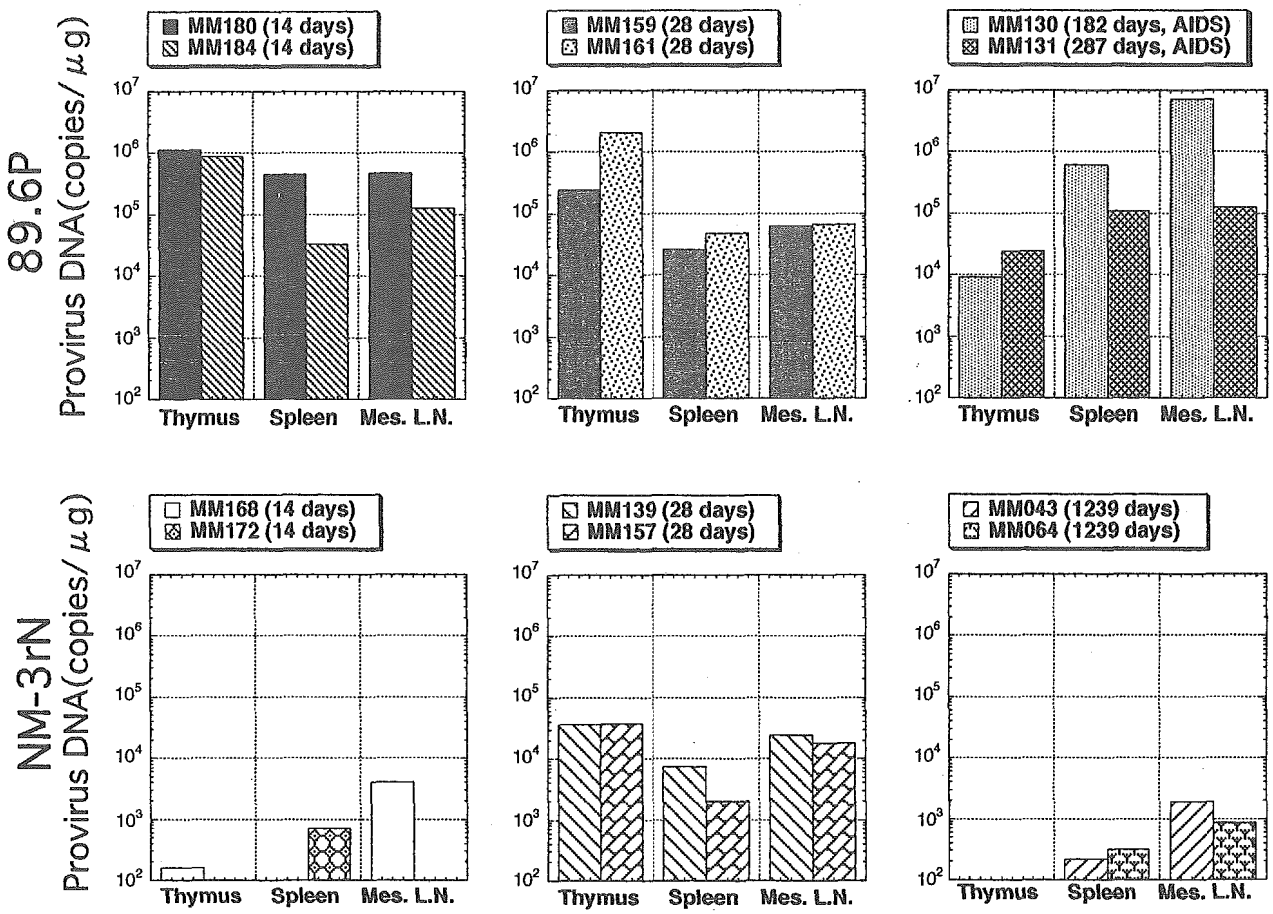

Fig. 6. Proviral DNA quantification in SHIV89.6P- or NM-3rN-infected lymphoid tissues (thymus, spleen, and mesenteric LN).

mal cells and known to play a key role in T cell development, in HIV-1-positive individuals correlates with CD4+ T cell depletion (Llano et al., 2001). Further investigations are needed to clarify the humoral factors involved in immune modulations.

As the thymic atrophy caused by the SHIV89.6P infection was so profound, the regeneration of the T cell pool would be expected to be impaired and the resultant T cell repertoire would be expected to be extremely limited. Such a limitation together with the paracortical depletion of the lymph nodes might explain the rapid, profound, and "continuous" T cell depletion in the SHIV89.6P-infected animals. This rapid and drastic clinical course is clearly different from that observed in HIV-1-infected humans. However, SHIV and HIV-1 share several properties: SHIV has several HIV-1 genes including *env,* the same CD4+ T lymphocyte subset that is depleted in HIV-1-infected humans is depleted in SHIV-infected monkeys, and the symptoms of the induced immunodeficiency in the SHIV-infected monkeys are also very similar to those observed in HIV-1-infected individuals (Douek et al., 1998). Furthermore, the infectivity of thymocytes was found to be closely associated with the CD4 depletion by HIV-1 in vivo (Stoddart et al., 2001). Therefore, the acute pathogenicity of SHIV89.6P in macaques can be viewed as a model of human AIDS with a markedly compressed clinical course and should be quite useful for further investigation of the in vivo mechanism of AIDS pathogenesis.

As we reported previously, NM-3rN replicated as fast as the parental SIVmac239 in human and macaque PBMCs and in several CD4+ human cell lines (Kuwata et al., 1995). Chronic persistent infections have been established in rhesus, pig-tailed, and cynomolgous monkeys (Hayami et al., 1999; Kuwata et al., 1995). In the very early stage of infection, the cell-associated virus loads of NM-3rN were comparable with those of SIVmac239 and they thereafter declined to a 10- to 100-fold lower level (Kuwata et al., 1995), while the plasma virus load, which is more sensitive than the cell-associated virus load, showed that NM-3rN has a lower growth ability in vivo (Fig. 2). Our study indicated that there was an activation of the infected LNs (as demonstrated by paracortical expansion with mixed cellular infiltration) at 28 dpi in both the NM-3rN- and SIVmac239-infected animals. The NM-3rN-infected monkeys subsequently showed follicular hyperplasia in the chronic state of infection. Nevertheless, the infected germinal centers did not show any involuted features and the frequencies of occurrence of VPCs and the amounts of proviral DNA were

significantly lower than those seen in the pathogenic SHIV89.6P-infected monkeys. The generalized lymphoid activation with the minimal involution and reduced frequency of VPCs in the lymphoid tissues seemed to be the main features of the NM-3rN infection. These results were similar to those observed in human long-term nonprogressors (Pantaleo et al., 1995). Thus, the in vivo NM-3rN proliferation did not cause extreme lymphoid activation such as persistent generalized lymphadenopathy, and therefore it may provide a good model for long-term nonprogressors. Further studies are needed to clarify the mode of infection at the early stage of long-term nonprogressive infection, which cannot be predicted in humans.

The tissue distributions of SHIV89.6P and SIVmac239 were clearly different although the viral load set points at 28 dpi for the two viruses were similar. The different tissue tropisms of these viruses seem to be due to a difference in the coreceptors that they use. That is, SIVmac239 uses CCR5, and SHIV89.6P uses both CCR5 and CXCR4. Other acute pathogenic SHIV strains causing rapid CD4+ cell depletion are also known to use CXCR4 only or both CXCR4 and CCR5, whereas infection with a pathogenic CCR5-specific enveloped virus, SHIVSF162P, caused a dramatic loss of CD4+ intestinal T cells followed by a gradual depletion in peripheral CD4+ T cells (Harouse et al., 1999; Igarashi et al., 1999; Joag et al., 1996). NM-3rN has the env of an HIV-1 strain that is derived from the NL432 strain and uses CXCR4 as a coreceptor but replication of NM-3rN in vivo is limited. The rapid CD4+ T cell depletion that occurs in peripheral blood at the early stage of infection may require the usage of the CXCR4 coreceptor and a high virus load set point.

In conclusion, the early stage of the SHIV89.6P infection was characterized by acute thymic involution and paracortical lymphadenitis which began within 14 dpi. Because of the severe pathological alterations and the profound peripheral CD4+ cell loss caused by SHIV89.6P, the infection in macaques is useful for analyzing the in vivo mechanism of AIDS pathogenesis. In contrast, NM-3rN showed generalized lymphoid activation with minimal involution and these pathological findings were similar to those seen in human long-term nonprogressors. NM-3rN induced strong immunological responses and resulted in minimal cell loss caused by apoptosis. Clarification of the mechanisms responsible for the disease-free state in NM-3rN infection might provide insights for preventing pathogenic virus infections. Also, the nonpathogenic features of NM-3rN might make this virus suitable as a prototype for live-attenuated vaccines.

Materials and methods

SHIV

The detailed protocol for generation of NM-3rN has been described (Kuwata et al., 1995). SHIV89.6P was kindly provided by Dr. K.A. Reimann and Dr. N.L. Letvin (Reimann et al., 1996a). The pathogenic molecular clone, SIVmac239, was kindly provided by Dr. R.C. Desrosiers (Naidu et al., 1988). The plasmid DNAs of NM-3rN and SIVmac239 were transfected into M8166 cells (Clapham et al., 1987) and infectious viruses were obtained. Rhesus macaque PBMCs were infected with these viruses and the supernatant with the highest reverse transcriptase activity level was used as the virus stock. The $TCID_{50}$ values of these stock viruses were determined using M8166 cells and were adjusted by adding an appropriate volume of medium.

Animal experiments

Monkeys were treated in accordance with the institutional regulations approved by the Committee for Experimental Use of Nonhuman Primate in the Institute for Virus Research, Kyoto University. As references for the AIDS state, two SHIV89.6P-infected monkeys (MM130 and MM131) and two SIVmac239-infected monkeys (MM082 and MM105) were observed until they showed the initial symptoms of the disease. Two NM-3rN-infected monkeys were followed up for more than 3 years (Table 1). A total of eight young rhesus monkeys (*Macaca mulatta,* 3 to 6 years old) were infected with either SHIV89.6P or NM-3rN. Two monkeys for each incubation period (14 and 28 days) were employed. For positive controls of viral infection, we examined three SIVmac239-infected monkeys for 28 days (Tables 2 and 3). All of these monkeys were confirmed to be free of SIV and simian T-cell leukemia/lymphoma virus type 1 (STLV-1). The animals were intravenously inoculated with 10 $TCID_{50}$ of SHIV89.6P or NM-3rN or with 10^5 $TCID_{50}$ of SIVmac239. The peripheral LNs were sequentially removed to examine the histopathological alterations. The blood samples were periodically monitored by CD4+ cell counts and by determining the plasma virus load as described (Kozyrev et al., 2001). At 14 and 28 dpi, the SHIV89.6P- and NM-3rN-infected animals were euthanized and necropsied for subsequent histopathological examinations. The thymus, spleen, tonsil, and peripheral LN (axillary, inguinal, and mesenteric) derived from an identical animal were either fixed in 4% paraformaldehyde (PFA)–PBS overnight at 4°C, and then embedded in paraffin wax for the histological examinations and histochemical in situ hybridization (HISH) studies, or directly frozen for DNA extraction.

Histopathological examination

The tissue sections were cut at a thickness of 4 μm and stained with hematoxylin–eosin (H&E) for the subsequent histopathological examinations. Histological alterations of the viral-infected macaques were classified according to a previous report (Ringler et al., 1989). To examine alterations in the tissue, some of the PFA-fixed and paraffin-

embedded thymic tissues were excised, deparaffinized, and subjected to transparent electron microscopy (TEM).

Histochemical in situ hybridization (HISH)

Digoxigenin-labeled antisense RNA probes were employed for the HISH detection of the viral genomes (DIG–RNA, Böehringer–Mannheim, Tokyo, Japan) (Bucy et al., 1994; Karr et al., 1994). Briefly, 4-μm-thick paraffin sections were deparaffined and subjected to 0.2 M HCl degeneration followed by Proteinase K (PK; Sigma–Aldrich Japan, Tokyo) digestion and acetylation. The conditions of PK treatment (4 μg/ml in PBS at 37°C for 30 min) were determined by preliminary tests. After postfixation, the sections were prehybridized for 1 h at room temperature and hybridized overnight at 42°C with 5 ng/section of the DIG–RNA probes. Following RNase treatment and extensive washing, the hybrids were incubated with alkaline phosphatase-conjugated antidigoxigenin antibody (Fab fractionated) and the resultant complexes were visualized by NBT/BCIP substrates (Böehringer–Mannheim, Tokyo, Japan) at 4°C for 40 h.

For an RNA probe, we synthesized and tested several candidates such as the *gag, pol,* or *nef* regions, which were raised against almost identical sequences among SIVmac239, NM-3rN, and SHIV89.6P. We eventually selected a 562-nucleotide region in *nef* because it gave the strongest and most reproducible signals. Viral distributions were estimated by the number of VPCs per high power (×400) field under the microscope.

Quantitative analysis of provirus DNA

The SHIV proviral DNA quantitative assay was performed with TaqMan DNA PCR (Perkin–Elmer, USA) for the SIV gag region using the primers SIVII-696F (5′-GGAAATTACCCAGTACAACAAATAGG-3′) and SIVII-784R (5′-TCTATCAATTTTACCCAAGGCATTTA-3′). DNA samples were extracted directly from frozen lymphoid tissues with a QIAGEN DNeasy Tissue kit (QIAGEN, Germany) according to the manufacturer's protocol. A labeled probe, SIVII-731T (5′-Fam-TGTCCACCTGCCATTAAGCCCG-Tamra-3′), was used for detection of the PCR product. For each run, a standard curve was generated from a plasmid DNA sample containing the full genome of SHIV NM-3rN, which was quantified with a UV spectrophotometer.

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

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

Introduction

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

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

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

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

Materials and methods

Construction of SHIVs

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

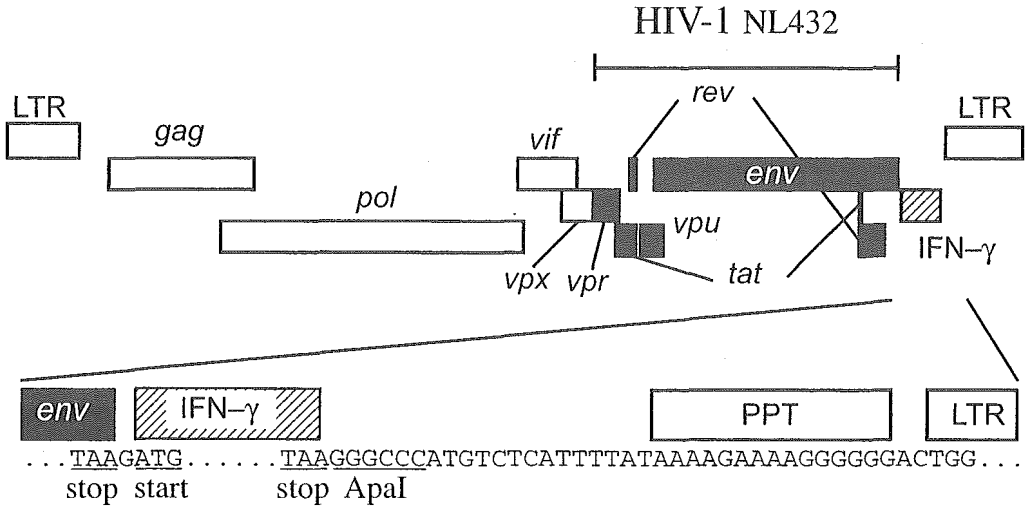

Fig. 1. Genetic structure of SHIV-IFN-γ. The black-colored regions represent sequences derived from HIV-1 (NL432 isolate), the white-colored regions represent SIVmac 239-derived sequences. Human IFN-γ gene DNA (hatched regions) was inserted between the EcoRV and ApaI endonuclease sites of SHIV-NI

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

Virus stock production and in vitro *infection*

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

Vaccinations of rhesus macaques

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

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

Virus inactivation procedure

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

Stimulation of PBMC and LN cells

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

Assay of cytokine production

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

Results

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

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

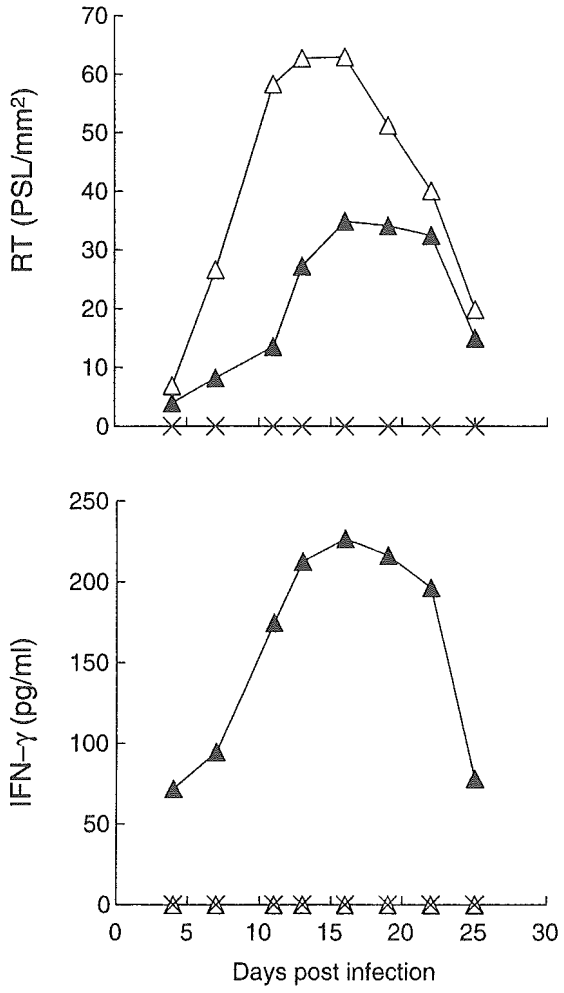

Fig. 2. *In vitro* SHIV infection. Kinetics of viral replication (top) and IFN-γ production (bottom) in macaque PBMC infected with SHIV-NI (△), SHIV-IFN-γ (▲) or mock (×). *PSL*: relative photon-stimulated luminescence

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

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

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

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

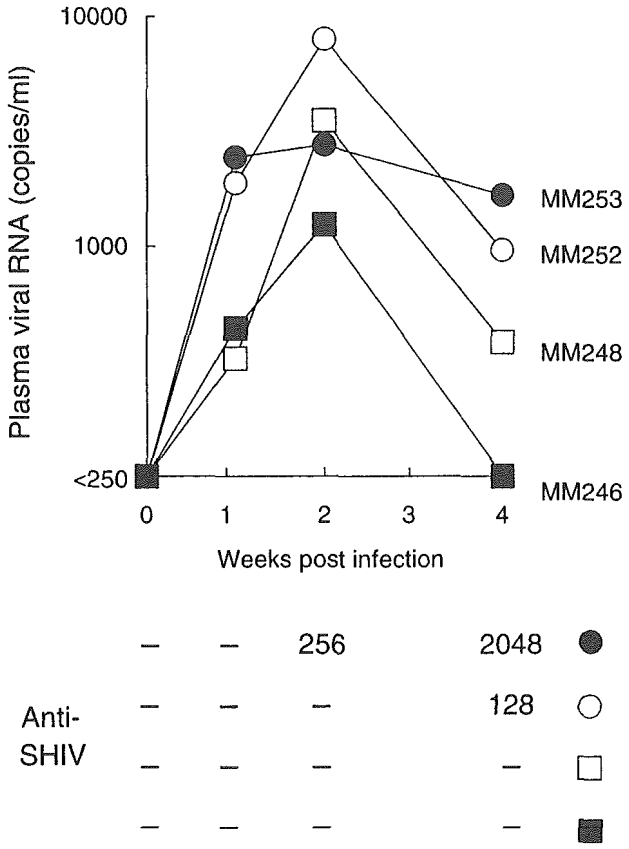

Fig. 3. *In vivo* SHIV vaccination. Changes in viral load (top) and antibody titer (bottom) in MM248 and MM252 after SHIV-NI vaccination, and in MM246 and MM253 after SHIV-IFN-γ vaccination. Antibody titers are expressed as the reciprocal to the maximum dilution in the particle agglutination test. –,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

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

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

Cytokine production in antigen-stimulated PBMC

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

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

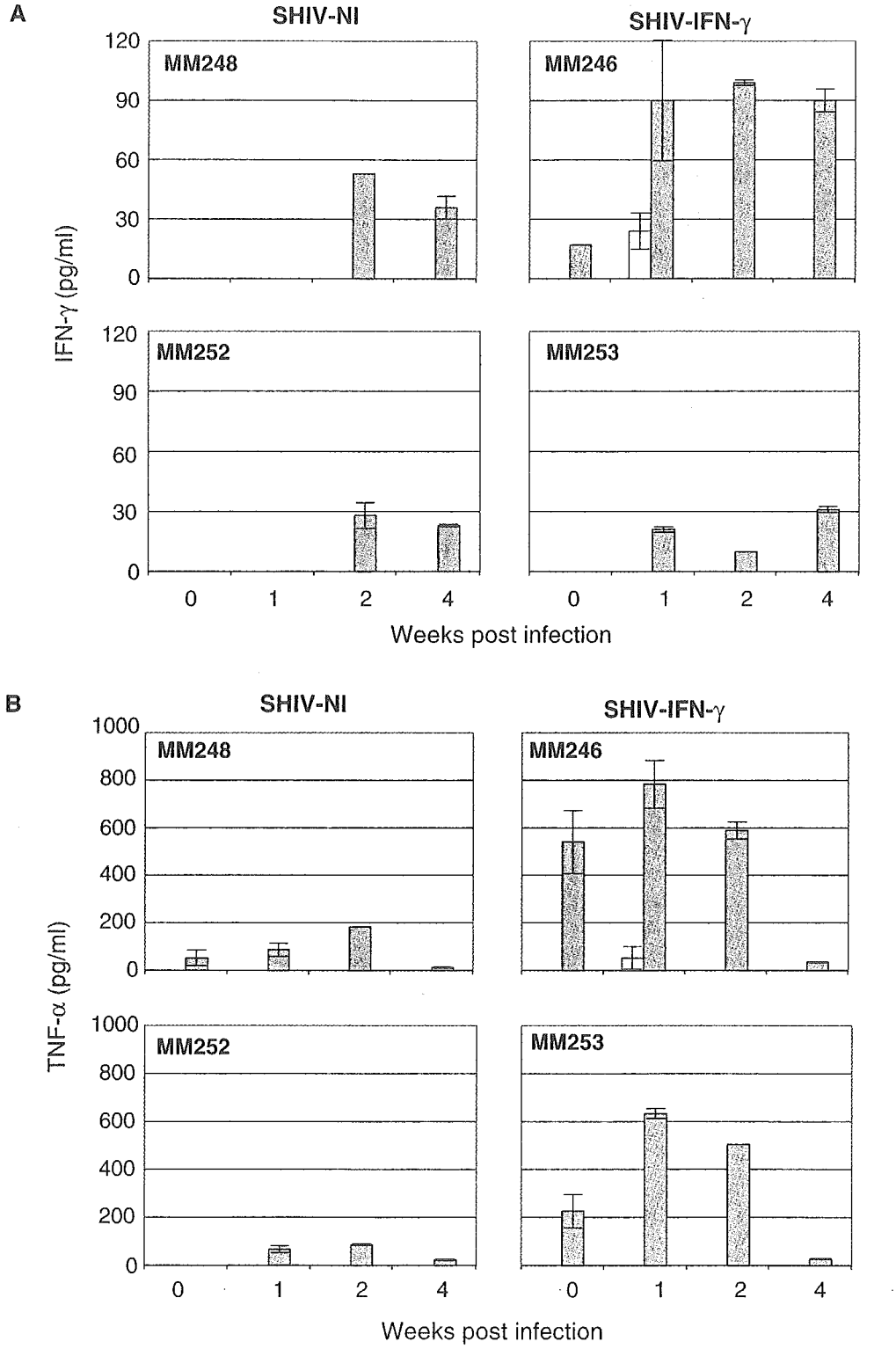

Fig. 4. Change in cytokine response of PBMC to viral antigen stimulation after SHIV vaccination. White columns indicate concentrations of IFN-γ (**A**) and TNF-α (**B**) in the culture supernatant of unstimulated cells. Shadowed columns indicate the corresponding concentrations in the supernatant of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

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

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

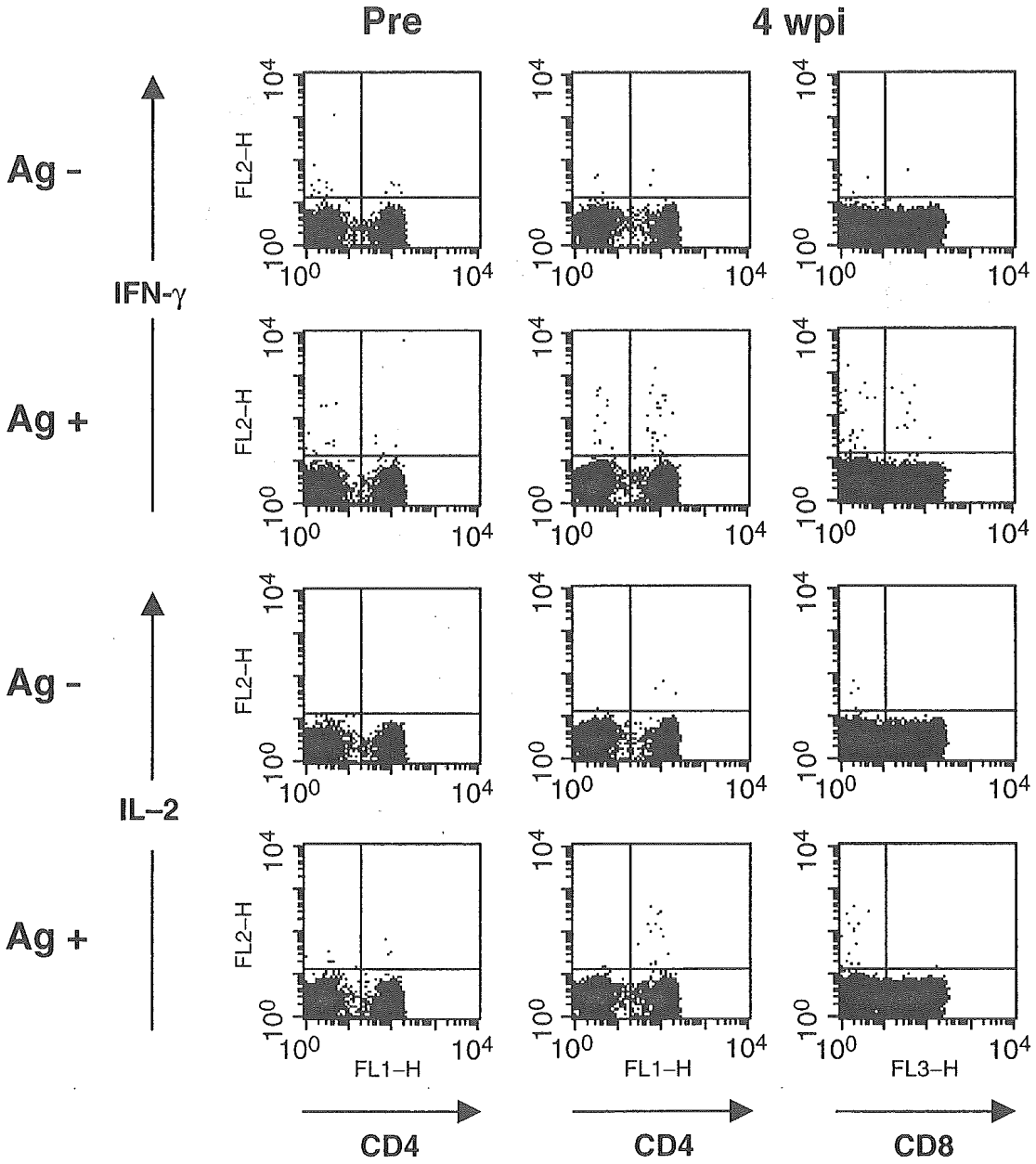

Fig. 5. Flow cytometry results showing intracellular cytokines in viral antigen-stimulated PBMC. Data show representative plots longitudinally obtained from MM246, which was infected with SHIV-IFN-γ. FL-1 represents CD4 or CD8 expression and FL-2 represents IFN-γ expression (top) or IL-2 expression (bottom).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

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

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

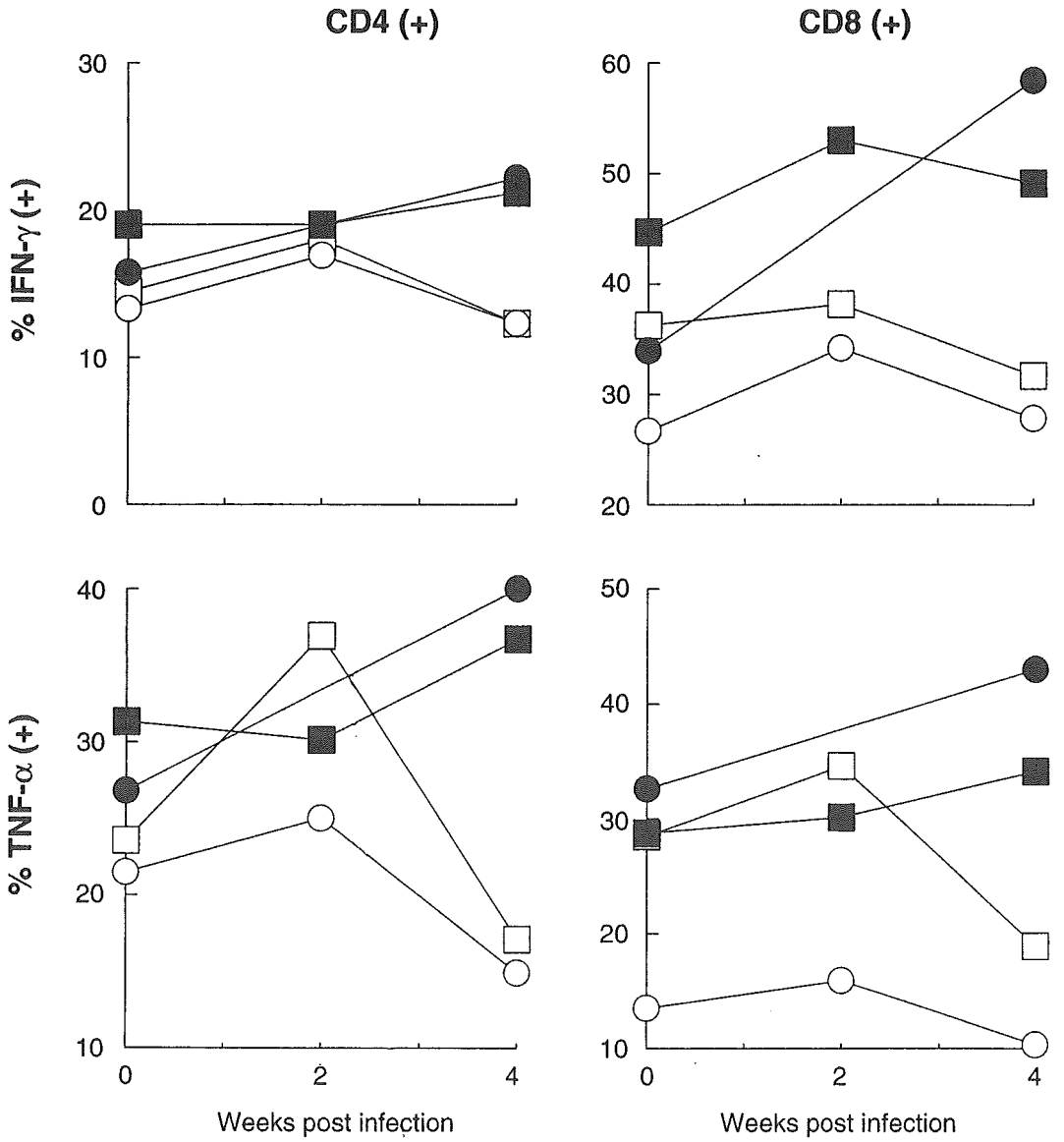

Fig. 6. Change in cytokine production in PMA/IM-stimulated PBMC after SHIV vaccination. Data show percentages of IFN-γ, IL-2, IL-4 and TNF-α producing cells in CD4+ (left) and CD8+ (right) population in PBMC from MM248 (□) and MM252 (○) infected with SHIV-NI, and MM246 (■) and MM253 (●) infected with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

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

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

Cytokine production in antigen-stimulated lymph nodes cells

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

Cytokine production in mitogen-stimulated PBMC

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

Discussion

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

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

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

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

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

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

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

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

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