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

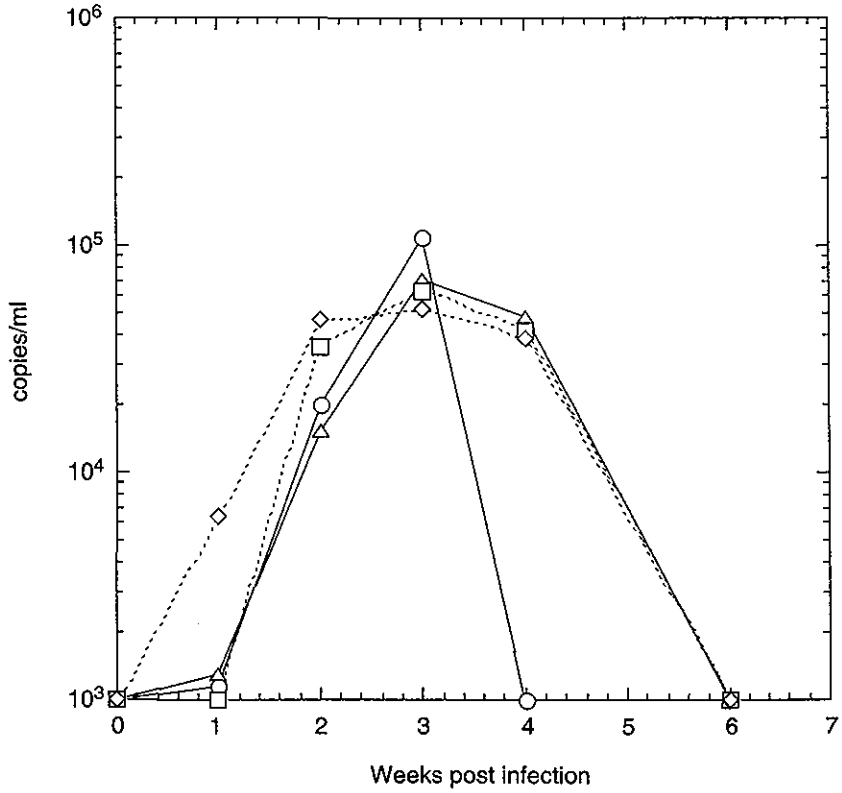

Fig. 1. Plasma viral RNA loads in SHIV IFN-γ- or SHIV-NI-infected monkeys; MM285 (○), MM286 (△), MM287 (□) and MM288 (◇). The detection limit of this assay was 10^3 copies/ml

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.

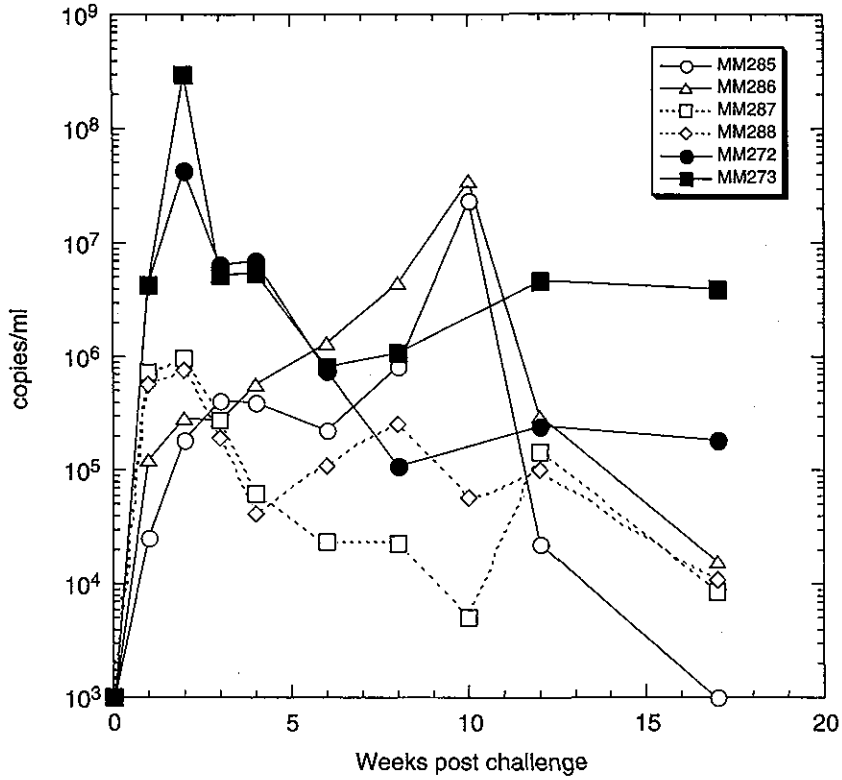

Fig. 2. SHIV-C2/1-specific viral RNA loads in plasma of SHIV IFN-γ- and SHIV-NI-infected monkeys after challenge. MM272 (●) and MM273 (■) were intravenously inoculated with SHIV-C2/1 as uninfected controls. The detection limit of this assay was 10^3 copies/ml

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.

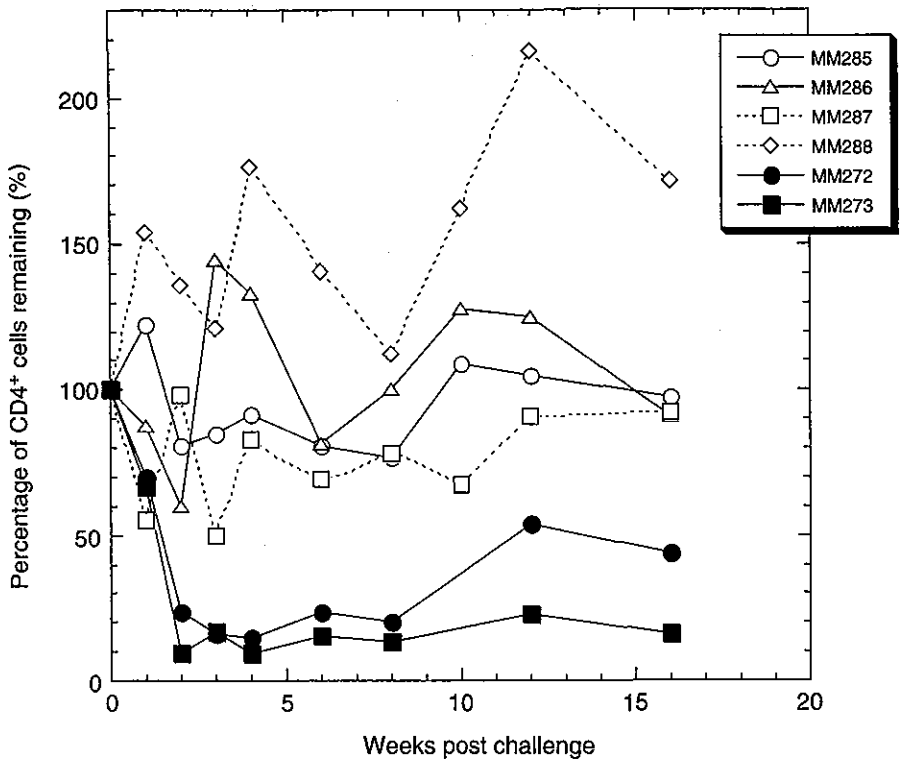

Fig. 2. Number of $CD4^+$ T cells in peripheral blood of SHIV IFN-γ- and SHIV-NI-infected monkeys after challenge. Data are expressed as the percentage based on the prechallenge values of CD3 and CD4 double positive cells in each monkey

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.

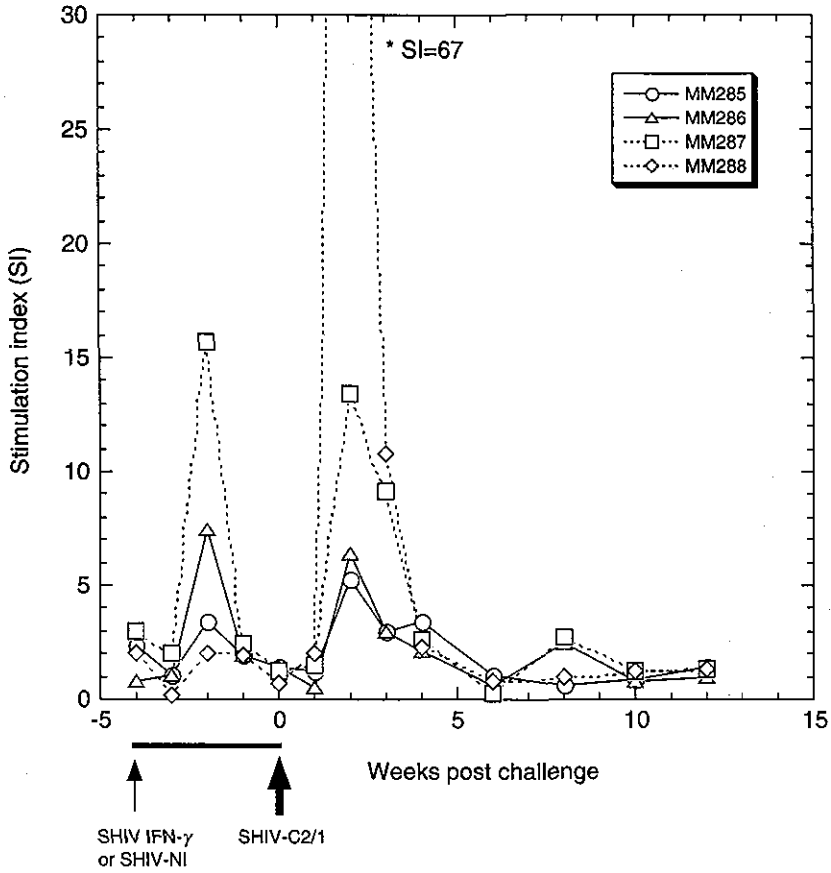

Fig. 3. SIV Gag-specific lymphocyte proliferation in PBMCs of SHIV IFN-γ- and SHIV-NI-infected monkeys before and after challenge. Data are expressed as a stimulation index, which is defined as the ratio of the stimulated cell counts to the unstimulated cell counts

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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