^+$ T cell depletion and an immunodeficiency syndrome [28]. The SHIV-C2/1 virus stock was prepared from the culture supernatants of COS-1 cells transfected with a SHIV-C2/1 molecular clone, pKS661 (GenBank accession number AF217181), and was stored at −80 °C until use.

Monkeys and viral infection

Six rhesus macaques (*Macaca mulatta*) were used in this experiment. All monkeys used in this study were housed in accordance with regulations approved by the Institutional Animal Care and Use Committee of the Institute for Virus Research, Kyoto University. Prior to viral infection, monkeys were anesthetized by intramuscular injection of ketamine chloride. Monkeys MM285 and MM286 were intravenously inoculated with 1×10^5 $TCID_{50}$ of SHIV-NI and monkeys MM287 and MM288 were intravenously inoculated with 1×10^5 $TCID_{50}$ of SHIV IFN-γ.

To assess the induced immunity to protect against heterologous viral infection, the four monkeys were intravenously challenged with 1.2×10^4 $TCID_{50}$ of the pathogenic SHIV-C2/1 at 4 weeks post infection (wpi). Two other unvaccinated monkeys (MM272 and MM273) were intravenously challenged with SHIV-C2/1 as controls.

Sample collection

Peripheral blood was periodically collected from all monkeys. Peripheral blood mononuclear cells (PBMCs) were separated from heparinized blood by Percoll density centrifugation. All plasma samples were frozen at −80 °C until analysis.

Virus isolation

To isolate the virus, $CD8^+$-depleted PBMCs from each monkey were cocultured with M8166 cells, a human T cell line, and monitored for at least one month. $CD8^+$-depleted PBMCs were obtained by using mouse anti-human CD8 monoclonal antibody (NU-Ts/c; Nichirei, Tokyo, Japan) and sheep anti-mouse IgG magnetic beads (Dynabeads M-450; Dynal A. S, Oslo, Norway). Virus recovery was confirmed by the appearance of syncytium formation and reverse transcriptase (RT) activity in the culture supernatants.

Polymerase chain reaction (PCR)

Cellular DNAs were extracted from PBMCs of the inoculated monkeys using DNeasy tissue kits (QIAGEN, Hilden, Germany) according to the manufacturer's recommendations. The proviral DNA fragments (569 bp of SHIV-NI and SHIV IFN-γ and 612 bp of SHIV-C2/1) covering the vpr gene of HIV-1 were amplified from each cellular DNA by nested PCR as previously described [7]. PCR products were electrophoretically separated on 1.5% agarose gels and differentiated by their lengths.

Quantification of plasma viral RNA loads

Plasma viral RNA loads were determined by quantitative RT-PCR [31]. Total RNAs were prepared from plasma with a QIAamp viral RNA kit (QIAGEN) according to the manufacturer's recommendations, and RT-PCR was performed using a Taqman RT-PCR kit (Perkin Elmer). RNAs of the attenuated viruses and the challenge virus were evaluated with primer pairs specific to SHIV NM-3rN and SHIV-C2/1, respectively, as previously described [7]. These reactions were performed with a Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA) and analyzed using the manufacturer's software. For each run, a standard curve was generated from duplicate samples at different dilutions whose copy numbers were known, and the RNA in the plasma samples were quantified based on the copy number of the standard samples.

Flow cytometry

The frequency of T lymphocyte subset in whole bloods was examined by flow cytometry. Blood samples of each monkey were stained with FITC-conjugated anti-monkey CD3 (FN-18; BioSource International, Inc, Nivelles, Belgium), phycoerythrin (PE)-conjugated anti-human CD4 (NU-TH/I; Nichirei) and PerCP-conjugated anti-human CD8 (Leu-2a; BD Pharmingen, San Diego, CA). After hemolysis of the whole blood using FACS^{TM} Lysing Solution (BD Pharmingen), each labeled lymphocyte was analyzed on a FACScan (Becton Dickinson, Mountain View, CA). Absolute lymphocyte counts in the blood were determined with an automated blood cell counter (F-820; Sysmex, Kobe, Japan).

Antibodies responses in plasma

Antibody levels in the plasma were detected by the passive agglutination (PA) method (Serodia HIV-1/2, Fujirebio Inc., Tokyo, Japan). The antibody titer was measured by a four-fold serial dilution of each sample, as recommended by the manufacturer.

Neutralizing antibodies were assessed by M8166 culture, as described previously [7]. A twofold serial dilution of each heat-inactivated plasma sample was incubated with an equal volume of SHIV-NM3rN or SHIV-C2/1 virus stock (100 $TCID_{50}$) for 90 min at 37 °C and was cultured with 2×10^4 M8166 cells in 96-well plates. RT activities in the culture supernatants were measured at 5 days post infection. The last dilution of each sample at which the virus replication was inhibited in less than 50% of the positive samples was defined as the neutralizing titer of the sample.

SIV Gag-specific lymphocyte proliferation

SIV Gag-specific lymphocyte proliferation was measured by incorporation of BrdU into the stimulated-lymphocytes. To remove monocytes from PBMCs of each monkey, non-adherent lymphocytes were collected after incubation of PBMCs on the tissue culture plate at 37 °C for 1 h. 2×10^5 lymphocytes obtained from each monkey were cultured in the presence of 10 μg/ml of SIVmac251 purified Gag p27 proteins (Advanced Biotechnologies, Inc.,