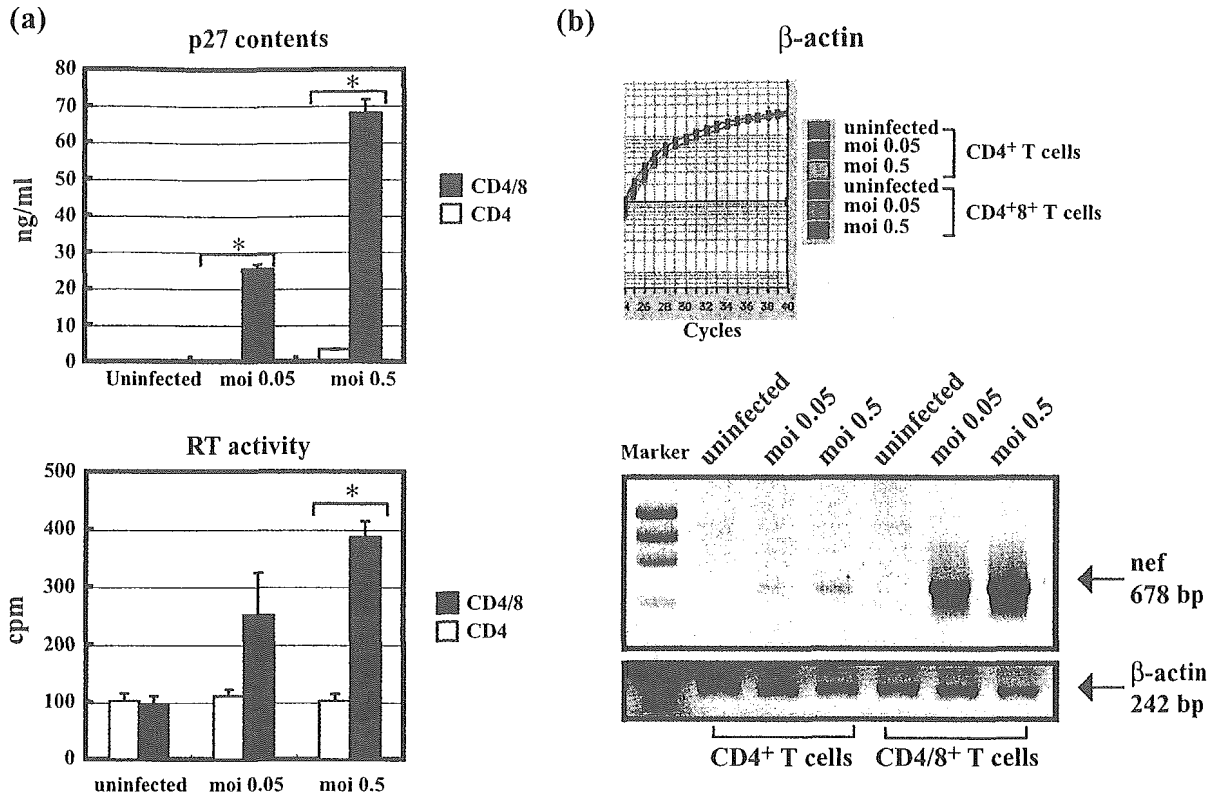

Fig. 2. Assessment of viral replication in $CD4^+$ and $CD4^+8^+$ T cells. $CD4^+$ (□) and $CD4^+8^+$ (■) T cells were infected with SIVmac239 (moi 0.05 or 0.5). After 4 days incubation; **a** the supernatants were harvested and subjected to measure the amount of p27 core antigen and RT activity. The data shown are the mean of triplicate cultures ±SD (*$P < 0.01$). **b** DNA was isolated from infected or uninfected cells and subjected to semi-quantitative PCR using SIVmac239 *nef* or β-actin primer. The thermal cycle was consisted of 30 s at 94 °C, 45 s at 60 °C for *nef* or 55 °C for β-actin, 45 s at 72 °C. The numbers of PCR cycles for *nef* were 35 cycles, and those of β-actin were 24 cycles to generate PCR products during the exponential phase of amplification

and higher level of the RT activity compared to that from infected $CD4^+$ T cells (Fig. 2a). Next, to determine the level of viral DNA synthesis in infected cells, we performed semi-quantitative PCR analysis using the primer pair for the *nef* gene of SIVmac239. As shown in Fig. 2b, low level of the *nef* DNA was detected in infected $CD4^+$ T cells, whereas an evident increase in the *nef* DNA was observed in infected $CD4^+8^+$ T cells.

Effect of SIV infection on CD3 and CD4 expression in $CD4^+$ and $CD4^+8^+$ T cells

It has been reported that HIV/SIV modulated the surface expression of various molecules [11]. Therefore, we analyzed the expression level of surface CD3 and CD4 molecules on both SIVmac239-infected $CD4^+$ and $CD4^+8^+$ T cells by

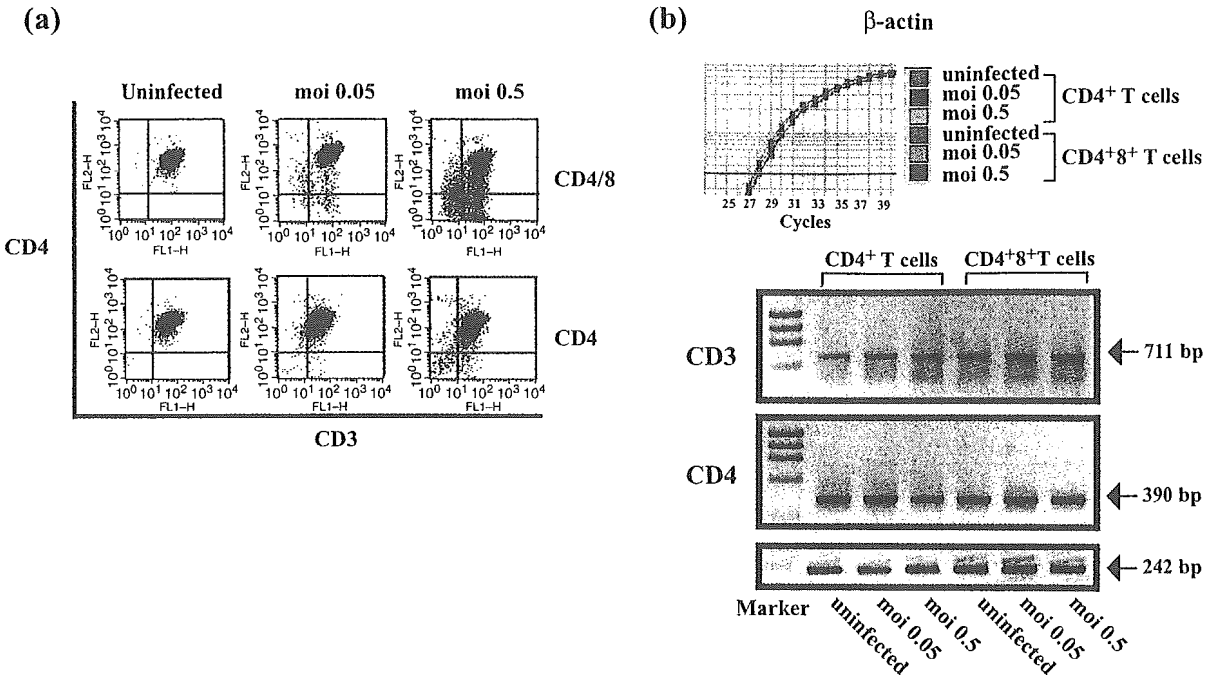

Fig. 3. Effect of SIVmac239 infection on CD3 and CD4 expression on $CD4^+$ and $CD4^+8^+$ T cells. $CD4^+$ and $CD4^+8^+$ T cells were infected with SIV (moi 0.05 or 0.5). After 4 days incubation; **a** Cells were stained with FITC-labeled anti-CD3 mAb and PE-labeled anti-CD4 mAb, followed by propidium iodide staining to exclude dead cells. **b** RNA was isolated from infected or uninfected cells and then reverse-transcribed into cDNA. cDNA was subjected to semi-quantitative PCR using CD3, CD4 or β-actin primer. The thermal cycle was consisted of 30 s at 94 °C, 45 s at 60 °C for CD3 and CD4, or 55 °C for β-actin, 45 s at 72 °C. The numbers of PCR cycles for CD3 and CD4 were 33 cycles, and those of β-actin were 24 cycles to generate PCR products during the exponential phase of amplification

flow cytometry. SIV-infected $CD4^+8^+$ T cells showed marked down-modulation for both CD3 and CD4 expression when compared with infected $CD4^+$ T cells (Fig. 3a). The down-modulation was dependent on the infectious doses of virus. Since semi-quantitative RT-PCR analysis demonstrated that the respective levels of CD3 and CD4 transcripts did not change between uninfected and infected cells, down-modulation is due to the internalization of these molecules induced by the virus infection (Fig. 3b).

Evaluation of receptor and co-receptor expression on $CD4^+$ and $CD4^+8^+$ T cells and virus entry

Since efficiency of SIV entry depends on the presence of appropriate receptor and co-receptor, we examined the expression levels of those receptors on uninfected $CD4^+$ T cells or $CD4^+8^+$ T cells by FACS and RT-PCR analysis. There seemed little difference in the expression levels of CD4, CXCR4 or CCR5. The transcript level of Bob was almost the same, whereas that of Bonzo was slightly decreased

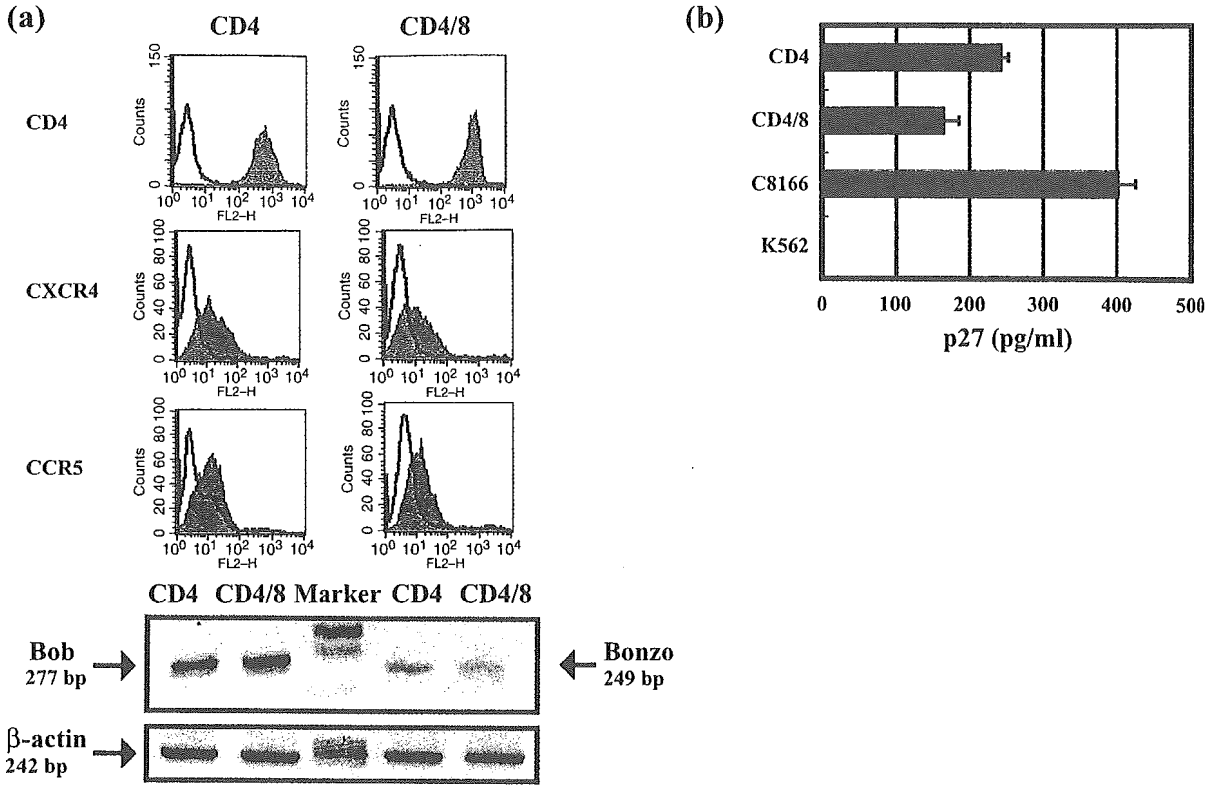

Fig. 4. Expression of receptor and co-receptor on $CD4^+$ and $CD4^+8^+$ T cells. **a** Uninfected cells were stained with PE-labeled anti-CD4 mAb, PE-labeled anti-CXCR4 mAb, or PE-labeled anti-CCR5 mAb, followed by propidium iodide staining to exclude dead cells. RNA was isolated from uninfected cells and then reverse-transcribed into cDNA. cDNA was subjected to semi-quantitative PCR using Bob, Bonso or β-actin primer. The thermal cycle consisted of 30 s at 94 °C, 45 s at 52 °C for Bob, 58 °C for Bonso or 55 °C for β-actin, 45 s at 72 °C. The numbers of PCR cycles for Bob and Bonso were 35 cycles, and those for β-actin were 24 cycles to generate PCR products during the exponential phase of amplification. **b** Entry efficiency of virus into $CD4^+$ and $CD4^+8^+$ T cells. Cells were incubated with virus (moi 1) for 2 h, followed by washing, trypsinizing, and lysed with lysis buffer. The p27 core antigen content in the lysate was monitored by ELISA. C8166 cells were used as positive control and K562 cells were used as negative one. The data shown are the mean of triplicate cultures ±SD

in $CD4^+8^+$ T cells (Fig. 4a). When we monitored the efficiency of virus entry into $CD4^+$ and $CD4^+8^+$ T cells at 2 h post-infection by the measurement of the intracellular p27 antigens, a slightly lower level of the amount of p27 antigens in $CD4^+8^+$ T cells was observed when compared with $CD4^+$ T cells (Fig. 4b). These observations indicate that the entry step of virus did not account for the increased amounts of virus accumulation in $CD4^+8^+$ T cells.

Efficiency of reverse transcription in $CD4^+$ and $CD4^+8^+$ T cells

To define at which step virus replication is accelerated in $CD4^+8^+$ T cells, we performed DNA-dependent PCR with primers that distinguish salient stages of

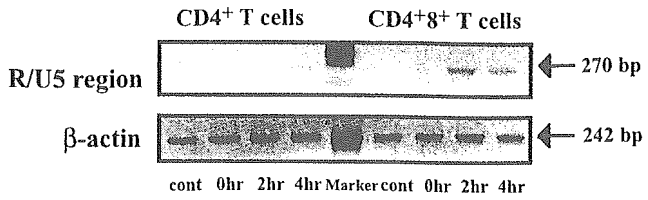

Fig. 5. Detection of the earliest reverse transcription product of SIV in $CD4^+$ and $CD4^+8^+$ T cells. DNA was isolated from infected or uninfected cells at indicated time and subjected to semi-quantitative PCR using R/U5 or β-actin primer. The thermal cycle consisted of 30 s at 94 °C, 45 s at 64 °C for R/U5 and 55 °C for β-actin, 45 s at 72 °C. The numbers of PCR cycles for R/U5 were 35 cycles, and those for β-actin were 25 cycles to generate PCR products during the exponential phase of amplification

reverse transcription. Cells were infected with SIV (moi 1) for 2 h. After washing to remove free virus, cells were further cultured and the genomic DNA was extracted from infected cells at 0, 2, and 4 h after incubation. The primer pair R/U5 can amplify the earliest reverse transcription product [1]. This earliest reverse transcription product was detected in both $CD4^+$ T cells and $CD4^+8^+$ T cells at 2 h after incubation, however, level of the product was higher in $CD4^+8^+$ T cells than in $CD4^+$ T cells (Fig. 5). Thus, after the entry of SIV, reverse transcription appeared to initiate more efficiently in $CD4^+8^+$ T cells than in $CD4^+$ T cells.

We speculated that some cellular components might act to function as an inhibitor or cofactor for viral reverse transcriptase in cells. We thus prepared the cell lysate from uninfected $CD4^+$ T cells or $CD4^+8^+$ T cells and performed RT assay in the presence of those cell lysate. In all cases, RT activity was decreased in the presence of cell lysate from both types of T cells, however, the lysate from $CD4^+$ T cells impaired the RT activity more strongly than that from $CD4^+8^+$ T cells (Table 1). These results indicate that $CD4^+8^+$ T cells possess a favorable environment for intracellular reverse transcription.

Table 1. Effect of cell lysates on RT activity

RT source	Cell lysates			
	$CD4^+$T cells	$CD4^+8^+$ T cells	No lysates	p value[c]
SIVmac239	332 ± 15[a]	765 ± 158	1665 ± 271	$p < 0.05$
SHIV3rN	802 ± 75	1086 ± 98	9346 ± 1883	$p < 0.05$
M-MLV-RT[b]	17039 ± 1218	53211 ± 1619	100713 ± 13490	$p < 0.01$

Cell lysates were prepared from $CD4^+$ and $CD4^+8^+$ T cells. RT assay was performed in the presence or absence of cell lysates

[a] cpm/10 μg protein

[b] Moloney Murine Leukemia Virus Reverse Transcriptase (Gibco): 8.3 U

[c] $CD4^+$ T cells versus $CD4^+8^+$ T cells

Discussion

We observed in this study that SIV replicated more efficiently in $CD4^+8^+$ T cells than in $CD4^+$ T cells, of which expression levels of receptors and turnover rate were almost the same. We therefore set out to focus at which steps SIV replication was accelerated in $CD4^+8^+$ T cells. We found that reverse transcription initiated more efficiently in $CD4^+8^+$ T cells than in $CD4^+$ T cells. Therefore, we presumed that intracellular environment in $CD4^+8^+$ T cells was optimal for reverse transcription. After the entry of virus, productive HIV-1/SIV infection requires overcoming several cellular blocks in the establishment of provirus and its replication. There have been significant advances in the identification of cellular cofactors that affect events in HIV-1 replication after entry of virus [23]. Kinoshita et al. demonstrated that a nuclear factor of activated T cells (NFAT) overcame a blockade at reverse transcription and permitted active HIV-1 replication [13]. Although the target genes for NFAT that can aid completed reverse transcription are unknown, NFAT appears to be involved in HIV-1 replication at pre- and post integrative steps of the HIV-1 life cycle. Although we have not yet determined whether NFAT expression was more greatly enhanced in $CD4^+8^+$ T cells, it is possible that $CD4^+8^+$ T cells contain either known or unknown factors that permit reverse transcription like NFAT, or fewer cellular factors that inhibit reverse transcription.

Many investigators have provided evidence for the widespread infection of CD8 T cells by HIV-1/SIV both in vitro and in vivo [5, 10]. In most cases, transient co-expression of CD4 during maturation or activation renders them susceptible to HIV-1/SIV infection and destruction [14, 29]. In our case, the efficiency of virus entry into $CD4^+8^+$ T cells was almost the same as into $CD4^+$ T cells. An important issue is whether the expression of CD8 molecules on $CD4^+8^+$ T cells is related to induce a higher permissive state for virus replication. To solve this question, we are currently designing the experiment using CD8 specific RNAi which offers a valuable opportunity to modulate the expression of cellular genes.

We have established $CD4^+8^+$ T cells and $CD4^+$ T cells from rhesus macaque PBMC. $CD4^+8^+$ T cells in PBMC are observed in approximately 3% of human and 5% of rhesus macaques [11, 22]. Several reports have shown that activation of $CD8^+$ T cells from peripheral blood resulted in the de novo expression of CD4 molecule and these peripheral $CD4^+8^+$ T cells were in the activated states [2, 14, 24]. Also, it has been reported that the majority of the intestinal T cells including $CD4^+8^+$ T cells are memory T cells and are in an activated state [12]. Since HIV-1/SIV is assumed to preferentially infect and kill the activated T cells [28], $CD4^+8^+$ T cells in the peripheral blood or the intestine may be readily infected by the virus and produce a large amount of virus. The high level of viral production disseminates infection systemically and provides access to large numbers of target cells for prolonged replication. The function of $CD4^+8^+$ T cell subset in these organs is not fully defined, however, we speculate that infected $CD4^+8^+$ T cells might play an important role for early viral dissemination and

serve as a potent viral reservoir in HIV-1/SIV infection. On the other hand, we observed that the number of viable cells in infected $CD4^+$ T cells were considerably more than that in infected $CD4^+8^+$ T cells, which indicated that the depletion of $CD4^+$ T cells by SIV infection were less. Previous studies have also shown that the depletion of $CD4^+$ T cells in PBMC was gradual in HIV-1/SIV infection [11]. The low level of viral replication could provide a mechanism for cells to escape the host defense surveillance and favor establishment of a persistent infection. Therefore, although there were little differences between $CD4^+$ T cells and $CD4^+8^+$ T cells in the expression level of receptors, both types of CD4 positive cells might play distinct roles in HIV-1/SIV infection.

In the present study, we compared the susceptibility to SIV infection in $CD4^+$ T cells and $CD4^+8^+$ T cells using HVS-transformed T cell lines. Although we have not confirmed our findings by ex vivo analysis using PBMC from rhesus macaques, our model may be helpful in understanding the viral replication and reservoirs in vivo.

Acknowledgments

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Protective Efficacy of Nonpathogenic Nef-Deleted SHIV Vaccination Combined with Recombinant IFN-γ Administration against a Pathogenic SHIV Challenge in Rhesus Monkeys

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Abstract: **We previously reported that a nef-deleted SHIV (SHIV-NI) is nonpathogenic and gave macaques protection from challenge infection with pathogenic SHIV-C2/1. To investigate whether IFN-γ augments the immune response induced by this vaccination, we examined the antiviral and adjuvant effect of recombinant human IFN-γ (rIFN-γ) in vaccinated and unvaccinated monkeys. Nine monkeys were vaccinated with nef-deleted nonpathogenic SHIV-NI. Four of them were administered with rIFN-γ and the other five monkeys were administered with placebo. After the challenge with pathogenic SHIV-C2/1, $CD4^+$ T-cell counts were maintained similarly in monkeys of both groups, while those of the unvaccinated monkeys decreased dramatically at 2 weeks after challenge. However, the peaks of plasma viral load were reduced to 100-fold in SHIV-NI vaccinated monkeys combined with rIFN-γ compared with those in SHIV-NI vaccinated monkeys without rIFN-γ. The peaks of plasma viral load were inversely correlated with the number of SIV Gag-specific IFN-γ-producing cells. In SHIV-NI-vaccinated monkeys with rIFN-γ, the number of SIV Gag-specific IFN-γ-producing cells of PBMCs increased 2-fold compared with those in SHIV-NI-vaccinated monkeys without rIFN-γ, and the NK activity and MIP-1α production of PBMCs were also enhanced. Thus, vaccination of SHIV-NI in combination with rIFN-γ was more effective in modulating the antiviral immune system into a Th1 type response than SHIV-NI vaccination alone. These results suggest that IFN-γ augmented the anti-viral effect by enhancing innate immunity and shifting the immune response to Th1.**

Key words: **Adjuvant, Cytokine, SHIV, IFN-γ**

Nef-deleted mutants of simian immunodeficiency virus (SIV) and simian and human immunodeficiency chimeric virus (SHIV) were found to be effective live-attenuated vaccine candidates in macaque models (9, 11, 24). We previously reported that a new nef-deleted SHIV-NI is nonpathogenic and that it totally protected macaques from challenge infection with a nef-intact parental NM-3rN and partially protected them from challenge infection with heterologous pathogenic SHIV-C2/1 (54, 55). To increase protective vaccine efficacy by immune-potentiation, a nef-deleted SIV or SHIV expressing IFN-γ was constructed and tried (13, 23, 49). In addition, the other researcher examined immunization with co-immunization of plasmid vectors

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Abbreviations: AIDS, acquired immunodeficiency syndrome; ELISPOT, enzyme-linked immunospot; ELISA, enzyme-linked immunosorbent assay; HIV, human immunodeficiency virus; MIP-1α, macrophage inflammatory protein-1α; NK, natural killer; PBMC, peripheral blood mononuclear cell; SFCs, spot-forming cells; SHIV, simian/human immunodeficiency virus; SIV, simian immunodeficiency virus; $TCID_{50}$, 50% tissue culture infectious dose.

expressing IFN-γ with SIV antigens (17, 30).

IFN-γ is a cytokine that possesses antiviral activity, including activity against HIV *in vitro* (18, 51). IFN-γ, in addition to having a direct effect on virus replication, also affects the course of infection and induction of protective immunity by modulating the antiviral immune response including macrophage activating activities and T cell growth promoting activity (8, 10, 18, 19, 21, 26). The release of IFN-γ early in an infection was reported to contribute to differentiation of T cells to Th1 cells (56). IFN-γ is critical for the induction of cell-mediated immunity, especially cytotoxic-T cell (CTL) responses (6, 33, 46), and was reported to be one of the main effector molecules released by CTLs after antigenic stimulation (4).

We have previously reported that an SHIV having human IFN-γ inserted into the deleted nef region (SHIV-IFN-γ) had a greater protective effect against a challenge infection with a heterologous pathogenic SHIV-C2/1 than nef-deleted SHIV having no IFN-γ (13). These results raised a possibility that IFN-γ increases the suppression of heterologous pathogenic SHIV-C2/1 replication.

In the present study, to investigate whether IFN-γ augments the immune response induced by vaccination with a live attenuated SHIV, we examined the antiviral effect of rIFN-γ administration and adjuvant effect of rIFN-γ in live attenuated SHIV-vaccinated or unvaccinated monkeys. An enzyme-linked immunospot (ELISPOT) assay was used to analyze SIV Gag-specific immune responses elicited in monkeys either by rIFN-γ administration alone or SHIV-NI vaccination combined with rIFN-γ administration. Moreover, to investigate whether IFN-γ affects the innate immunity, we examined the natural killer (NK) cell activity and production of CC-chemokines and cytokines.

Our results show that SHIV-NI vaccination combined with rIFN-γ administration is effective for inducing a stronger protective cellular immune response against SHIV-C2/1, suggesting that it has promise as a potential vaccine adjuvant. In addition, the enhancement of NK activity and MIP-1α production may act together as a functional unit enhancing the innate and adaptive immunity to drive a type 1 immune reaction in SHIV-NI-vaccinated monkeys with rIFN-γ.

Materials and Methods

Monkeys. Rhesus macaques (*Macaca mulatta*) about 4 kg in weight, were used in this experiment. All monkeys were maintained according to the institutional animal care and use guidelines of the Institute for Virus Research, Kyoto University.

Virus preparation. SHIV-C2/1 is an SHIV-89.6 variant isolated by passage at the peak of initial plasma viremia from an infected cynomolgus macaque (47). SHIV-C 2/1 virus stock was prepared from the culture supernatants of COS-7 cells transfected with an SHIV-C2/1 molecular clone, pKS661 and was stored in liquid nitrogen until use. SHIV-NI obtained by deleting the nef gene from SHIV-NM-3rN, was used as an attenuated vaccine virus (13, 23). SHIV-NM-3rN is a chimeric simian and human immunodeficiency virus, having the envelope gene of HIV-1 pNL432. These virus stocks were prepared from supernatants of a human T cell line, M8166, transfected with pSHIV-NI.

Interferon-γ. Imunomax^{R}-γ, which contains rIFN-γ 3×10^{6} U/ml, maltose 52.6 mg/ml and L-cysteine 0.44 mg/ml was a kind gift from Shionogi & Co., Ltd., Osaka, Japan. As solutes, maltose 52.6 mg/ml and L-cysteine 0.44 mg/ml which was used as placebo. Plasma IFN-γ concentrations were determined using a commercially available ELISA kit (BioSource International, Camarillo, Calif., U.S.A.).

Pharmacokinetics of exogenous IFN-γ in rhesus macaques. Plasma rIFN-γ levels were assessed sequentially after single intramuscular administration of 1×10^{6} U/ml rIFN-γ to two macaques. Subsequently, plasma rIFN-γ levels in rhesus macaques were measured with an immunoassay kit (BioSource International). The rIFN-γ concentrations in rhesus macaques remained at high levels (100 pg/ml to 80 pg/ml) until 10 hr after injection. This level is similar to the level that was found to be effective against cancer (53).

Immunization and challenge. Four treatments were given (Table 1). To examine the antiviral effect of rIFN-γ administration against a pathogenic SHIV-C2/1 infection, three of five unvaccinated monkeys were intramuscularly administered with rIFN-γ (group A) dissolved in 5% auto-monkey serum and the other two (group B) were given placebo dissolved in solvent of rIFN-γ. rIFN-γ was administered at a dose of 1×10^{6} U/ml per monkey three times per week for 4 weeks. To investigate whether IFN-γ augments the immune response induced by immunization with a live attenuated SHIV, nine monkeys were intravenously inoculated with 1×10^{5} $TCID_{50}$ of SHIV-NI. Four of them were given rIFN-γ (group C) and the other five were given placebo (group D). Subsequently all groups were intravenously challenged with 200 $TCID_{50}$ of the pathogenic SHIV-C2/1. One monkey of group C (MM411) died by accident after the challenge. This monkey did not show complete loss of $CD4^{+}$ T cells which is a symptom of AIDS.

Sample collection. Peripheral blood mononuclear cells (PBMCs) were prepared by Percoll density gradi-

Table 1. Immunization and treatment

Group	Monkeys (MM nos.)	Treatment		
		rIFN-γ[a]	Placebo[b]	SHIV-NI[c]
A	348, 352, 295	×		
B	316, 328		×	
C	371, 291, 413, 414	×		×
D	367, 369, 405, 411, 412		×	×

[a] 1×10^6 U, i.m. three times per week for 4 weeks.
[b] i.m.
[c] 1×10^5 $TCID_{50}$, i.v.

ent centrifugation. Freshly isolated PBMCs were used for ELISPOT assay and NK assay as described below. All plasma samples were frozen at −80 C until use.

Quantification of plasma viral RNA loads. Plasma viral RNA loads were determined by quantitative RT-PCR (50). 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 *Taq*man RT-PCR kit (Perkin Elmer). RNAs of attenuated viruses and challenge virus were evaluated with primer pairs specific to SHIV NM-3rN and SHIV-C2/1, respectively, as previously described (14). These reactions were performed with a Prism 7700 Sequence Detector (Applied Biosystems, Foster City, Calif., U.S.A.) 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.

Measurement of antibodies to HIV-1 Env protein in plasma. HIV-1 Env-specific IgG antibodies in the plasma were detected by ELISA using the following method developed at Japan Immunoresearch Laboratories, Takasaki, Japan (2). In brief, a 96-well microplate (Nunc-Immuno™Modules, Maxisorp™, Nalge-Nunc, Rochester, N.Y., U.S.A.) was coated with HIV-1 IIIB gp160 recombinant viral protein (Advanced Biotechnologies, Inc., Columbia, Md., U.S.A.) at 1 μg/ml in 0.1 M Na_2CO_3-$NaHCO_3$ buffer (pH 9.6), incubated overnight at 4 C, washed with 0.15 M NaCl containing 0.05% Tween 20 and treated with 25% Block Ace™ (Nacalai Tesque, Inc., Kyoto, Japan) for 2 hr at room temperature, incubated overnight with plasma diluted with staining buffer (10% Block Ace) at 4 C, washed, incubated with peroxidase-conjugated goat anti-monkey IgG (1 μg/ml) (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Nd., U.S.A.) for 2 hr at room temperature, washed, treated with *O*-phenylenediamine dihydrochloride (OPD, Sigma, St. Louis, Mo., U.S.A.) for 10 min and immersed in 2 N H_2SO_4 to stop the reaction. Specific absorbances were read at 490 nm with a microplate reader as described (2). The amounts of antibody were expressed as the optical density. Plasmas of SHIV-infected monkeys and naïve monkeys were used as positive and negative controls, respectively.

IFN-γ ELISPOT assay. The number of antigen specific cytokine rIFN-γ-producing cells was determined with an IFN-γ ELISPOT kit (Mabtech, Nacka, Sweden) using an MAHAS4510 multi-screen 96-well plate (Millipore, Bedford, Mass., U.S.A.), anti-monkey IFN-γ antibody, biotinylated anti-IFN-γ antibody and streptavidin-alkaline phosphatase conjugate antibody, according to the manufacturer's instructions. SIV Gag p27 protein (soluble native p27, Advanced biotechnologies) was added to the cultures at a final concentration of 10 μg/ml. Medium alone was used as a negative control. As a positive control, concanavalin A was added to control wells at a final concentration of 5 μg/ml. Cells were tested at 1×10^6 cells per well in duplicate and incubated undisturbed at 37 C, 5% CO_2, for 36 hr. The resulting spots were counted with a microscope. Date in the figures show the mean number of spot-forming cells (SFC) per 1×10^6 PBMCs.

Flow cytometry. An absolute cell count was determined from samples of PBMCs as described previously (13). PBMCs of each monkey were stained with FITC-conjugated anti-monkey CD3 (FN-18; BioSource International, Belgium), phycoerythrin (PE)-conjugated anti-human CD4 (NU-TH/I; Nichirei), PerCP-conjugated anti-human CD8 (leu-2a; BD Pharmingen, San Diego, Calif., U.S.A.) or FITC-conjugated anti-human CD29 (4B4; Beckman Coulter). After hemolysis of the whole blood using FACS TM Lysing Solution (BD Pharmingen), each type of labeled lymphocytes was measured on a FACScan (Becton Dickinson, Mountain View, Calif., U.S.A.) by using Cell Quest software (Becton Dickinson). Absolute lymphocyte counts in the blood were determined with an automated blood cell counter (F-820; Sysmex, Japan).

NK cytotoxicity assay. NK cell activities of PBMCs in each monkey were periodically measured by a chromium (^{51}Cr) release assay using K562 cells as target cells (12). Briefly, PBMCs obtained from each mon-

key were used as effector cells. Serial dilutions of effector cells were cultured with 1×10^4 cells of ^{51}Cr-labeled K562 cells dispensed in triplicate into each well of 96-well plates. After 4 hr, the culture supernatants of each well were monitored with a γ counter. Cytotoxic activity was calculated as the percent specific lysis at an effector cells to target cells ratio of 50:1. Percent specific lysis was calculated from (experimental release−spontaneous release)/(total release−spontaneous release)×100. Total release was determined by treating the target cells with 2.5% Triton X-100.

Measurement of cytokines and CC-chemokines. PBMCs obtained from each monkey were incubated in a tissue-culture dish for 1 hr and were separated into adherent and non-adherent cells. We used peripheral blood lymphocytes (PBLs), prepared as non-adherent cells, in the cytokine and CC-chemokine detection assays. After the stimulation of non-adherent cells with the SIV Gag p27 protein (Advanced Biotechnologies) at a final concentration of 10 μg/ml for 72 hr, the productions of IFN-γ, IL-4, IL-10, MIP-1α, MIP-1β, RANTES were measured in the culture supernatants using an immunoassay kit (BioSource International) and Quantikine (R & D Systems, Minneapolis, Minn., U.S.A.).

Results

No Antiviral Effect of rIFN-γ Administration to Unvaccinated Naïve Monkeys against SHIV-C2/1 Infection

First, to evaluate the antiviral effects of rIFN-γ without vaccination against a challenge virus, SHIV-C2/1, three of five unvaccinated monkeys (MM348, MM352 and MM295) were administered with rIFN-γ (group A) and the other two (MM316 and MM328) were administered with placebo (group B). rIFN-γ was administered at a dose of 1×10^6 U per monkey three times per week for 4 weeks. Then these monkeys were challenged intravenously with 200 $TCID_{50}$ of a pathogenic SHIV-C2/1. The five challenged monkeys were monitored for viral load for 15 weeks.

As shown in Fig. 1-A, in placebo-administered naïve monkeys (group B) that were infected with SHIV-C2/1, the viral RNA loads in plasma dramatically increased to about 6.3×10^7 to 8.7×10^8 at 2 weeks post challenge (w.p.c.). On the other hand, in rIFN-γ-administered monkeys (group A), the viral RNA loads in plasma increased to about 1.6×10^7 at 2 w.p.c. Thus, the peaks of plasma viral load were reduced about 5-fold in group A monkeys with rIFN-γ compared with the levels in naïve group B monkeys. In both group A and group B, almost all monkeys developed a rapid and complete loss of $CD4^+$ T cells, but only one monkey in group A (MM 295) did not show complete loss of $CD4^+$ T cells. Viral set point levels of 4.6×10^6 to 5.0×10^5 copies/ml were maintained in these unvaccinated monkeys. These results show that rIFN-γ administration had little or no antiviral effects.

Enhanced Protective Effect of rIFN-γ Administration to Vaccinated Monkeys against SHIV-C2/1 Replication

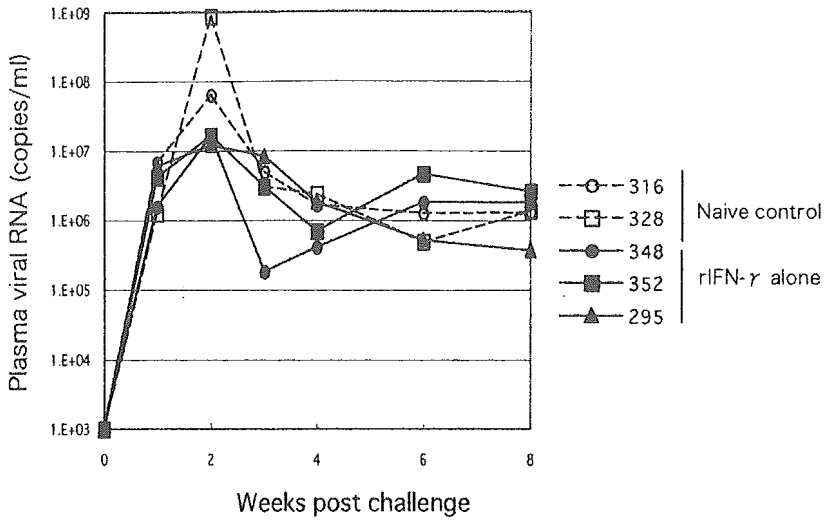
To investigate the adjuvant effect of IFN-γ, nine monkeys were immunized with SHIV-NI. Four of them (MM371, MM291, MM413 and MM414) were administered with rIFN-γ (group C) and the other five monkeys (MM367, MM369, MM405, MM411, MM412) were administered with placebo (group D). The dose and schedule of rIFN-γ administration were the same with those of group A. Then these monkeys were challenged with SHIV-C2/1.

Neither the rIFN-γ administered monkeys (group C) nor the placebo-administered monkeys (group D) experienced the severe $CD4^+$ T cell loss after SHIV-C2/1 challenge that occurred in the unvaccinated monkeys (Fig. 2-B). However, the plasma viral RNA loads after the challenge were different in the two groups. In one rIFN-γ-administered monkey (MM413, group C), viral loads were undetectable after the challenge. In the three other rIFN-γ-administered monkeys (group C), the viral RNA loads in plasma quantified by PCR specific for a challenge SHIV-C2/1 were about 1×10^3 to 2×10^5 at 2 w.p.c. (Fig. 1-B). On the other hand, the plasma viral RNA loads increased to about 3×10^7 to 1×10^6 at 2 w.p.c. in placebo-administered monkeys (group D). Thus, in the vaccinated monkeys with rIFN-γ (group C), the peaks of plasma viral load were reduced 100- to 1,000-fold compared to those in the vaccinated monkeys without rIFN-γ (group D). The significant difference of the peak values of plasma viral load between groups C and D showed that vaccination of SHIV-NI in combination with rIFN-γ administration could induce strong resistance to SHIV-C2/1 replication.

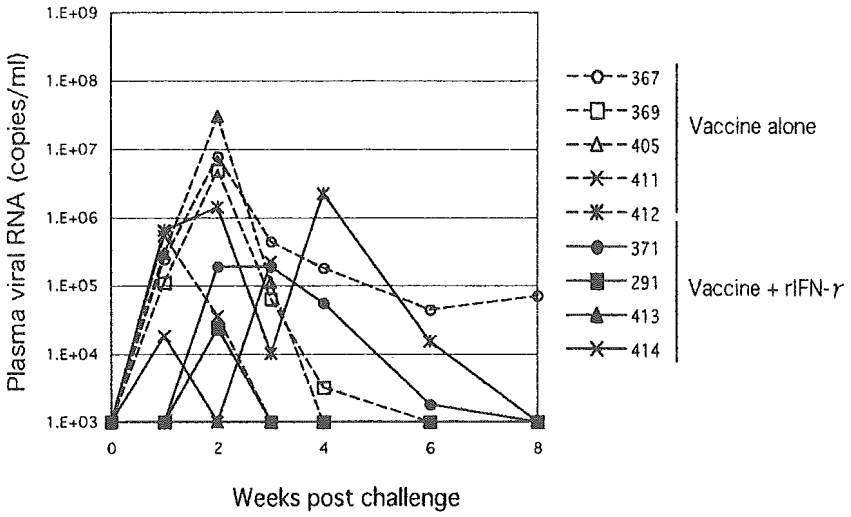
Enhancement of IFN-γ Producing Cells in the Vaccinated Monkeys with rIFN-γ

To investigate whether IFN-γ augments the immune response induced by vaccination with a live attenuated SHIV, the number of virus specific spot forming cells (SFC) secreting IFN-γ of PBMCs in each monkey groups were measured by ELISPOT assays.

SIV Gag-specific SFC induced in the SHIV-NI vaccinated monkeys receiving rIFN-γ (group C) was higher than that induced in the vaccinated monkeys without rIFN-γ. Two weeks after the challenge, group C with rIFN-γ reached the highest number of Gag-specific IFN-γ SFC (Fig. 3-B). On the other hand, in the unvac-



(A) Unvaccinated monkeys.



(B) Vaccinated monkeys.

Fig. 1. Kinetics of plasma viral RNA of the SHIV C2/1-infected monkeys. (A) Unvaccinated monkeys; (B) vaccinated monkeys. The experimental schedule of each group of monkeys is presented in Table 1.

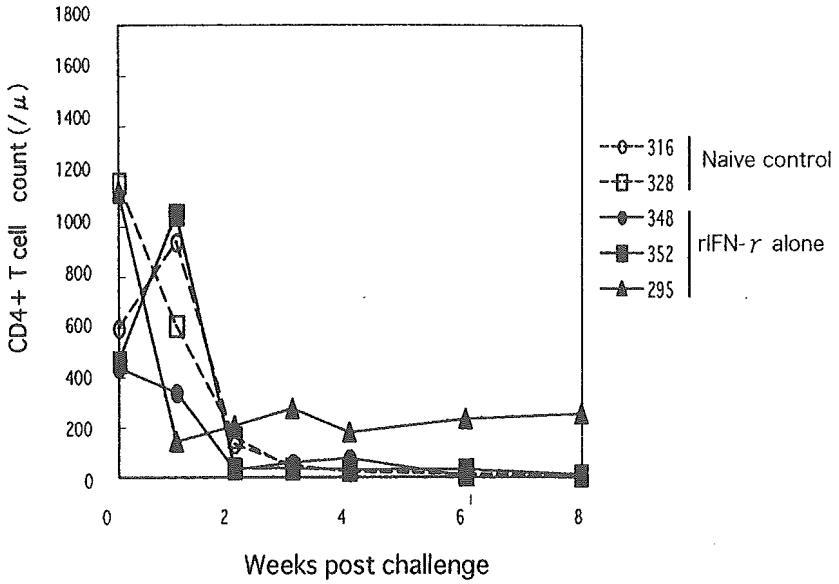
cinated monkeys with and without rIFN-γ (groups A and B), the number of Gag-specific IFN-γ SFC were hardly detectable (Fig. 3-A). These results demonstrated that vaccination of SHIV-NI in combination with rIFN-γ administration augments the cellular immune (Th1) response.

Delayed Antibody Response in the SHIV-NI Vaccinated Monkeys Receiving rIFN-γ

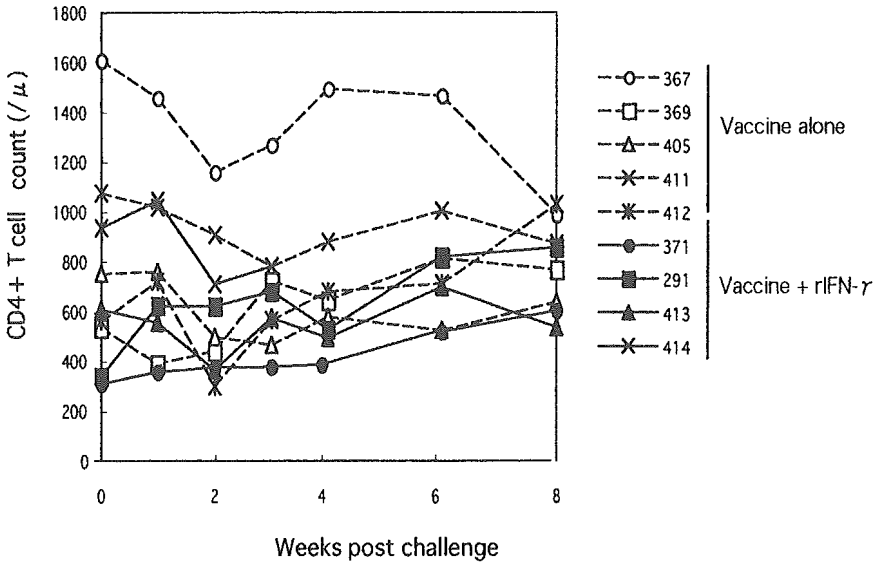
The time of appearance of HIV-1 Env-specific IgG antibodies detected by ELISA was different in vaccinated macaques with and without rIFN-γ after the challenge (Fig. 4). The SHIV-NI-vaccinated monkeys that received the placebo (group D) exhibited HIV-1 gp160-specific IgG antibody responses gradually from 1 w.p.c. On the other hand, at 1 w.p.c. no antibody was detected in the SHIV-NI vaccinated monkeys that received rIFN-γ (MM291, MM413 and MM414) (group C). At 2 w.p.c. the titer of antibody dramatically increased in both monkey groups but was slightly higher in group D without rIFN-γ than in group C with rIFN-γ, which showed a higher Th1 response as shown in the previous section. No HIV-1 Env-specific IgG antibodies were detected in any of the vaccinated macaques before challenge (the cut off value was 0.11).

Enhanced Chemokine MIP-1α Production in the SHIV-NI Vaccinated Monkeys Receiving rIFN-γ

To assess the production of CC-hemokines and cytokines by antigen-stimulated PBMCs from the vacci-



(A) Unvaccinated monkeys.



(B) Vaccinated monkeys.

Fig. 2. Changes in $CD4^+$ T cell counts in PBMCs of unvaccinated monkeys (A) and SHIV-NI-vaccinated monkeys (B) after SHIV C2/1 challenge.

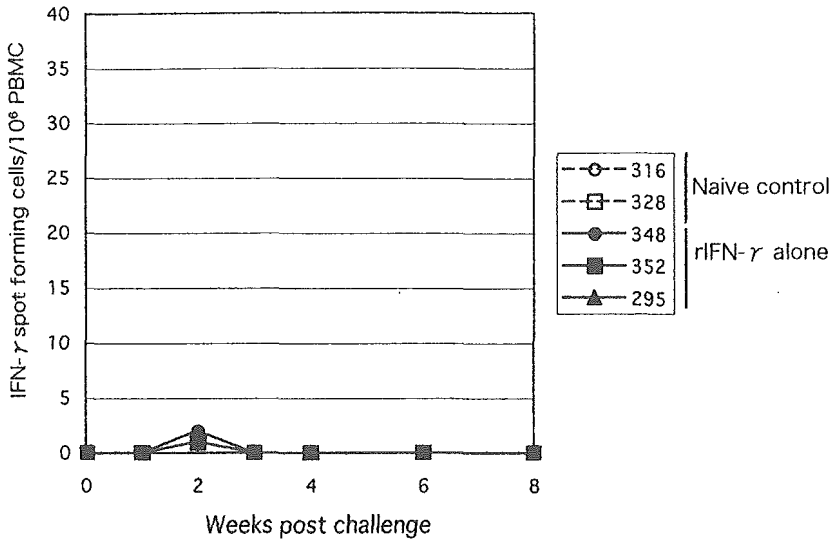
nated monkeys (groups C and D), the production of MIP-1α, MIP-1β, RANTES, IFN-γ, IL-4 and IL-10 by specific ELISAs was analyzed. PBMCs of nine vaccinated monkeys (groups C and D) were stimulated with SIV Gag and their presence in cell culture supernatants were measured before and at 2 weeks after vaccination.

No substantial differences were seen in the production of MIP-1β, RANTES, IFN-γ, IL-4 or IL-10 by PBMCs stimulated with SIV Gag in the SHIV-NI vaccinated monkeys with and without rIFN-γ during the time of follow-up (data not shown). However, the levels of MIP-1α secreted by PBMCs in SHIV-NI-vaccinated monkeys receiving rIFN-γ (group C) were dramatically higher at 2 week post vaccination (w.p.v.), than those of vaccinated monkeys without rIFN-γ (group D) (Fig. 5).

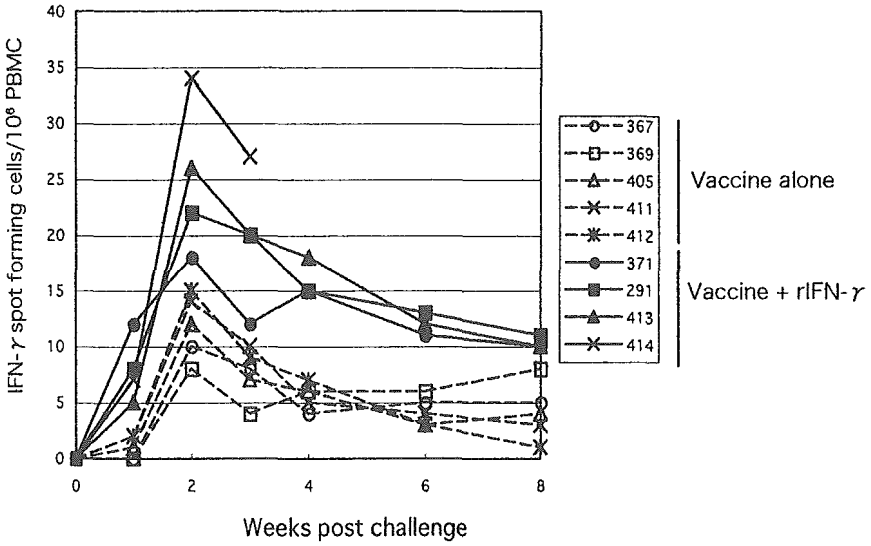
Augmentation of NK Activity by rIFN-γ Administration

To evaluate innate immune response that contributed to the protection against SHIV-C2/1, we assayed NK cell activity of PBMCs of the SHIV-NI vaccinated monkeys with (group C) and without (group D) rIFN-γ.

In all the group C monkeys except MM291, NK cell



(A) Unvaccinated monkeys.



(B) Vaccinated monkeys.

Fig. 3. Numbers of SIV Gag-specific IFN-γ-producing cells in PBMCs of unvaccinated monkeys (A) and vaccinated monkeys (B). PBMCs were stimulated with SIV Gag protein. IFN-γ-producing cells were detected by IFN-γ-specific ELISPOT assays, and data are expressed as the number of SFC per 10^6 cells. Each point represents the mean number of IFN-γ spots in duplicate wells.

activities at 1 w.p.v. were approximately 30 to 45% greater than the activities before rIFN-γ treatment (Fig. 6). These augmented NK activities were observed during the 3-week rIFN-γ treatment period. On the other hand, no augmentation was observed in the vaccinated monkeys without IFN-γ (group D). After the challenge, The NK activities of these monkeys remained at low levels in both groups. These results demonstrate that rIFN-γ administration augmented NK cell activities in the vaccinated monkeys.

Discussion

We previously reported that monkeys immunized with a nef-deleted SHIV-NM-3rN (SHIV-NI) or an SHIV-NI expressing human IFN-γ (SHIV-IFN-γ) could control the replication of a heterologous pathogenic virus and prevent the loss of $CD4^+$ T cells. SHIV-IFN-γ vaccinated monkeys showed resistance to the SHIV C2/1 challenge, even though only 4 weeks had passed

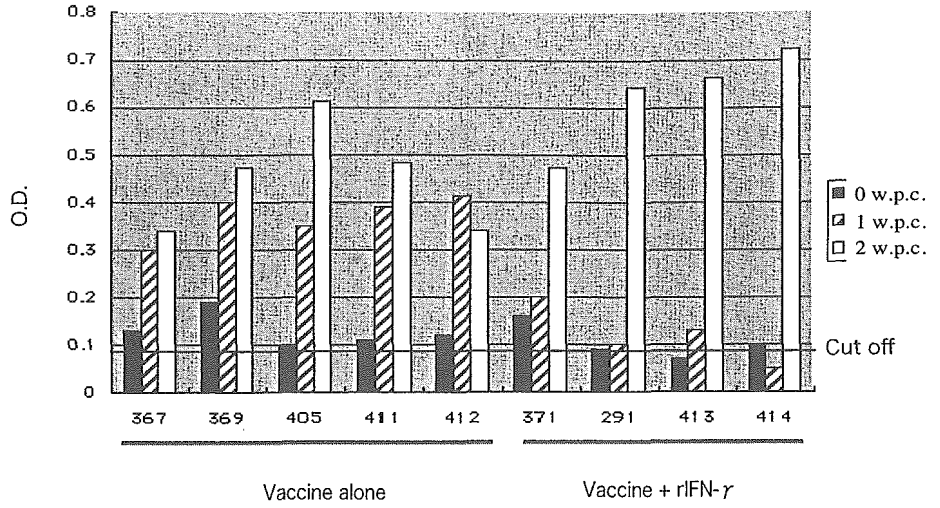


Fig. 4. HIV-1 Env-specific IgG antibodies in plasma of SHIV-NI vaccinated monkeys combined with rIFN-γ or placebo administration after challenge.

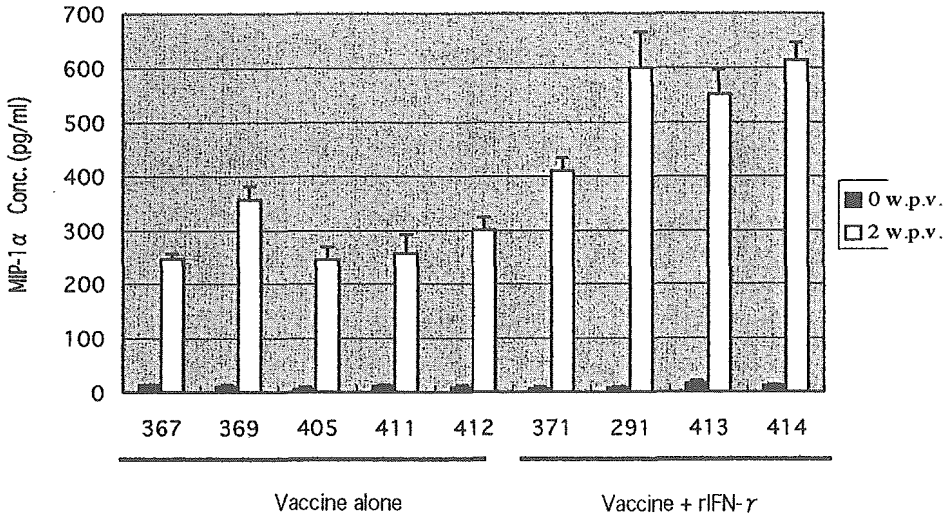


Fig. 5. Production of MIP-1α by SIV Gag-stimulated PBMCs of SHIV-NI vaccinated monkeys combined with rIFN-γ or placebo. PBMCs were obtained prior to vaccination (0) and 2 weeks after vaccination.

since the immunization. Moreover, the peak value of the plasma viral load in the early phase of the challenge with SHIV-C2/1 was reduced in the SHIV-IFN-γ-vaccinated monkeys compared with those in the SHIV-NI vaccinated monkeys. These results raise the possibility that IFN-γ makes a strong contribution to the suppression of SHIV-C2/1 replication (13, 23). The other study has been reported that HIV p24 antigen was decreased in plasma samples obtained from six of nine rIFN-γ-administered patients with initially detectable HIV protein (19). These data suggest that rIFN-γ should be considered as a therapeutic agent, possibly with other antiviral, in the treatment of patients with AIDS. However, whether exogenous IFN-γ administration induces protective immunity in the SHIV/monkey model which is useful for clarifying the mechanism of IFN-γ-induced protection is unknown.

The aim of the present study was to investigate whether exogenous IFN-γ administration could augment the immune response induced by vaccination with a live attenuated SHIV-NI. In the vaccinated monkey groups, SHIV-NI vaccination combined with rIFN-γ administration resulted in significantly limiting the peak value of the plasma viral load and maintaining $CD4^+$ T cells after SHIV-C2/1 challenge. In this group, the peak of plasma viral load was reduced approximately 100- to 1,000-fold and the number of SIV Gag-specific IFN-γ-producing cells increased 2-fold compared with those in the SHIV-NI-vaccinated monkeys without rIFN-γ (group D). On the other hand, in the unvaccinat-

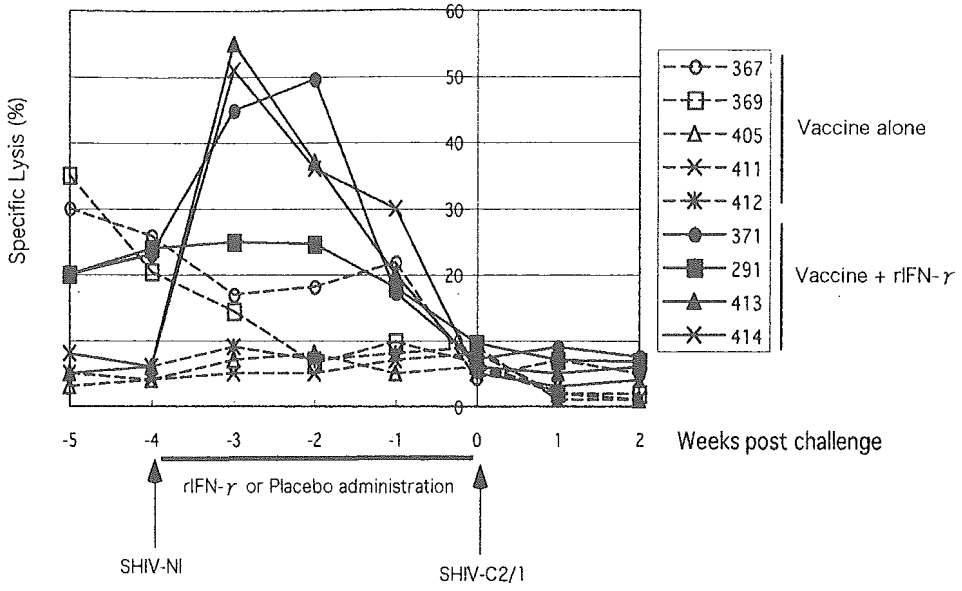


Fig. 6. NK cell activity of PBMCs in the SHIV-NI vaccinated monkeys combined with rIFN-γ or placebo administration before and after challenge.

ed monkey groups, $CD4^+$ T cells declined after SHIV-C2/1 infection almost equally in monkeys with rIFN-γ (group A) and without (group B). SIV Gag-specific IFN-γ-producing cells were undetectable in unvaccinated monkeys of both groups. The increase in the number of IFN-γ-producing cells in the vaccinated monkeys was closely correlated with the decrease in the peak value of plasma viral load of SHIV-C2/1.

These data suggest that vaccination with SHIV-NI in combination with rIFN-γ administration is more effective at modulating the immune system into the Th1-type response than either rIFN-γ administration alone or SHIV-NI vaccination alone. This modulation to a Th1-type response indicates that rIFN-γ administration could induce the strong cellular immune response against SHIV infection. The augmented Th1-type responses assessed by SIV Gag protein-specific IFN-γ-producing cell activity might be associated with the activity of cytotoxic T lymphocytes (CTLs). Antigen-specific Th1-mediated immune responses and CTLs have been reported to provide protection and reduce disease progression (16, 22, 32, 36, 39). Th1/$CD8^+$ T cell responses have been shown to play an important role in controlling HIV-1 replication (5, 27, 34, 35, 37, 38, 40, 42–44). Th1/$CD4^+$ T cell responses (antigen-specific $CD4^+$ T helper cells) also may promote CTL activity either by a CD4-antigen-presenting cell (APC)-CD8 pathway or by IL-2 secretion (20, 41, 57). The present observations are encouraging in light of the hypothesis that Th1-mediated immunity is associated with resistance to virus infection and suppression (3, 28).

To investigate whether IFN-γ affect the innate immunity, we examined production of CC-chemokines and cytokines, and the NK activity of freshly isolated PBMCs of these monkeys. The levels of RANTES, MIP-1α, MIP-1β, IFN-γ, IL-4 and IL-10 of supernatants from SIV Gag-stimulated PBMCs were measured by ELISA. In result of these, NK activity and MIP-1α production were enhanced in SHIV-NI-vaccinated monkeys with rIFN-γ (group C). Moreover, no HIV-1 Env-specific antibody was detected at 1 w.p.c. in SHIV-NI-vaccinated monkeys with rIFN-γ. On the other hand, SHIV-NI vaccinated monkeys without rIFN-γ (group D) exhibited HIV-1 Env-specific antibody responses at 1 w.p.c. The lower IgG Ab response generated in SHIV-NI vaccinated monkeys with rIFN-γ might have resulted from the predominant Th1>Th2 cytokine response modulated probably by both NK activity and MIP-1α production. Several studies have shown that MIP-1α stimulation enhances IFN-γ production, which is essential for the induction of Th1-derived HIV-specific cell-mediated immunity (25). MIP-1α was reported to activate NK cells and the activated NK cells produce MIP-1α (29, 31, 45). NK cells are a critical component of the host innate immune response to a variety of viruses, fungi, parasites and bacteria (35, 45, 52). After activation, NK cells release various cytokines and chemokines including MIP-1α that induce the inflammatory response, modulate hematopoiesis, control monocyte and granulocyte growth and function, and influence the type of adaptive immune responses (52). With regard to HIV infection, the nonspecificity of NK cell activity might be relevant to the maintenance of a degree of antiviral activity in the

face of a high level of virus replication that negatively impacts HIV-specific cellular and humoral immune responses (7, 15). Recently, Enose et al. (13) showed vaccination of monkeys with live attenuated SHIV having IFN-γ effectively suppressed the peak value of plasma viral loads from 1 to 3 weeks after SHIV-C2/1 challenge. Cellular immune responses were augmented by SHIV-IFN-γ as compared to SHIV-NI without the IFN-γ gene insert. From 4 to 12 weeks after the challenge, however, these monkeys showed a transient increase in viral load. IFN-γ mediated inflammation has been reported to be associated with a lack of protection from SIV challenge in vaccinated rhesus macaques (1). It is possible that IFN-γ-driven inflammation promotes SHIV-C2/1 replication in SHIV-IFN-γ immunized monkeys at later stage, because SHIV-IFN-γ immunization may increase IFN-γ mRNA levels in lymphoid tissues (1). In a vaccine study using a combination of DNA immunization with plasmid adjuvants expressing GM-CSF and IFN-γ (30), both humoral and cellular immune responses to SIV antigens were augmented by cytokine expression plasmids as compared to mock plasmid without a cytokine gene insert, but the plasma viral loads at set points were not significantly different between both the immunized group and the non-immunized control group. On the other hand, in this present study, the SHIV-NI-vaccinated monkeys that were treated with rIFN-γ (group C) exhibited various responses including augmentation of HIV-1 Env-specific antibody responses. In addition, the innate and cellular immune responses in these monkeys significantly reduced the peak value of the plasma viral load without a transient increase in viral RNA load after the challenge. To obtain better protection against a pathogenic virus, it may be necessary to develop a vaccine that induces both innate and cellular immune responses.

In conclusion, our data show that SHIV-NI vaccination combined with rIFN-γ administration induces strong SIV Gag-specific Th1-type cellular immune response, which might contribute to the control of a heterologous pathogenic SHIV challenge infection. Since the magnitude of the induced immunity is considered enough for protection against viral infection and disease progression, rIFN-γ could be used as an adjuvant for attenuated vaccine candidates against SHIV infection, probably against HIV infection.

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