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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.,

Columbia, MD) for 72 hours. After the incubation with BrdU for 24 h more, lymphocyte proliferation was assayed using Cell Proliferation ELISA, BrdU (Roche Diagnostics, Basel, Switzerland) following the manufacturer's recommendations. The stimulation index for cell activity was calculated using the following formula: (OD value in the stimulated PBMC – blank) / (OD value in unstimulated PBMC – blank).

Results

Intravenous immunization of SHIV-NI and SHIV IFN-γ

Each SHIV IFN-γ and SHIV-NI was intravenously inoculated into two rhesus macaque monkeys. After the inoculation, viral RNAs in plasma of all monkeys transiently increased with the peak at 3 wpi and were below the detectable level at 4 to 6 wpi (Fig. 1). Proviral DNAs in PBMCs were also transiently detected at 1 to 3 wpi and the infectious viruses were isolated at 1 to 3 wpi from coculture of PBMCs with M8166 (Table 1). The viruses that were reisolated from SHIV IFN-γ-infected monkeys at 1 and 2 wpi continued to release more than 100 pg/ml of IFN-γ into the culture supernatant of M8166 cells at 3 days after infection

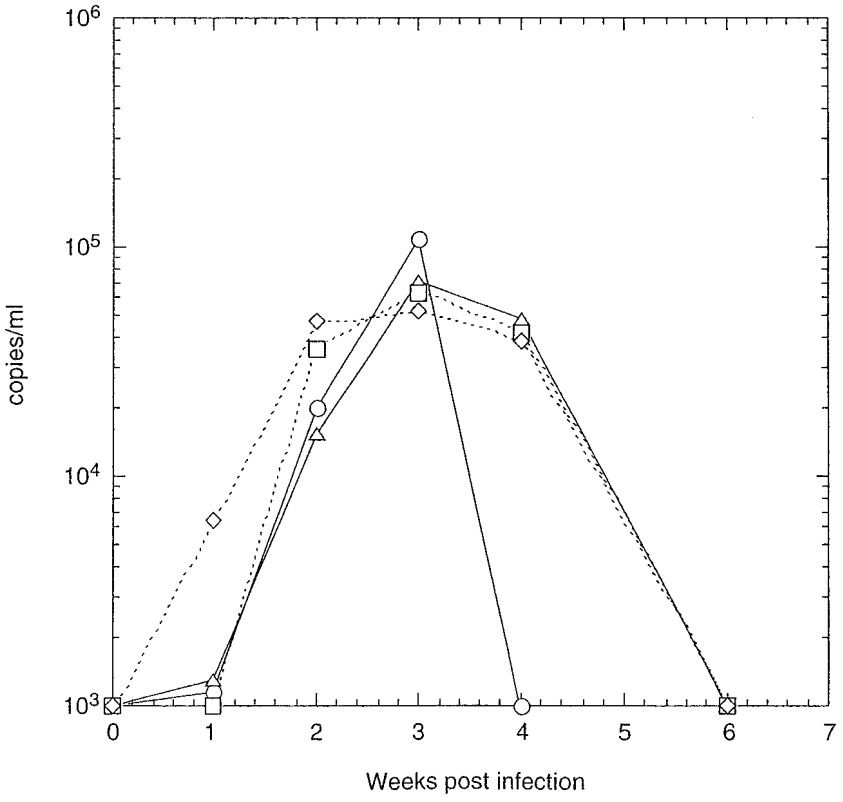

Fig. 1. Plasma viral RNA loads in SHIV IFN-γ- or SHIV-NI-infected monkeys; MM285 (○), MM286 (△), MM287 (□) and MM288 (◇). The detection limit of this assay was 10^3 copies/ml

Table 1. Virological status of SHIV IFN-γ or SHIV-NI inoculated monkeys after challenge

Monkey		Weeks post inoculation				
		0	1	2	3	4
SHIV IFN-γ						
MM285	Proviral DNA[a]	−	+	+	−	−
	Virus isolation[b]	−	+	+	ND	+
MM286	Proviral DNA[a]	−	+	−	−	−
	Virus isolation[b]	−	+	+	−	−
SHIV-NI						
MM287	Proviral DNA[a]	−	−	+	+	−
	Virus isolation[b]	−	+	+	−	−
MM288	Proviral DNA[a]	−	−	+	+	−
	Virus isolation[b]	−	−	+	+	−

[a]Proviral DNAs in PBMCs (1×10^6 cells) of SHIV-infected monkeys were detected by nested PCR

[b]Virus isolation was performed by coculture with human T cell lines, M8166, and $CD8^+$ cells-depleted PBMCs. ND – not done

(data not shown). After the infection, all monkeys clinically remained healthy and showed no change of $CD4^+$ T cell number in PBMCs. Thus, there was no significant difference in the extent of pathogenicity between SHIV IFN-γ and SHIV-NI infection to macaque monkeys.

Intravenous challenge of the SHIV-NI- or SHIV IFN-γ-immunized monkeys with SHIV-C2/1

To assess the protective effects against heterologous viral infection, four monkeys infected with SHIV IFN-γ or SHIV-NI were intravenously challenged with 1.2×10^4 $TCID_{50}$ of the pathogenic SHIV-C2/1 at 4 wpi. Two other monkeys (MM272 and MM273) were intravenously inoculated with SHIV-C2/1 as naive controls. In these unimmunized monkeys, the viral RNA loads in the plasma dramatically increased with a peak of 10^7 to 10^8 copies/ml at 2 wpi and were systemically detected at 10^5 to 10^6 copies/ml in both monkeys (Fig. 2).

After challenge of SHIV-C2/1, the plasma viral RNA loads of SHIV-NI-immunized monkeys (MM287 and MM288) were suppressed to about 1/10 of those of the naive controls at 2 weeks post challenge (wpc), at the peak of viral RNA loads (Fig. 2). Both monkeys maintained low viral RNA loads (around 10^4 copies/ml) in the plasma during the observation period. Thus, SHIV-NI-infected monkeys could partially suppress the challenge virus.

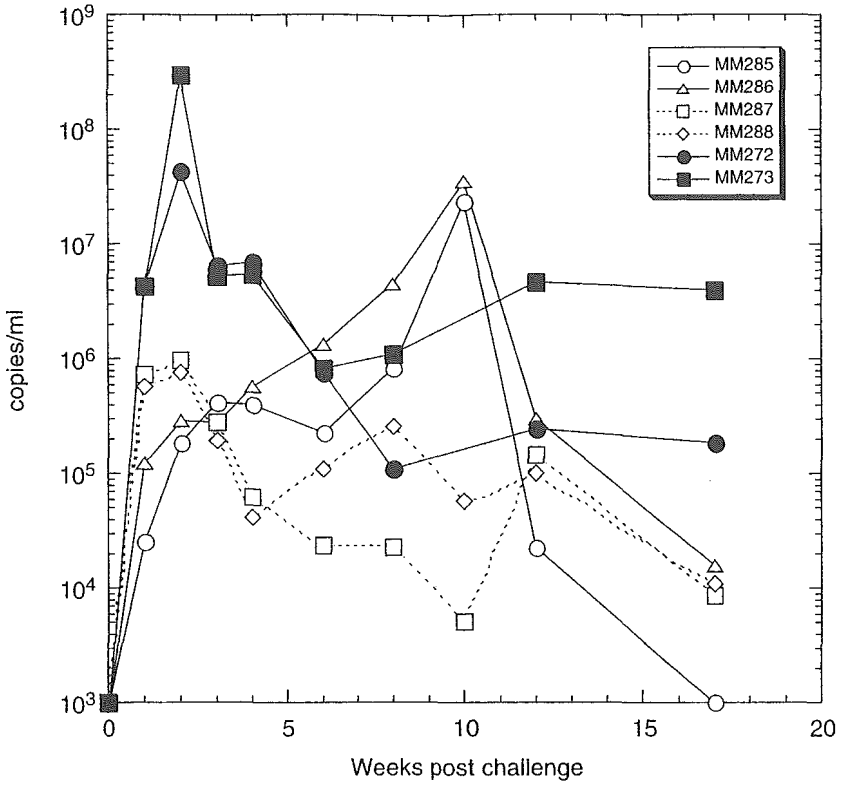

Fig. 2. SHIV-C2/1-specific viral RNA loads in plasma of SHIV IFN-γ- and SHIV-NI-infected monkeys after challenge. MM272 (●) and MM273 (■) were intravenously inoculated with SHIV-C2/1 as uninfected controls. The detection limit of this assay was 10^3 copies/ml

On the other hand, the SHIV IFN-γ-immunized monkeys had viral RNA loads of SHIV-C2/1 at 2 wpc that were about 1/100 of those of the naïve controls, which is a greater reduction than was observed in the SHIV-NI-immunized monkeys (Fig. 2). However, the infection pattern of the SHIV IFN-γ-immunized monkeys was different from that of the SHIV-NI-immunized monkeys after 4 wpc. The SHIV-C2/1 RNA loads in plasma of the SHIV IFN-γ-immunized monkeys gradually went up again from 4 wpc and peaked at about 10^7 copies/ml at 10 wpc. After the transient increase of the viral RNA, the plasma viral loads in both monkeys remained lower than those of the naïve controls at 12 wpc. Moreover, both the detection of proviral DNA in PBMCs and the isolation of infectious virus were simultaneously examined in all the immunized monkeys after the challenge (Table 2). Once SHIV-C2/1 infection was established, neither SHIV IFN-γ nor SHIV-NI was the major viral population in any of the monkeys. Although the proviral DNAs of SHIV IFN-γ and SHIV-NI were transiently detected again in MM285, MM286 and MM288 after SHIV-C2/1 challenge, the plasma viral RNA levels of SHIV IFN-γ and SHIV-NI in all the monkeys were below the detectable level (data not shown). These results showed that the plasma viral RNA loads

Table 2. Virological status of SHIV IFN-γ - or SHIV-NI-inoculated monkeys after SHIV-C2/1 challenge

Monkey		Weeks post challenge									
		0	1	2	3	4	6	8	10	12	16
SHIV IFN-γ											
MM285	Proviral DNA[a]	IFN	IFN	C2/1	C2/1	IFN	C2/1	C2/1	C2/1	C2/1	C2/1
	Virus isolation[b]	+	+	+	+	–	–	ND	–	–	–
MM286	Proviral DNA[a]	–	–	C2/1	IFN, C2/1	C2/1	–	IFN	C2/1	C2/1	C2/1
	Virus isolation[b]	–	+	+	+	–	–	ND	–	–	–
SHIV-NI											
MM287	Proviral DNA[a]	–	C2/1	C2/1	C2/1	C2/1	C2/1	C2/1	C2/1	C2/1	C2/1
	Virus isolation[b]	–	+	+	+	–	+	ND	–	–	–
MM288	Proviral DNA[a]	NI	NI	NI	NI	NI, C2/1	C2/1	C2/1	C2/1	C2/1	C2/1
	Virus isolation[b]	–	+	–	–	–	–	ND	–	–	–

[a]Proviral DNAs in PBMCs (1×10^6 cells) of SHIV-infected monkeys were detected by nested PCR for differentiation of SHIV IFN-γ, SHIV-NI or SHIV-C2/1. IFN, NI and C2/1 represent the detection of proviral DNA of SHIV IFN-γ, SHIV-NI or SHIV-C2/1

[b]Virus isolation was performed by coculture with human T cell lines, M8166, and $CD8^+$ cells-depleted PBMCs. ND – not done

were suppressed in all immunized monkeys after challenge although none of the monkeys could completely inhibit SHIV-C2/1 infection.

$CD4^+$ T cell counts in SHIV IFN-γ - and SHIV-NI-infected monkeys after challenge

Since SHIV-C2/1 caused a severe $CD4^+$ T cell depletion in cynomolgus macaques early after infection [28], we periodically examined $CD4^+$ T cell counts in whole blood of all monkeys. In this study, $CD4^+$ T cells drastically decreased in the peripheral blood of both naive controls early after infection and remained at approximately 10 to 50% of the normal range (Fig. 3). However, SHIV-C2/1 infection did not cause the depletion of $CD4^+$ T cells in SHIV IFN-γ- and SHIV-NI-immunized monkeys (Fig. 3). Interestingly, the $CD4^+$ T cell counts in the SHIV IFN-γ-immunized monkeys remained within the normal range in spite of the transient high level of plasma viremia. $CD4^+$ T cell counts in the SHIV-NI-immunized monkeys, like those in SHIV IFN-γ-immunized monkeys, remained in the normal range after challenge. Thus, vaccination by both SHIV IFN-γ and SHIV-NI could prevent a severe $CD4^+$ T cell depletion by SHIV-C2/1 challenge. Moreover, the result obtained in the SHIV IFN-γ immunized monkeys showed that the increase of plasma viremia is not always correlated with the depletion of $CD4^+$ T cell counts in peripheral blood of monkeys immunized with an attenuated virus.

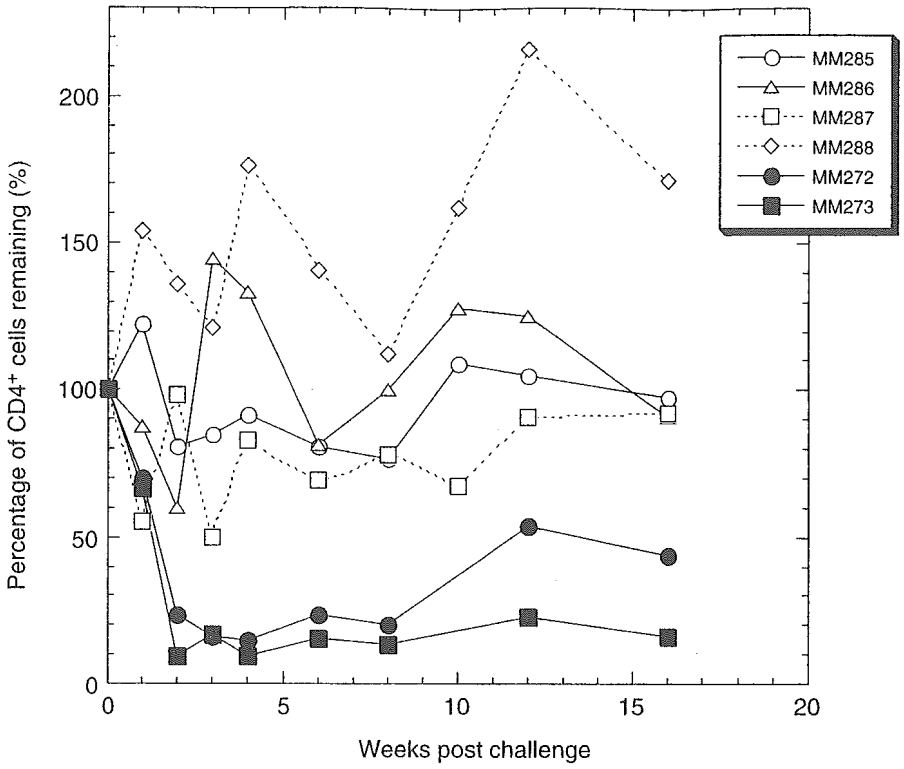

Fig. 2. Number of $CD4^+$ T cells in peripheral blood of SHIV IFN-γ- and SHIV-NI-infected monkeys after challenge. Data are expressed as the percentage based on the prechallenge values of CD3 and CD4 double positive cells in each monkey

Antibody responses in the SHIV-NI- and SHIV IFN-γ-immunized monkeys

To assess the humoral immune responses against the challenge virus in the SHIV IFN-γ- and SHIV-NI-immunized monkeys, HIV-1 Env-specific antibodies in the plasma were measured by the PA method, and the neutralizing abilities in the plasma were measured by the inhibition effects of the parental and the challenge viruses. Whereas the HIV-1 Env-specific antibody titer in the plasma of MM288 at 0 wpc was below 32 fold, which is the limit of detection, two SHIV IFN-γ-immunized monkeys (MM285 and MM286) and one SHIV-NI-immunized monkey (MM287) had an antibody titer of 64 fold at 0 wpc. However, no monkeys had neutralizing antibodies to the parental virus, SHIV-NM3rN (Table 3).

After the challenge, antibodies to HIV-1 Env were gradually detected by the PA method and neutralized SHIV-C2/1 at a titer of 20-160 fold. Although neutralization against SHIV-NM-3rN was observed in three of four monkeys (MM285, MM286 and MM287) but not in one monkey (MM288), the neutralizing antibody titers became less than those against SHIV-C2/1 with the progress of the SHIV-C2/1 infection. These results showed that there were no significant differences

Table 3. Antibody titers in plasma of SHIV IFN-γ or SHIV-NI inoculated monkeys after challenge

Monkey		Weeks post immunization			
		0	4	8	12
SHIV IFN-γ					
MM285	Anti-HIV-1 Env[a]	64	2048	2048	2048
	Neutralization to NM-3rN[b]	–	80	40	40
	Neutralization to C2/1[b]	–	–	–	20
MM286	Anti-HIV-1 Env[a]	64	4098	2048	2048
	Neutralization to NM-3rN[b]	–	40	40	40
	Neutralization to C2/1[b]	–	–	–	20
SHIV-NI					
MM287	Anti-HIV-1 Env[a]	64	2048	1024	1024
	Neutralization to NM-3rN[b]	–	80	40	20
	Neutralization to C2/1[b]	–	–	80	160
MM288	Anti-HIV-1 Env[a]	–	512	512	512
	Neutralization to NM-3rN[b]	–	–	–	–
	Neutralization to C2/1[b]	–	–	–	20

[a]Antibody responses for HIV-1 Env were measured by PA methods. –, <32
[b]Neutralizing antibodies were measured against SHIV NM-3rN or SHIV-C2/1. –, <20

between the SHIV IFN-γ-immunized and SHIV-NI-immunized monkeys in their abilities to neutralize the challenge virus.

Cellular immune responses in the SHIV-NI- or SHIV IFN-γ-immunized monkeys

To evaluate the cellular immune responses that contributed to the protection against SHIV-C2/1, we assayed antigen-specific lymphocyte proliferations in PBMCs of the SHIV IFN-γ- and SHIV-NI- immunized monkeys. During the immunization, SIV Gag-specific lymphocyte proliferation was transiently induced at 2 wpi in one SHIV IFN-γ-immunized monkeys (MM286) and one SHIV-NI-immunized monkey (MM287) (Fig. 4). After the SHIV-C2/1 challenge, all the immunized monkeys showed the transient increase of SIV Gag-specific lymphocyte proliferation. Surprisingly, the proliferation responses in SHIV-NI-immunized monkeys (MM287 and MM288) were much greater than those in SHIV IFN-γ-immunized monkeys (MM285 and MM286) (Fig. 4). These results suggest that the immune reactions in the attenuated SHIVs-immunized monkeys were strongly boosted after the challenge, even though the challenge was only four weeks after the immunization.

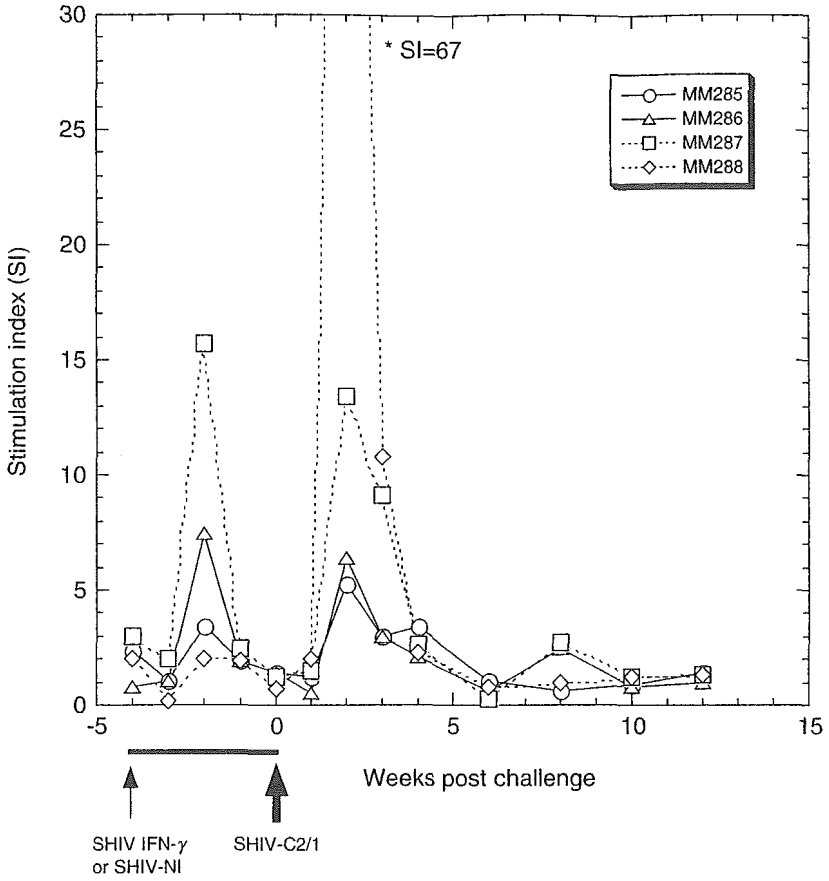

Fig. 3. SIV Gag-specific lymphocyte proliferation in PBMCs of SHIV IFN-γ- and SHIV-NI-infected monkeys before and after challenge. Data are expressed as a stimulation index, which is defined as the ratio of the stimulated cell counts to the unstimulated cell counts

Discussion

The monkeys immunized with the attenuated SHIVs were able to develop protective immunity from the disease progression by a pathogenic virus infection early after vaccination. Previous studies reported that monkeys vaccinated with a nef-deleted SIV experienced a transient reduction in viremia against a pathogenic SIV challenge early after immunization and stronger protection was observed when challenged after a longer period [4, 36]. In the present study, the monkeys immunized with the attenuated SHIVs showed protection to the SHIV C2/1 induced disease even though only four weeks had passed since the immunization. These immunized monkeys had no neutralizing antibodies against the parental and challenge viruses at the time of challenge. SIV Gag-specific lymphocytes proliferation was transiently induced in only one of each attenuated virus-immunized monkey. Although some virus-specific immune responses might be elicited in all the monkeys, there was no evidence of strong development of highly acquired immune responses against the challenge virus in any of the monkeys. However, the

protective effects against the challenge virus were observed in all the immunized monkeys, which were shown by a reduction of the peak value of the viral load and the maintenance of $CD4^+$ T cell counts. If highly acquired antigen-specific immune responses were not strongly involved in this viral protection, primitive non-specific immunity might have contributed to the protection to SHIV-C2/1. Giavedoni et al. [8] reported the activation of NK cells during acute infection of a pathogenic SIV. The importance of NK cell activity in the protection against pathogenic viruses has been well demonstrated. NK cells could preferentially lyse virus-infected target cells lacking cell surface MHC class I expression, although the expression of MHC class I molecules on the cell surface is down-regulated by Nef protein [2, 3, 14, 23, 24, 26, 32]. Although we attempted to examine the involvement of NK cells, the activities of these cells were unfortunately not evaluated in this study (data not shown).

Antigen-specific immune responses against the challenge virus were observed in all the monkeys after the challenge. Neutralizing antibodies against the challenge virus developed at 8 to 12 weeks after challenge in the immunized monkeys. SIV Gag-specific lymphocyte proliferation was transiently detected in all the immunized monkeys. Unexpectedly, the proliferative responses in the SHIV-NI-infected monkeys were higher than those in SHIV IFN-γ-infected monkeys after the challenge. Difference in the immune responses resulted in difference in the ability to regulate plasma viremia in each immunized monkey after the challenge. While SHIV-NI-immunized monkeys could control the viral RNA load of SHIV-C2/1, SHIV IFN-γ-immunized monkeys were unable to maintain low plasma viral RNA loads from 4 to 12 wpc. Thus, the stronger immune responses in SHIV-NI-immunized monkeys might help to control plasma viremia after the challenge, although this hardly explains why the immune responses were stronger in the SHIV-NI-immunized monkeys than in SHIV IFN-γ immunized monkeys after the challenge.

We previously reported that SHIV IFN-γ-infected monkeys could produce IFN-γ more rapidly than SHIV-NI-infected monkeys, and that IFN-γ induced a Th1 type immune response [16]. Although SHIV IFN-γ-immunized monkeys effectively suppressed the peak value of plasma viral RNA loads from 1 to 3 weeks after the challenge, these monkeys showed transient increases in viral loads from 4 to 12 wpc. Several studies have demonstrated that insertion of a cytokine in a SIV can boost the immunogenicity of the SIV and increase protection [9, 10, 12, 13, 29]; on the other hand, there are doubts about the safety of such SIVs because they have occasionally led to the emergence of a virulent virus with an increase of viral load [11, 25]. Our results did not show that the insertion of IFN-γ to SHIV-NI supported the protective effect better than SHIV-NI. However, there is little possibility that more virulent viruses caused the high level of viremia after challenge, because the viremia was transient and the severe $CD4^+$ T cell loss was not observed in the SHIV IFN-γ-immunized monkeys. Change of immunological environment, which was dramatically stimulated after challenge, might actively induce the virus production. IFN-γ has important roles in antiviral immunity, such as in activating CTLs and NK cells and in directly inhibiting viral replication [18,

33]. On the other hand, abnormal production of IFN-γ might alter the integrity of immune regulation and lead to the development of inflammatory conditions in the host. In fact, Selin et al. [27] showed that memory T cells specific to a prior virus might clear a second virus more rapidly but they also exacerbate IFN-γ-dependent immunopathogenesis by reactivation of a second virus. Furthermore, IFN-γ has been reported to have a potentiating effect on macrophages, which accelerate viral production from latently infected cells [37]. In this study, the SHIV IFN-γ-immunized monkeys did not show severe $CD4^+$ T cell depletion in spite of the transient increase of viral RNA loads from 4 to 12 weeks after the challenge, which might be due to an altered T cell response or activation of the infected macrophages. It is necessary to further understand how the over expression of IFN-γ had an influence on the virus-infected monkeys.

Thus, we demonstrated that the monkeys immunized by nef-deleted, attenuated SHIV and that having IFN-γ could resist to the disease progression by a heterologous pathogenic virus and prevent the loss of $CD4^+$ T cells, even when the monkeys were challenged 4 weeks after immunization. Further studies about involvement of non-specific immunity are needed to evaluate more effective protection against virus infection.

Acknowledgments

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Comparison of susceptibility to SIVmac239 infection between $CD4^+$ and $CD4^+8^+$ T cells

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Summary. CD4-bearing T cells are the primary targets for human immunodeficiency virus type 1(HIV-1)/simian immunodeficiency virus (SIV) infection. However, it is unclear whether the susceptibility of CD4-bearing T cells including CD4 single positive and CD4/8 double positive T cells to HIV/SIV infection is the same or not. In this study, we compared the susceptibility to SIV infection between $CD4^+$ and $CD4^+8^+$ T cells, using Herpesvirus saimiri (HVS)-transformed $CD4^+$ and $CD4^+8^+$ T cells established from peripheral blood mononuclear cells (PBMC) of rhesus macaques. Although there was little difference between the two CD4-bearing T cell population in the expression level of CD4 molecules and chemokine receptors such as CXCR4 and CCR5, SIV replicated more efficiently in $CD4^+8^+$ T cells than in $CD4^+$ T cells. Moreover, we found that reverse transcription initiated more efficiently in $CD4^+8^+$ T cells than in $CD4^+$ T cells and that the cell lysates from $CD4^+$ T cells impaired the RT activity more strongly than that from $CD4^+8^+$ T cells. These findings suggest that intracellular environment in $CD4^+$ 8^+ T cells is better for reverse transcription and that the infection of those $CD4^+8^+$ T cells might play critical and different roles in HIV-1/SIV infection and dissemination.

Introduction

CD4 molecule is the major receptor used by human immunodeficiency virus type 1(HIV-1)/simian immunodeficiency virus (SIV) for binding and entry into target cells. The HIV-1/SIV have a strong cytopathic action on the $CD4^+$ T cells, and the significant depletion of those $CD4^+$ T cells in the blood as well as the peripheral lymphoid organs of infected patients are the hallmarks of progressive immune

impairment leading to AIDS [17, 20]. The chemokine receptors CCR5 and CXCR4 have been identified as co-receptors for HIV-1/SIV [6, 8]. Distribution of these receptors permits the virus to infect to not only $CD4^+$ T cells but also macrophages and dendritic cells (DCs) [7, 12]. After the infection, HIV-1/SIV integrates into the DNA of the host cells. Therefore, the replicating capacity of the integrated viral genome, or provirus, may be greatly influenced by the metabolic and activation state of the host cells [9, 18].

CD4-bearing T cells include both CD4 single positive T cells and CD4/8 double positive T cells. $CD4^+$ T cells broadly distribute in the body, whereas $CD4^+8^+$ T cells are localized in the particular organs such as thymus and small intestine. $CD4^+8^+$ thymocytes, which are situated in the relatively immature stage of differentiation, have been shown a marked susceptibility to HIV-1/SIV infection and replication [3, 22]. Severe depletion of $CD4^+8^+$ T cells in intraepithelial lymphocytes (IELs) was also observed in SIV-infected rhesus macaques [19, 21]. These observations indicate that $CD4^+8^+$ T cells are highly permissive to HIV-1/SIV infection and replication. Thus we speculated that $CD4^+8^+$ T cells might be equally or more susceptible to HIV-1/SIV infection with higher replication capacity in comparison with $CD4^+$ T cells. To address this question, we compared the susceptibility to SIV infection in $CD4^+$ T and $CD4^+8^+$ T cells using Herpesvirus saimiri (HVS)-transformed $CD4^+$ T and $CD4^+8^+$ T cells established from peripheral blood mononuclear cells (PBMC) of rhesus macaques.

Here we show that SIV replicated more efficiently in $CD4^+8^+$ T cells than in $CD4^+$ T cells and such SIV replicating capacity in $CD4^+8^+$ T cells was enhanced via progressed reverse transcription.

Materials and methods

Cell culture and virus

Two $CD4^+$ T cell clones (133, 135-1-3) and two $CD4^+8^+$ T cell clones (135-1, 136-1) were established by HVS-immortalized method [16, 27] from PBMC of three rhesus macaques (*Macaca mulatta*) MM133, MM135 or MM136. Briefly, PBMC were separated from whole blood by Ficoll-Hypaque (Nacalai Tesque, Kyoto, Japan) centrifugation, stimulated for 2 to 3 days with 10 μg/ml of phytohemagglutinin-P (Sigma-Aldrich, St. Louis, MO, USA) and then infected in bulk with HVS strain C-488 (kindly provided by Dr. Yasukawa). After 6 to 8 weeks in culture in RPMI 1640-based medium [25] supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, a mixture of vitamins, 1 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-ME, heat-inactivated 10% FCS, and 100 units/ml of human recombinant IL-2, viable cells were recovered by Ficoll-Hypaque separation and $CD4^+$ T cells or $CD4^+8^+$ T cells were cloned by limiting dilution. A molecular clone of SIV (SIVmac239) was used in the present study and was grown on C8166 cells [4] and titrated with M8166 cells (a subclone of C8166). The titer of virus stock was 10^+ $TCID_{50}$/ml.

Infection of $CD4^+$ and $CD4^+8^+$ T cells with SIV

Cells were infected with SIV (moi 0.05-1) in 48 well plates and incubated for 2 h at 37 °C. After washing twice with phosphate buffered saline (PBS) to remove free virus, cells were cultured for 4–7 days.

Reverse transcriptase (RT) assay and detection of p27

The RT assay was performed as described previously [26]. The amount of p27 core antigen in the culture supernatant was measured using the SIV Core Antigen ELISA kit (Beckman Coulter, Fullerton, CA, USA).

Semi-quantitative PCR

DNA was isolated from cultured cells using QIAamp DNA Blood kits (Qiagen, Valencia, CA, USA). Quantification of DNA was performed by real-time PCR with β-actin primers as internal control. Real-time PCR was carried out in a 20 μl containing template DNA, primers for β-actin (forward, 5′-tcacccacactgtgcccatctacga-3′; reverse, 5′-cagcggaaccgctcattgccaatgg-3′), and SYBR Green PCR master mix (Applied Biosystems Foster City, CA, USA). Fluorescence was measured after each cycle using ABI Prism 7700 Sequence Detection System (Applied Biosystems). DNA estimated by real-time PCR was subjected to PCR with the following primers specific for the *nef* gene of SIVmac239 (forward, 5′-ctcactctcttgtgagggacagaaa-3′; reverse, 5′-ccccgtaacatccccttgtggaaagtccc-3′) and the R/U5 region of SIVmac239 LTR (forward, 5′-ttctctccagcactagcaggtagagcctgggtgttccctg-3′; reverse, 5′-caggcgccaatctgcta gggattttcctgcttc-3′). To demonstrate that equal amounts of DNA were subjected to PCR, we showed the electrophoresis data of β-actin PCR product during the exponential phase of amplification.

RNA was prepared from cultured cells using RNAeasy (Qiagen). One microgram of RNA was incubated for 1 h at 42 °C after adding 20 U of RNase inhibitors (Takara, Otsu, Japan), 0.2 mM deoxynucleoside triphosphates, 2.5 nM random primers, 11 U of Rous associated virus 2 reverse transcriptase (Takara) and reverse transcriptase buffer to a final volume of 20 μl. Quantification of cDNA was performed by real-time PCR with β-actin primers as mentioned above. cDNA estimated by real-time PCR was subjected to PCR with the following respective primers specific for CD3 (forward, 5′-tctgctggcctccgccatctt-3′; reverse, 5′-ggagacctgggccagcgggag-3′), CD4 (forward, 5′-ccttcccactcgcctttaca-3′; reverse, 5′-tcagcacccacaccgccttc-3′), Bob (forward, 5′-aaggacagactgtgcatatgtagtctg-3′; reverse, 5′-tcttcagctttttgttgtgctttcctg-3′), and Bonzo (forward, 5′-ggatgacctggggcaaggtcaccagc-3′; reverse, 5′-gtgcttctggaagcctccagcatgaag-3′).

Cell staining

Cells were pelleted and resuspended at a concentration of 5×10^5 cells in 50 μl of PBS with 0.1% NaN_3 containing FITC-labeled mouse anti-CD3 mAb (clone SP34; BD PharMingen, San Diego, CA, USA), PE-labeled mouse anti-CD4 mAb (clone L200; BD PharMingen), FITC-labeled mouse anti-CD8 mAb (clone RPA-T8; BD PharMingen), PE-labeled anti-CXCR4 mAb (clone 12G5; BD PharMingen), or PE-labeled anti-CCR5 mAb (clone 3A9; BD PharMingen). After 30 min incubation on ice, cells were washed and resuspended in PBS for analysis by FACScan (Becton Dickinson, Franklin Lakes, NJ, USA).

Measurement of cell growth

Single cell suspensions were seeded at a density of 1×10^5 cells per well on 96-well microtiter plates. After 7 days of incubation, colorimetric determination of cell growth was performed using XTT Cell Proliferation kit (Roche Diagnostics, Indianapolis, IN, USA).

Virus entry assay

Cells were infected with virus (moi 1) for 2 h at 37 °C. After washing with PBS, cells were treated with 0.05% trypsin containing 0.02% EDTA. After washing extensively, cells were

lysed with lysis buffer (10 mM Tris-HCl (pH 7.5), 10 mM NaCl, 0.5% NP-40). Internalized virion Gag p27 protein was measured using the SIV Core Antigen ELISA kit (Beckman Coulter).

Results

Comparison of viable cell counts after SIV infection

We could successfully establish $CD4^+$ and $CD4^+8^+$ T cell clones from monkey's PBMC by HVS-immortalized method (Fig. 1a). As shown in the right panel of Fig. 1a, the obtained $CD4^+8^+$ T cell clones appeared to contain two subpopulations, $CD4^{high}CD8^{high}$ T cells and $CD4^{high}CD8^{low}$ T cells. It is interesting to note that, when a single cell from either $CD4^{high}CD8^{high}$ or $CD4^{high}CD8^{low}$T cell clones was further cultured, those two promiscuous populations were always emerged. Therefore, the expression level of CD8 molecules on $CD4^+8^+$ T cells might fluctuate. Taking advantage of these $CD4^+$ T cell clones, we then examined their susceptibility to SIVmac239. SIVmac239-infected or uninfected T cells were plated in culture for 7 days and were estimated the viable cell counts by XTT colorimetric method. After 7 days of incubation, a total number of viable cells were significantly decreased in infected $CD4^+8^+$ T cells, whereas that was slightly decreased in infected $CD4^+$ T cells (Fig. 1b).

Assessment of viral replication in $CD4^+$ and $CD4^+8^+$ T cells

To determine viral protein production, the amount of p27 antigens and RT activity in the culture supernatants were measured. The supernatants from SIVmac239-infected $CD4^+8^+$ T cells contained large amounts of p27 antigens

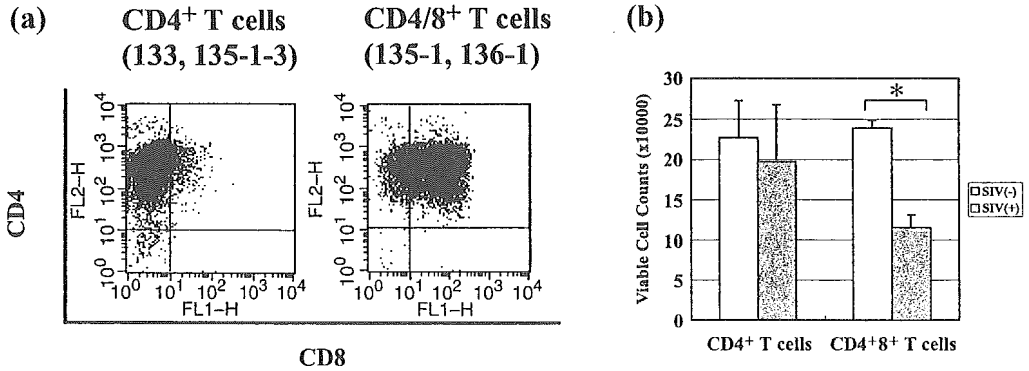

Fig. 1. **a** HVS-transformed $CD4^+$ and $CD4^+8^+$ T cells established from PBMC of rhesus macaques. Cells were stained with PE-labeled anti-CD4 mAb and FITC-labeled anti-CD8 mAb, followed by propidium iodide staining to exclude dead cells. **b** Determination of viable cell counts in $CD4^+$ and $CD4^+8^+$ T cells after SIV infection. Cells were inoculated with (□) or without (□) SIVmac239 (moi 0.5) and estimated the viable cells by XTT colorimetric method after 7 days incubation. The data shown are the mean of triplicate cultures ±SD (*$P < 0.01$)