fragments were suspended in RPMI 1640 medium (Gibco, Grand Island, NY, USA) containing 10% FCS, and after agitation for 30 minutes at RT the supernatants were removed. The fragments were resuspended in RPMI 1640 medium containing 10% FCS and 0.2 mg/ml of collagenase (type II; Sigma, St Louis, MO, USA) and agitated for 90 minutes at RT. The suspensions (containing LPL) were filtered through the glass wool columns, and cells were enriched by Percoll density gradient centrifugation as described above for IEL. The cells obtained from each organ were immediately used in the infectious plaque assay and flow cytometry analysis.

Quantification of plasma viral RNA

Viral RNA loads in plasma were determined by quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) [19]. Total RNA was prepared from plasma with a QIAamp Viral RNA kit (QIAGEN, Hilden, Germany). RT-PCR was performed with a Taqman EZ RT-PCR kit (Perkin Elmer, Wellesley, MA, USA) for the SIV gag region using the following primers: SIV2-696F (5′-GGA AAT TAC CCA GTA CAA CAA ATA GG-3′) and SIV2-784R (5′-TCT ATC AAT TTT ACC CAG GCA TTT A-3′). A labeled probe, SIV2-731T (5′-Fam-TGT CCA CCT GCC ATT AAG CCC G-Tamra-3′; Perkin Elmer), was used for detection of the PCR products. These reactions were performed with a Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA, USA) and analyzed using the manufacturer's software. For each run, a standard curve was generated from dilutions whose copy numbers were known, and the RNA in the plasma samples was quantified based on the standard curve.

Quantification of proviral DNA

The proviral DNA loads in tissues were determined by quantitative PCR. DNA samples were extracted directly from frozen tissues with a QIAGEN DNeasy Tissue kit (QIAGEN). PCR was performed with a Taqman PCR Reagent kit (Perkin Elmer) using the same primer set and probe that were used in RT-PCR. 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.

Infectious plaque assay

Infectious viruses were quantified and isolated using the infectious plaque assay [6, 11]. An agarose gel bilayer containing RPMI 1640 medium was made in plastic culture dishes of 100 mm diameter; the lower layer consisted of 12 ml of 1.2% agarose (Agarose NA; Pharmacia) and the upper layer consisted of 12 ml of 0.4% low-gelling-temperature agarose (SeaPlaque Agarose; FMC, Rockland, ME, USA). The dishes were incubated at 37°C in 5% CO_2 overnight. The following day, 2×10^6 cells of each sample and 8×10^6 cells of M8166 were suspended in 3 ml of a 0.4% low-gelling-temperature agarose solution containing the culture medium and the mixture was immediately overlaid on the agarose gel layer previously prepared. After the gel was hardened, the plates were covered with 12 ml of culture medium and incubated at 37°C in 5% CO_2 for 10 days. The medium over the plates was replaced with fresh medium every day. After removal of the medium in the plates at 10 days, the plates were stained with 2 ml of 0.7% MTT for 2 hour to count the number of plaques.

Flow cytometry

The frequency of CD4 single-positive and CD4CD8 double-positive T cells in PBMCs and the various tissues were examined by flow cytometry. Lymphocytes were treated with anti-CD3 (FN-18-FITC; Biosource, Camarillo, CA, USA), anti-CD4 (Nu-TH/I-PE; NICHIREI, Tokyo, Japan) and anti-CD8 (SK1; Becton Dickinson Biosciences) monoclonal antibody and examined on a FACScan analyzer (Becton Dickinson Biosciences). The absolute number of lymphocytes in the blood was determined by using an automated blood cell counter (F-820; Sysmex, Kobe, Japan).

Results

Intrarectal infection of newborn macaques with SHIV-C2/1 KS661c

Two newborn rhesus macaque monkeys were intrarectally inoculated with SHIV- C2/1 KS661c and were killed at 13 and 26 days after inoculation, respectively. The plasma viral RNA load was about $1–3 \times 10^8$ copies/ml at 13 or 14 days after inoculation, and then decreased slightly but remained at 5×10^6 copies/ml at 26 days after inoculation (Fig. 1). These levels were almost the same as the levels we measured in adult monkeys in our previous study [Miyake A, Ibuki K, Enose Y, Suzuki H, Horiuchi R, Suzuki M, Saito N, Nakasone T, Honda M, Watanabe T, Miura T, Hayami M, unpublished data]. In the adults, the plasma viral RNA load peaked at about 10^8 to 5×10^9 copies/ml

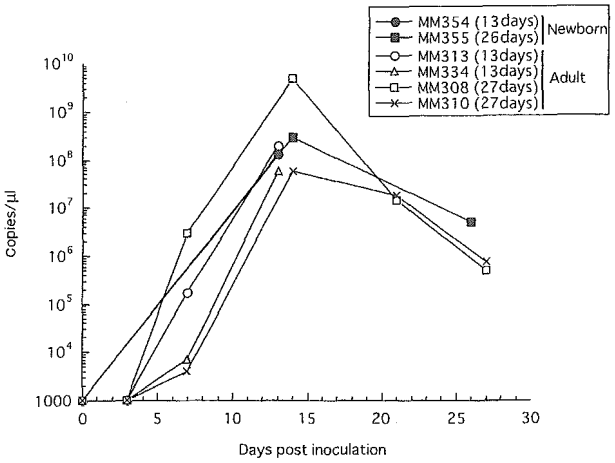

Fig. 1. Plasma viral RNA loads of two newborn and four adult monkeys intrarectally inoculated with SHIV-C2/1-KS661c as determined by quantitative RT-PCR. The detection limit of this assay was 1×10^3 copies/ml.

at 13 days after inoculation, and remained 5×10^5 to 10^6 copies/ml at 27 days after inoculation (Fig. 1). Before the inoculation, $CD4^+$ T cell counts in peripheral blood in the newborn monkeys (1700–2500 cells/μl) were higher than those in the adult monkeys (500–1200 cells/μl; Fig. 2). However, the $CD4^+$ T cell count in newborn monkeys dropped to <500 cells/μl by 13 or 14 days after inoculation, which is the same level as was observed in the adult monkeys, and remained at this low level until 26 days after inoculation.

Detection of proviral DNA in various tissues soon after intrarectal inoculation

To investigate viral distribution to the systemic tissues early after mucosal infection, proviral DNAs

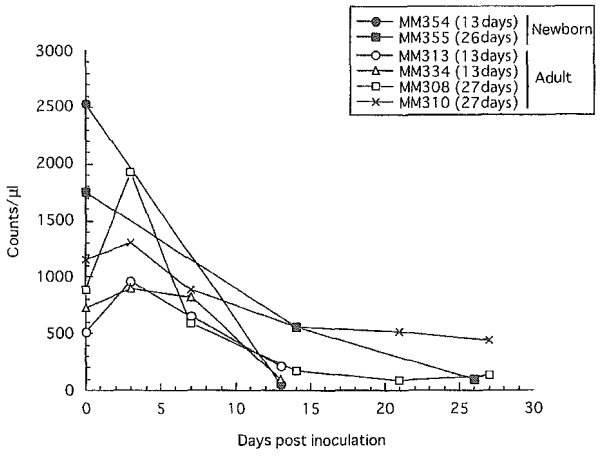

Fig. 2. Number of $CD4^+$ T cells in peripheral blood of two newborn and four adult monkeys inoculated with SHIV-C2/1-KS661c.

in various tissues were quantified by quantitative PCR. In the monkey that was examined at 13 days after inoculation (MM354), proviral DNA was detected in all the tissues examined at high levels (Fig. 3). The levels of proviral DNA in the PBMCs, spleen, lymph nodes and intestinal tract were each about 10^3 to 10^4 copies/μg. The level of proviral DNA in the thymus (10^5 copies/μg) was about 10 times higher than the levels in the other tissues examined. The levels of proviral DNA in the non-lymphoid tissues, including lung, liver, kidney and brain were about 20 to 2×10^3 copies/μg (data not shown). The titers of proviral DNA remained high in all the tissues of the monkey examined at 26 days after inoculation (MM355; Fig. 3). These results indicated that the viral loads in various tissues were amplified within 13 days after inoculation and that the virus stocks remained in each tissue until 26 days after inoculation. Similar results were obtained for the adult monkeys at 13 and 27 days after inoculation (MM313 and MM310; Fig. 3). High titers of proviral DNA were detected in all examined tissues of adult monkeys at both 13–27 days after inoculation.

Detection of infectious virus in various tissues early after intrarectal infection

Although proviral DNA was present in each tissue early after the mucosal infection, it was not clear whether these tissues released infectious viruses, which are considered to play a major role in the spread of virus in the body. To observe the release of infectious viruses from the various tissues, we quantified the infectious viruses in those tissues using the plaque assay. As shown by plaque assays, infectious viruses were more abundantly in the thymus than that in the other tissues (Fig. 4). At 13 days after inoculation, 170 plaque forming units (pfu)/10^6 cells were detected in the thymus. This high titer of infectious virus remained until 26 days after inoculation (220 pfu/10^6 cells). However, in the other lymphoid tissues examined, the titers at 13 and 26 days after inoculation were <63 and 55.5 pfu/10^6 cells, respectively. In contrast, the level of infectious viruses in the lymphoid tissues of the adult monkeys were high at 13 days after inoculation, and those infectious viruses decreased profoundly by 27 days after inoculation (Fig. 4). However, the level of infectious viruses in the intestinal tract (jejunum IEL and LPL) were low in both the newborn and adult monkeys. In the newborn monkeys, the titers of infectious viruses in the intestinal tracts at 13 and 26 days after inoculation were <7.5 pfu/10^6 cells and <2 pfu/10^6 cells, respectively (Fig. 4), while in the adult

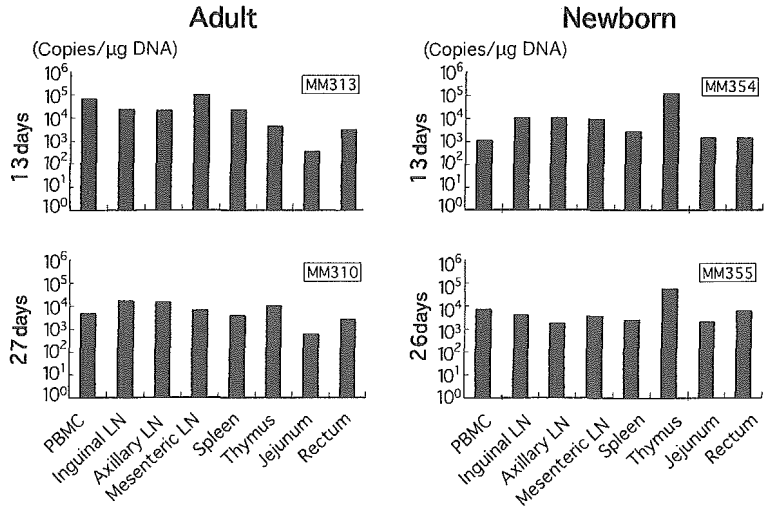

Fig. 3. Proviral DNA loads in various tissues of newborn and adult monkeys inoculated with SHIV-C2/1-KS661c. Viral loads were determined by quantitative PCR and are expressed as viral DNA copy numbers per microgram of total DNA extracted from tissue homogenates.

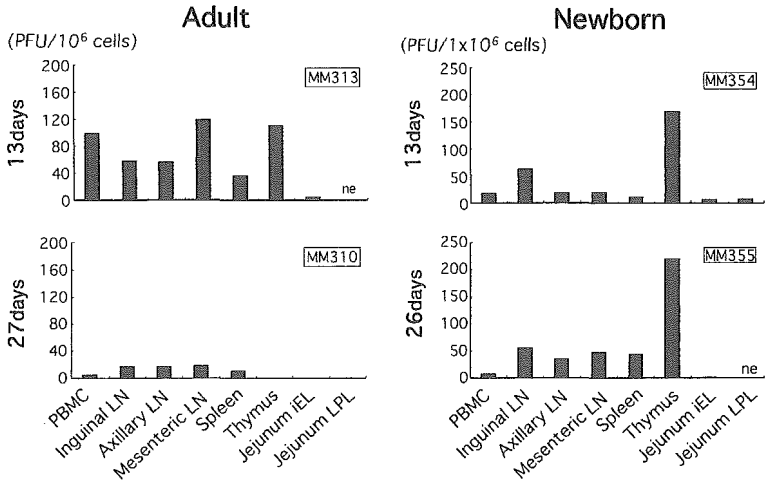

Fig. 4. Infectious virus loads in various tissues of newborn and adult monkeys inoculated with SHIV-C2/1-KS661c. Viral loads were determined by infectious plaque assay and are expressed as the number of plaque forming units (pfu) per 10^6 cells. 'ne' means the culture was not evaluated because of contamination.

monkeys, they were not detectable throughout the infection.

Sequential changes in the proportion of $CD4^+$ T cells in thymus

Infectious viruses were detected at extremely high levels in the thymus at 13 and 26 days after inoculation. The extent of virus replication in a tissue might be correlated with the proportion of $CD4^+$ T cells in the tissue, as $CD4^+$ T cells have been reported to be the main target and source for amplification of HIV, SIV and SHIV [1, 7, 14, 16]. Therefore, sequential changes in the proportion of $CD4^+$ T cells in the thymus were examined using flow cytometry. The thymus is a site of T cell development, in which T cells progress through four stages in the following order: CD3 negative ($CD3^-$) CD4 SP, $CD3^-$ CD4CD8 DP, CD3 positive ($CD3^+$) CD4CD8 DP, and $CD3^+$ CD4 SP. As shown by flow cytometry, the thymuses of both uninoculated adult and newborn monkeys had many more immature $CD3^-$ CD4CD8 DP T cells (white bars in Fig. 5A) and $CD3^+$ CD4CD8 DP T cells (light gray bars) than mature $CD3^+$ CD4 SP T cells (black bars; Fig. 5A). The percentage of $CD3^-$ CD4 SP T cells (hatched bars) were less in both uninoculated adults and newborns. In the adult monkeys, total $CD4^+$ T cells

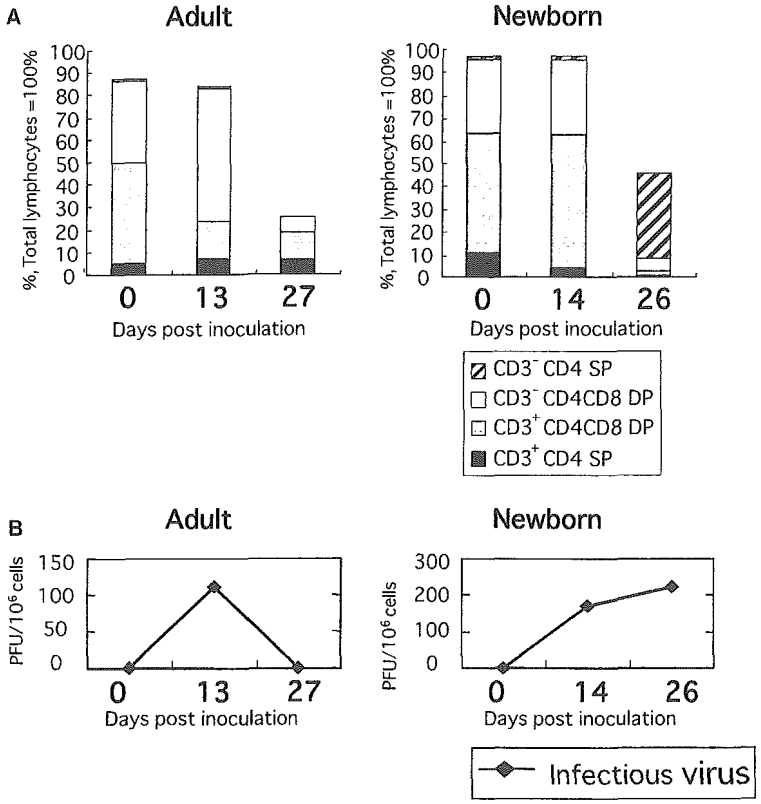

Fig. 5. (A) Changes in the proportions of $CD4^+$ T cells in the thymus of uninoculated and SHIV-inoculated monkeys. The percentage of $CD3^-$ CD4 single positive (SP), $CD3^-$ CD4CD8 double positive (DP), $CD3^+$ CD4CD8 DP, and $CD3^+$ CD4 SP cells in total lymphocytes was determined by flow cytometry. (B) Changes in the amount of infectious virus in the thymus of uninoculated and SHIV-inoculated monkeys.

(Fig. 5A) and the number of infectious viruses (Fig. 5B) decreased concurrently between 13 and 27 days after inoculation. In the newborn monkeys, mature $CD3^+$ CD4 SP T cells (black bars in right panel of Fig. 5A) and immature $CD3^-$ CD4CD8 DP and $CD3^+$ CD4CD8 DP T cells (white bars and light gray bars, respectively) also decreased between 13 and 26 days after inoculation. However, immature $CD3^-$ CD4 SP cells (hatched bars) were still present at 26 days after inoculation. The thymus of newborn monkeys also had high levels of infectious viruses at 26 days after inoculation (Fig. 5B, right panel). These results suggest that the immature $CD3^-$ CD4 SP T cells in the thymus of newborn monkeys were not destroyed by the virus infection and continued to produce infectious viruses.

Sequential changes in the proportion of $CD4^+$ T cells in various tissues

Sequential changes in the proportion of $CD4^+$ T cells were also examined in various tissues other than the thymus. The lymphoid tissues of the uninfected newborn had more $CD4^+$ T cells than those of adult monkeys (Fig. 6). In the newborn, these cells were already depleted at 13 days after inoculation. The decrease of $CD4^+$ T cells in the adult monkeys was more moderate and some of them remained even at 27 days after inoculation. On the contrary, the intestinal tracts of both the uninfected and virus-infected newborn monkeys did not have many $CD4^+$ T cells (Fig. 6). This suggests that the intestinal tracts of the newborn monkeys are not a major source for virus replication.

Discussion

Here, we show that, in newborn monkeys, the thymus is the main site of virus replication early after mucosal infection and that this replication was sustained by the immature $CD4^+$ T cells in the thymus. In the newborn monkey examined 13 days after intrarectal inoculation, the infectious viruses were detected more abundantly in the thymus than in the other tissues. Moreover, this high titer of infectious virus in the thymus was also present in the monkey examined 26 days after inoculation. These results were different from the results obtained with adult monkeys, which were analyzed

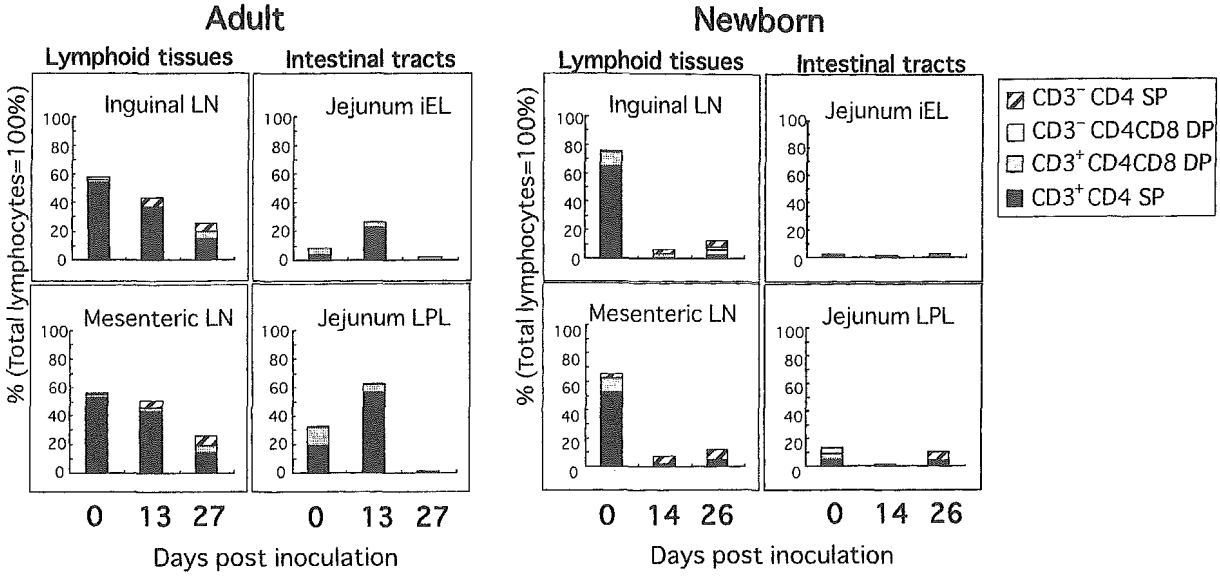

Fig. 6. Changes in the proportions of $CD4^+$ T cells in the lymphoid tissues and intestinal tracts of uninoculated and SHIV-inoculated monkeys. The percentage of $CD3^-$ CD4 single positive (SP), $CD3^-$ CD4CD8 double positive (DP), $CD3^+$ CD4CD8 DP, and $CD3^+$ CD4 SP cells in total lymphocytes was determined by flow cytometry.

in our previous research [Miyake A, Ibuki K, Enose Y, Suzuki H, Horiuchi R, Suzuki M, Saito N, Nakasone T, Honda M, Watanabe T, Miura T, Hayami M, unpublished data]. First, in the adult monkeys examined at 13 days after inoculation, the high levels of infectious virus were detected not only in the thymus but also in the other lymphoid tissues. These differences between adult and newborn monkeys might result from differences in the proportion of $CD4^+$ T cells in total lymphocytes of each tissue of newborn and adult monkeys. $CD4^+$ T cells have been reported as the main target and source for amplification of HIV, SIV and SHIV [1, 7, 14, 16]. Therefore, the percentage of remaining $CD4^+$ T cells might be correlate with the level of virus produced there, whereas the decrease of $CD4^+$ T cells might reflect the extent of virus replication. In the adult monkeys, the proportion of $CD4^+$ T cells in total lymphocytes of all the lymphoid tissues slightly decreased after virus infection but some of them remained until 13 days after inoculation. However, in the newborn monkey examined at 13 days after inoculation, the $CD4^+$ T cells in each of the lymphoid tissues except the thymus were already depleted. The rapid depletion of $CD4^+$ T cells in newborn monkeys might explain the rapid progression to disease in newborn monkeys. However, the reason for the depletion of $CD4^+$ T cells is not clear because there are no data for the first 12 days after inoculation. Moreover, the small proportion of $CD4^+$ T cells in the lymphoid tissues of newborn monkey at 13 days after inoculation might explain the low titers of infectious virus that were observed in these tissues. In contrast, the thymus of newborn monkeys, like the thymus of adult monkeys, had many $CD3^-$ CD4CD8 DP and $CD3^+$ CD4CD8 DP cells in addition to $CD3^+$ CD4 SP cells. In addition, the proportion of whole $CD4^+$ cells in the thymus of the newborn monkeys at 13 days after inoculation was similar to that in the uninfected newborn monkey. $CD3^-$ CD4CD8 DP and $CD3^+$ CD4CD8 DP cells have been proposed to represent the immature stages in T cell development, whereas $CD3^+$ CD4 SP cells are regarded as mature $CD4^+$ T cells [3, 9]. These immature T cells were also reported to be susceptible to HIV-1 [15, 18, 21]. Therefore, the number of $CD4^+$ T cells including immature cells in the thymus of newborn monkey at 13 days after inoculation appeared to be sufficient to account for the high titers of infectious virus found in the thymus.

Infectious viruses in the thymus of the newborn monkey examined at 26 days after inoculation were also at a high titer. However, the infectious viruses in the lymphoid tissues including thymus of the adult monkeys profoundly decreased by 27 days after inoculation. In the adults, the proportion of $CD4^+$ T cells also decreased significantly in these lymphoid tissues by 27 days after inoculation. The decrease occurred in not only mature $CD3^+$ CD4 SP T cells but also immature $CD3^-$ CD4CD8 DP and $CD3^+$ CD4CD8 DP T cells in the thymus of the adults. A similar decrease

was detected in the thymus of the newborn monkey examined at 26 days after inoculation. However, $CD3^-$ CD4 SP cells, which represent a more immature stage of T cell development than $CD3^-$ CD4CD8 DP and $CD3^+$ CD4CD8 DP T cells, were still present in the thymus of the newborn monkey examined at 26 days after inoculation. Like immature $CD3^-$ CD4CD8 DP and $CD3^+$ CD4CD8 DP T cells, immature $CD3^-$ CD4 SP T cells were reported to be susceptible to HIV-1 [18]. Therefore, the immature $CD3^-$ CD4 SP cells in the thymus of newborn monkeys did not appear to decrease in number by the virus infection even after the depletion of mature $CD4^+$ T cells and appeared to sustain production of infectious viruses.

In the adults, the proportion of immature CD4CD8 DP T cells decreased earlier than that of mature CD4 SP T cells in the thymus after virus infection [Miyake A, Ibuki K, Enose Y, Suzuki H, Horiuchi R, Suzuki M, Saito N, Nakasone T, Honda M, Watanabe T, Miura T, Hayami M, unpublished data]. However, a similar decrease was not detected in the newborns. Because the thymus of newborns is more active than it is in adults, and has a larger potential for *de novo* generation of T cells than that in adults, it may be able to more readily supply T cells to replace those lost by the virus infection. Moreover, because immature $CD4^+$ T cells are just as susceptible to virus infection as mature $CD4^+$ T cells, the thymus of newborns may produce the virus more abundantly than the thymus of adults. In our study, the thymus of newborn monkeys had a significantly larger number of infectious viruses than the thymus of adult monkeys. These infectious viruses might eventually cause the rapid progression to disease.

Acknowledgments

We thank James Raymond for editing this manuscript.

References

1. Dalgleish AG, Beverley PC, Clapham PR, Crawford DH, Greaves MF, Weiss RA: The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. Nature 312:763–767, 1984.
2. de Martino M, Tovo PA, Balducci M, Galli L, Gabiano C, Rezza G, Pezzotti P: Reduction in mortality with availability of antiretroviral therapy for children with perinatal HIV-1 infection. Italian Register for HIV Infection in Children and the Italian National AIDS Registry. JAMA 284:190–197, 2000.
3. Galy A, Verma S, Barcena A, Spits H: Precursors of CD3+CD4+CD8+ cells in the human thymus are defined by expression of CD34. Delineation of early events in human thymic development. J Exp Med 178:391–401, 1993.
4. Haynes BF, Denning SM, Le PT, Singer KH: Human intrathymic T cell differentiation. Semin Immunol 2:67–77, 1990.
5. Hirokawa K, Utsuyama M, Kasai M, Kurashima C: Aging and immunity. Acta Pathol Jpn 42:537–548, 1992.
6. Kato S, Hiraishi Y, Nishimura N, Sugita T, Tomihama M, Takano T: A plaque hybridization assay for quantifying and cloning infectious human immunodeficiency virus type 1 virions. J Virol Methods 72:1–7, 1998.
7. Klatzmann D, Champagne E, Chamaret S, Gruest J, Guetard D, Hercend T, Gluckman JC, Montagnier L: T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. Nature 312:767–768, 1984.
8. Kourtis AP, Nesheim SR, Thea D, Ibegbu C, Nahmias AJ, Lee FK: Correlation of virus load and soluble L-selectin, a marker of immune activation, in pediatric HIV-1 infection. AIDS 14:2429–2436, 2000.
9. Kraft DL, Weissman IL, Waller EK: Differentiation of CD3-4-8- human fetal thymocytes in vivo: characterization of a CD3-4+8- intermediate. J Exp Med 178:265–277, 1993.
10. Lundqvist C, Baranov V, Hammarstrom S, Athlin L, Hammarstrom ML: Intra-epithelial lymphocytes. Evidence for regional specialization and extrathymic T cell maturation in the human gut epithelium. Int Immunol 7:1473–1487, 1995.
11. Miyake A, Enose Y, Ohkura S, Suzuki H, Kuwata T, Shimada T, Kato S, Narayan O, Hayami M: The quantity and diversity of infectious viruses in various tissues of SHIV-infected monkeys at the early and AIDS stages. Arch Virol 149:943–955, 2004.
12. Pikora CA, Sullivan JL, Panicali D, Luzuriaga K: Early HIV-1 envelope-specific cytotoxic T lymphocyte responses in vertically infected infants. J Exp Med 185:1153–1161, 1997.
13. Ritter MA, Palmer DB: The human thymic microenvironment: new approaches to functional analysis. Semin Immunol 11:13–21, 1999.
14. Sattentau QJ, Clapham PR, Weiss RA, Beverley PC, Montagnier L, Alhalabi MF, Gluckmann JC, Klatzmann D: The human and simian immunodeficiency viruses HIV-1, HIV-2 and SIV interact with similar epitopes on their cellular receptor, the CD4 molecule. AIDS 2:101–105, 1988.
15. Schnittman SM, Denning SM, Greenhouse JJ, Justement JS, Baseler M, Kurtzberg J, Haynes BF, Fauci AS: Evidence for susceptibility of intrathymic T-cell precursors and their progeny carrying T-cell antigen receptor phenotypes TCR alpha beta + and TCR gamma delta + to human immunodeficiency virus infection: a mechanism for CD4+ (T4) lymphocyte depletion. Proc Natl Acad Sci U S A 87:7727–7731, 1990.
16. Shibata R, Kawamura M, Sakai H, Hayami M, Ishimoto A, Adachi A: Generation of a chimeric human and simian immunodeficiency virus infectious to monkey peripheral blood mononuclear cells. J Virol 65:3514–3520, 1991.
17. Shinohara K, Sakai K, Ando S, Ami Y, Yoshino N, Takahashi E, Someya K, Suzaki Y, Nakasone T, Sasaki Y, Kaizu M, Lu Y, Honda M: A highly pathogenic simian/human immunodeficiency virus with genetic changes in cynomolgus monkey. J Gen Virol 80:1231–1240, 1999.
18. Su L, Kaneshima H, Bonyhadi M, Salimi S, Kraft D, Rabin L, McCune JM: HIV-1-induced thymocyte depletion is associated with indirect cytopathogenicity and

infection of progenitor cells in vivo. Immunity 2:25–36, 1995.
19. Suryanarayana K, Wiltrout TA, Vasquez GM, Hirsch VM, Lifson JD: Plasma SIV RNA viral load determination by real-time quantification of product generation in reverse transcriptase-polymerase chain reaction. AIDS Res Hum Retroviruses 14:183–189, 1998.
20. Tovo PA, de Martino M, Gabiano C, Cappello N, D'Elia R, Loy A, Plebani A, Zuccotti GV, Dallacasa P, Ferraris G, Caselli D, Fundaro C, D'Argenio P, Galli L, Principi N, Stegagno M, Ruga E, Palomba E and the Italian Register for HIV Infection in Children. Prognostic factors and survival in children with perinatal HIV-1 infection. The Italian Register for HIV Infections in Children. Lancet 339:1249–1253, 1992.
21. Valentin H, Nugeyre MT, Vuillier F, Boumsell L, Schmid M, Barre-Sinoussi F, Pereira RA: Two subpopulations of human triple-negative thymic cells are susceptible to infection by human immunodeficiency virus type 1 in vitro. J Virol 68:3041–3050, 1994.
22. Veazey RS, Lifson JD, Pandrea I, Purcell J, Piatak M Jr, Lackner AA: Simian immunodeficiency virus infection in neonatal macaques. J Virol 77:8783–8792, 2003.
23. Weiblen BJ, Lee FK, Cooper ER, Landesman SH, McIntosh K, Harris JA, Nesheim S, Mendez H, Pelton SI, Nahmias AJ, *et al.*: Early diagnosis of HIV infection in infants by detection of IgA HIV antibodies. Lancet 335:988–990, 1990.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
Virology 343 (2005) 151 – 161
VIROLOGY
www.elsevier.com/locate/yviro

Induction of immune response in macaque monkeys infected with simian–human immunodeficiency virus having the TNF-α gene at an early stage of infection

Yuya Shimizu [a], Yasuyuki Miyazaki [b], Kentaro Ibuki [b], Hajime Suzuki [b], Kentaro Kaneyasu [b], Yoshitaka Goto [a], Masanori Hayami [b], Tomoyuki Miura [b], Takeshi Haga [a,*]

[a] *Department of Veterinary Microbiology, University of Miyazaki, Miyazaki 889-2192, Japan*
[b] *Laboratory of Primate Model, Experimental Research Center for Infectious Disease, Institute for Virus Research, Kyoto University, Kyoto 606-8507, Japan*

Received 23 April 2005; returned to author for revision 7 June 2005; accepted 18 August 2005
Available online 16 September 2005

Abstract

TNF-α has been implicated in the pathogenesis of, and the immune response against, HIV-1 infection. To clarify the roles of TNF-α against HIV-1-related virus infection in an SHIV-macaque model, we genetically engineered an SHIV to express the TNF-α gene (SHIV-TNF) and characterized the virus's properties in vivo. After the acute viremic stage, the plasma viral loads declined earlier in the SHIV-TNF-inoculated monkeys than in the parental SHIV (SHIV-NI)-inoculated monkeys. SHIV-TNF induced cell death in the lymph nodes without depletion of circulating $CD4^+$ T cells. SHIV-TNF provided some immunity in monkeys by increasing the production of the chemokine RANTES and by inducing an antigen-specific proliferation of lymphocytes. The monkeys immunized with SHIV-TNF were partly protected against a pathogenic SHIV (SHIV-C2/1) challenge. These findings suggest that TNF-α contributes to the induction of an effective immune response against HIV-1 rather than to the progression of disease at the early stage of infection.

Keywords: SHIV; TNF-α; Immune response; Cytokines; Proliferation; RANTES; HIV-1; SIV

Introduction

Cytokines mediate important immunoregulatory functions, and changes in their relative levels play key roles in the immune response against human immunodeficiency virus type-1 (HIV-1) infection and the progression of HIV-1 infection to clinical AIDS (Fauci, 1996; Matsuyama et al., 1991). Increased levels of proinflammatory cytokines, especially tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6), were detected in plasma and tissue of patient with AIDS (Fauci, 1993; Navikas et al., 1995). TNF-α has been strongly implicated in the progression of HIV-1 infection (Aukrust et al., 1994). A possible mechanism for the progression of HIV-1 infection is that TNF-α enhances HIV-1 replication (Folks et al., 1989; Poli et al., 1990). Another possibility is that TNF-α induces apoptosis of lymphocytes (Zheng et al., 1995), which destroys T cells in the peripheral lymphoid systems (Badley et al., 1997; Herbein et al., 1998).

On the other hand, TNF-α is also involved in controlling the immune responses in some viral infections (Herbein and O'Brien, 2000). Pretreatment of TNF-α inhibited replication of the monocyte-tropic strain of HIV-1 (Herbein and Gordon, 1997). Thus, as a pleiotropic cytokine, TNF-α might contribute not only to the pathogenesis of HIV-1 infection but also to the immune response against HIV-1 infection. Despite the importance of TNF-α, little information is available on its role in the pathogenesis of HIV-1 infection in vivo. The early events of infection are especially unclear because it is difficult to monitor humans immediately after they are infected.

Chimeric simian and human immunodeficiency virus (SHIV) clones containing the HIV-1 *tat*, *rev*, *vpu*, and *env* genes on a simian immunodeficiency virus (SIV) strain mac239 background readily infected several susceptible macaques. Inoculation of macaque monkeys with SHIV is an

* Corresponding author. Fax: +81 985 58 7575.
E-mail address: a0d518u@cc.miyazaki-u.ac.jp (T. Haga).

0042-6822/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2005.08.025

important tool for studying the early stage of an HIV-1-like infection, for elucidating the etiology of AIDS, and for evaluating the protective effects of vaccine candidates in vivo. We previously reported the in vivo properties of one of the SHIVs, SHIV-NM3rN (Kuwata et al., 1995). To assess the role of cytokines in the pathogenesis of HIV-1-related virus infection, the SHIV-*nef* vector having unique restriction enzyme sites (designated as SHIV-NI) was constructed from SHIV-NM3rN. Gene-deleted SHIVs can serve as vectors that express cytokine genes in infected macaque monkeys (Enose et al., 2004; Kozyrev et al., 2002). SHIV vectors containing a cytokine gene appear to be appropriate tools for observing the role of local production of a cytokine on virus replication, pathogenesis, and immunogenicity, especially because the inserted cytokine gene is expressed in the region where the SHIV vector replicates.

To study the role of TNF-α against HIV-1-related virus infection in an SHIV-macaque model, we constructed an SHIV in which the *nef* gene is replaced with a human TNF-α coding sequence (SHIV-TNF). In a previous in vitro study (Haga et al., 2002), we showed that SHIV-TNF (i) expressed a significant amount of biologically active TNF-α, (ii) replicated better than the parental SHIV-NI in monkey peripheral blood mononuclear cells (PBMCs), and (iii) induced a more severe cell death in monkey PBMCs than did the parental SHIV-NI.

In this study, we demonstrate that inoculation of SHIV-TNF in macaque monkeys induced cell death in peripheral lymph nodes without a depletion of circulating $CD4^+$ T cells. After the acute viremic stage, the plasma viral load declined earlier in the SHIV-TNF-inoculated monkeys than in the parental SHIV-NI-inoculated monkeys. SHIV-TNF was found to produce some immunity in macaque monkeys, leading to the elimination of SHIV. Based on these results, we propose that TNF-α contributes to the induction of an effective immune response rather than to the progression of disease at the early stage of HIV-1 infection.

Results

Virus load in rhesus macaques with SHIV-NI and SHIV-TNF

A recombinant SHIV was engineered to express TNF-α (SHIV-TNF) in place of *nef* in SHIV-NI (Fig. 1A). To investigate the in vivo properties of an SHIV-TNF, three rhesus monkeys (MM343, MM350, and MM351) were intravenously inoculated with 10^5 tissue culture 50% infectious dose ($TCID_{50}$) of SHIV-TNF, and two animals (MM346 and MM349) were inoculated with 10^5 $TCID_{50}$ of SHIV-NI as a control. All monkeys were viremic within 2 weeks post-inoculation (WPI) (Fig. 2A). This was also the case for two monkeys (MM287 and MM288) that were inoculated with same dose of SHIV-NI in a previous report (Enose et al., 2004). The plasma viral RNA loads were about the same in all SHIV-TNF- and SHIV-NI-inoculated monkeys, reaching a peak value of 10^4 RNA copies/ml during 1 to 2 WPI. Up-regulation of the plasma viral load in the SHIV-TNF-inoculated monkeys was not more than that in the SHIV-NI-inoculated monkeys.

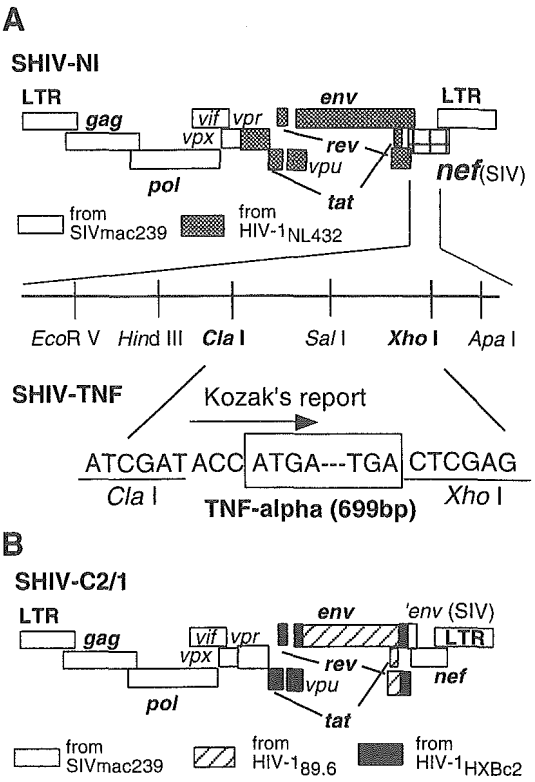

Fig. 1. Genetic structures of SHIVs used in this study. (A) Parental SHIV-NI and SHIV-TNF. SHIV-NI has some unique restriction enzyme sites in place of the *nef* gene of SHIV-NM3rN. SHIV-NM3rN was constructed from HIV-1 NL432 (dot regions) and SIV mac239 (white regions). In the SHIV-TNF, the *Cla*I and *Xho*I region of the parental SHIV-NI was replaced by the human TNF-α gene. The open reading frame including the initiation (ATG) and stop (TGA) codons of the TNF-α gene is shown in the box. The flanking sequence of the TNF-α initiation codon (ACCATGA) is an effective ribosomal initiation sequence based on Kozak's report (effective ribosomal initiation sequence: ANNATGN or GNNATGR). (B) Challenge virus SHIV-C2/1. SHIV-C2/1 (GenBank accession number AF217181) was generated by in vivo passage of SHIV-89.6 through cynomolgus monkeys. SHIV-89.6 was constructed from HIV-1 HXBc2, HIV-1 89.6, and SIV mac239.

However, the kinetics of the viral loads was different between the two groups. At the post-acute phase, the plasma viral loads declined earlier in the SHIV-TNF group (becoming undetectable at about 4 WPI) than in the SHIV-NI group (becoming undetectable at 6–8 WPI). After the acute viremic stage, the plasma virus load declined to undetectable levels earlier in the SHIV-TNF group than in the SHIV-NI group ($P < 0.05$ at 3 WPI).

Proviral DNAs in PBMCs from macaques infected with SHIV-TNF were isolated, and the stability of the inserted TNF-α gene in SHIV-TNF was studied by PCR with primer pairs that recognized the site where the TNF-α gene had been inserted. Due to the limited viral replication, proviral DNAs in PBMCs of the SHIV-TNF inoculated monkeys were detected up to 3 or 4 WPI. The full-length of the inserted TNF-α was observed in the proviral DNAs (Fig. 2B).

Lymphocyte phenotyping of PBMC and lymph nodes

After the infection, the number of peripheral $CD4^+$ lymphocytes remained within the normal level in both the

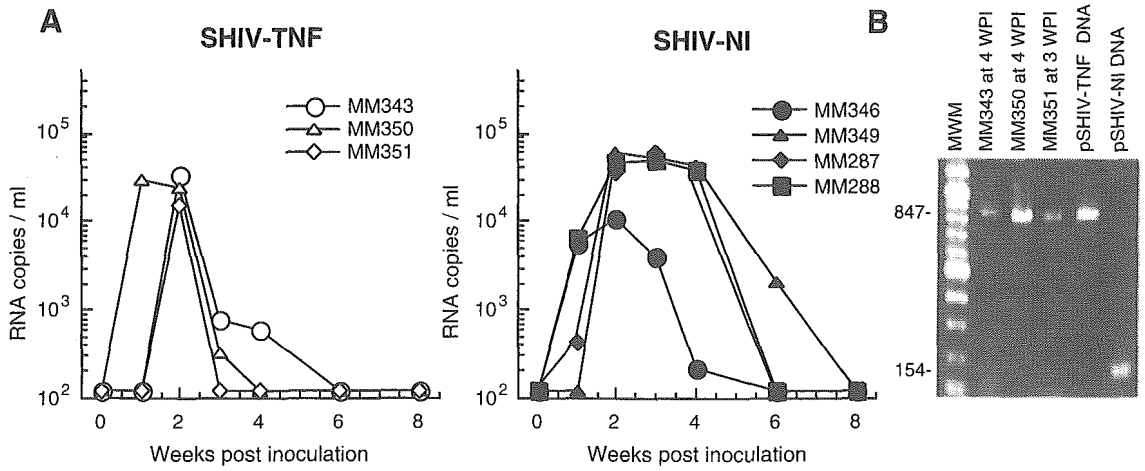

Fig. 2. Viral loads of SHIVs and TNF-α gene stability. (A) Viral RNA loads in plasma of monkeys inoculated with SHIV-TNF and SHIV-NI. Data for MM287 and MM288 are from a previous report (Enose et al., 2004). Plasma viral RNA loads were measured by RT-PCR. The detection limit of this assay was 1.2×10^2 copies/ml. (B) Stability of the inserted TNF-α gene in SHIV-TNF, as shown by PCR. DNA plasmids of SHIV-TNF (pSHIV-TNF DNA) and SHIV-NI (pSHIV-NI DNA) were used as the templates of the control. MWM, molecular weight markers of 100-bp ladder.

SHIV-NI- and SHIV-TNF-inoculated monkeys (Fig. 3A). No clinical changes were observed in any of the monkeys during the observation period. These results showed that SHIV-TNF did not cause an AIDS-like disease. No significant differences were observed between the SHIV-NI- and SHIV-TNF-inoculated monkeys in the cell surface markers of PBMCs. In contrast, the percentage of $CD20^+$ B cells in the lymph nodes changed dramatically in the SHIV-TNF-inoculated macaques after the inoculation of virus (Fig. 3B). After the inoculation of SHIV-TNF, development of germinal centers in the lymph nodes was observed with hematoxylin and eosin staining (data not shown). These data suggest that the inoculation of SHIV-TNF induced the activation of $CD20^+$ B cells in the lymph nodes at the early stage of the infection, without disturbing the immunological status.

Plasma levels of TNF-α and RANTES

We next examined the plasma levels of some cytokines by enzyme-linked immunosorbent assay (ELISA). The levels of TNF-α in the plasma of macaques infected with SHIV-TNF and SHIV-NI were below the detection limit (15.6 pg/ml) of the ELISA at all time points (data not shown).

TNF-α inhibited HIV-1 replication by inducing the production of CC-chemokines such as regulated-on-activation-normal-T-cell-expressed-and-secreted (RANTES) (Lane et al., 1999). To determine whether SHIV-TNF induces the expression of RANTES, plasma levels of RANTES were investigated (Fig. 3C). Interestingly, the plasma RANTES levels in the SHIV-TNF-infected monkeys were higher (about 3 times higher) than those in SHIV-NI-infected monkeys at 2 WPI, when the plasma viral load was about the same in the two groups. Plasma RANTES levels in the parental SHIV-NM3rN-inoculated monkeys (Kwofie et al., 2000) and two other SHIV-NI-inoculated monkeys (2.7 ng/ml for MM287 and 2.0 ng/ml for MM288, respectively, at 2 WPI) from our previous study were similar to those in the SHIV-NI-infected monkeys. The difference of the plasma RANTES levels between the SHIV-TNF-infected monkeys and the SHIV-NI-infected monkeys is statistically significant ($P < 0.05$ at 2 WPI). After 8 WPI, the plasma level of RANTES declined to the pre-infection level in all SHIV-TNF-infected monkeys.

Antibody titer

The humoral immune response was assessed with an HIV-1 particle agglutination assay. Induction of anti-HIV-1 antibodies was first detected in two of three SHIV-TNF-infected monkeys (MM343 and MM350) at 3 WPI (Fig. 3D). The antibody titers for the other SHIV-TNF-inoculated monkey (MM351), SHIV-NI-inoculated monkey, and the two SHIV-NI-infected monkeys in our previous experiment (MM287 and MM288) (data not shown) all slightly increased at 4 WPI. Although the data were not statistically significant ($P = 0.08$ at 3 WPI), the results showed that some SHIV-TNF-infected monkeys immediately developed an antiviral antibody response during the early stage of infection.

Induction of cell death in lymph nodes of SHIV-TNF-inoculated monkeys

TNF-α induces apoptosis of lymphocytes (Zheng et al., 1995). Spontaneous- or activation-induced ex vivo apoptosis of lymphocytes was reported in infections of HIV-1-related viruses (Gougeon et al., 1996; Iida et al., 2000; Reinberger et al., 1999). Thus, we investigated the induction of cell death in biopsy samples from lymph nodes by Annexin V assay and terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay. In two of the SHIV-TNF-inoculated monkeys (MM343 and MM351), the percentages of lymphocytes undergoing cell death increased 24.2% and 17.5%, respectively, at 2 WPI (Fig. 4A). In the SHIV-NI-inoculated monkeys

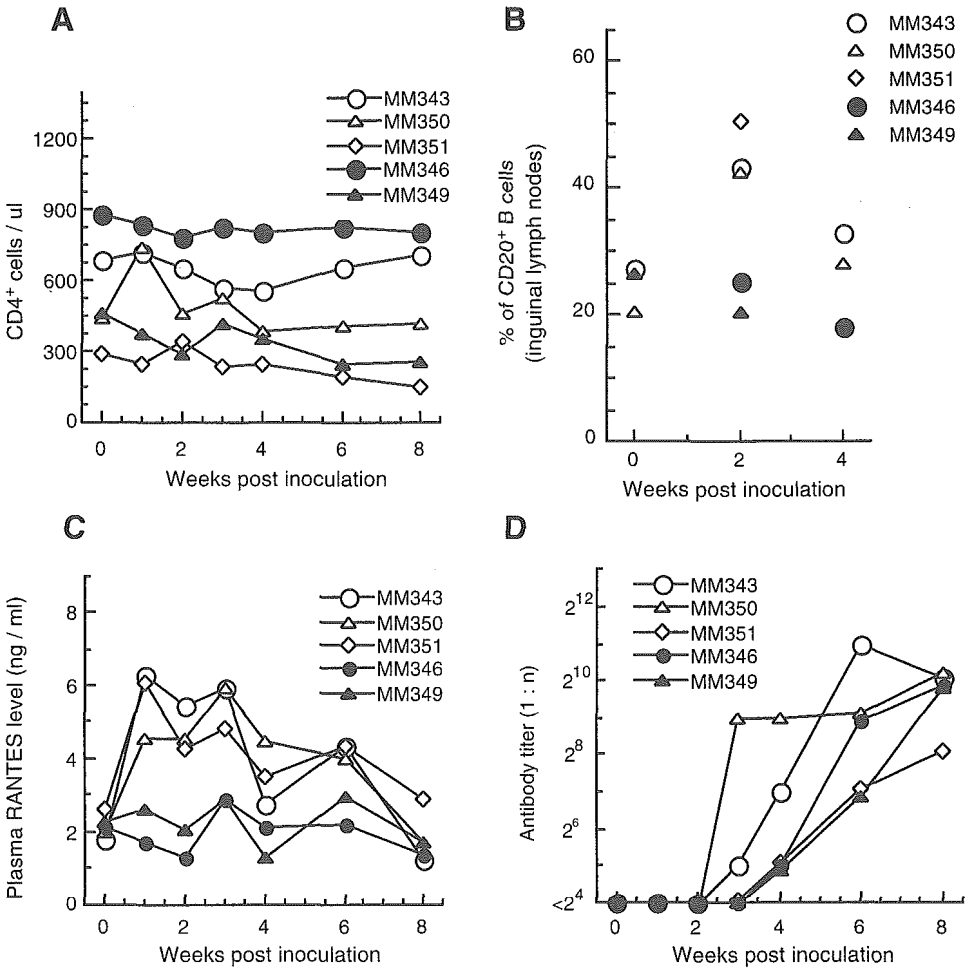

Fig. 3. Change in immune responses in rhesus monkeys after inoculation with SHIV-TNF (open symbols) and SHIV-NI (closed symbols). (A) $CD4^+$ T lymphocyte counts. Values were calculated from the percentage $CD3^+CD4^+$ cells measured by flow cytometry. The absolute number of lymphocytes was obtained by automated blood cell counter. (B) Percentage of $CD20^+$ B cells ($CD3^-CD20^+$ cells) in the inguinal lymph nodes. Data were not obtained for all monkeys at each time. (C) Plasma RANTES levels, as measured by ELISA. (D) Humoral immune responses, as expressed by anti-HIV-1 antibody titer. Antibody titer was analyzed by particle agglutination assay and is expressed as the maximum dilution of plasma to give a positive result.

(MM346 and MM349), the percentages of Annexin-V-positive lymphocytes after the virus inoculation remained at 4.1% and 7.5%, respectively, which were the pre-inoculation levels. The percentage of Annexin-V-positive cells in the lymph nodes was markedly higher in the SHIV-TNF-inoculated macaques than in the SHIV-NI-inoculated macaques. Induction of cell death in the SHIV-TNF group was also observed by the TUNEL assay (Fig. 4B). These results show that induction of cell death in inguinal lymph nodes in the SHIV-TNF group was significantly higher than that in SHIV-NI group, suggesting that TNF-α expressed by the SHIV-TNF-infected cells induced apoptosis in lymph nodes in the virus-inoculated monkeys.

Induction of antigen-specific lymphocyte proliferative responses in SHIV-TNF-inoculated macaques

To determine whether inoculation of the SHIV-TNF elicited antigen-specific lymphocytes in the animals, proliferative responses to SIV Gag were measured with PBMCs by uptake of 5-bromo-2′-deoxyuridine (BrdU). During the experimental period, antigen-specific T cell proliferation was induced especially in the SHIV-TNF-inoculated monkeys (Fig. 5A). Proliferative responses to Gag in the SHIV-TNF group were observed as early as 2 WPI. Subsets of proliferative lymphocytes were also characterized by combination of anti-BrdU and surface marker staining. In the SHIV-TNF-inoculated monkeys, the antigen-specific proliferative response was observed both in CD4-positive and in CD8-positive cells (Fig. 5B). These results show that antigen-specific T cell proliferation was induced markedly in the SHIV-TNF group at the early stage of infection.

The proliferative responses to a mitogen (Concanavalin A; ConA) were low or absent in the SHIV-NI-inoculated monkeys at 3 and 4 WPI, and then the response to ConA recovered to the normal level afterward (Fig. 5C). In contrast, the SHIV-TNF-infected macaques showed a continuous response to ConA.

Challenge with a pathogenic SHIV

To evaluate the protective effects provided by SHIV-TNF, monkeys were challenged intravenously with 10^5 $TCID_{50}$ of heterologous pathogenic SHIV-C2/1 KS661 (Fig. 1B) at 8 WPI.

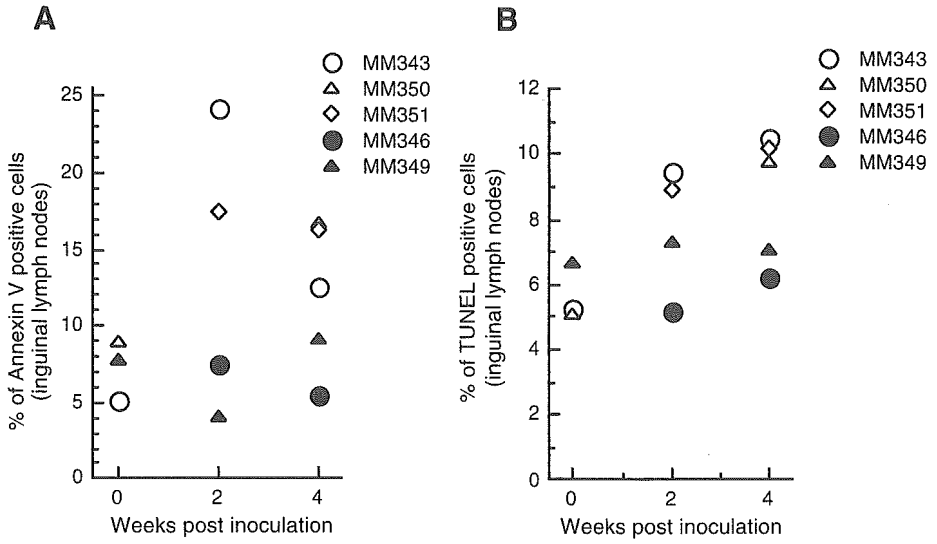

Fig. 4. Induction of cell death in inguinal lymph node mononuclear cells following infection with SHIV-TNF (open symbols) and SHIV-NI (closed symbols). Cells were collected at the indicated times and incubated for 24 h before analyses. (A) Percentage of Annexin-V-positive cells. (B) Percentage of fragmentation of chromosomal DNA, measured by flow cytometry with TUNEL assay. Data were not obtained for all monkeys at each time.

As controls, four naive monkeys (MM298, MM299, MM338, and MM339) were infected intravenously with the same dose (10^5 $TCID_{50}$) of SHIV-C2/1 KS661. Circulating $CD4^+$ lymphocytes decreased in all of the control monkeys, reaching less than 50 cells/μl in accordance with a previous report (Shinohara et al., 1999) (Fig. 6A). The viral RNA in the plasma of the control monkeys increased to above 10^7 copies/ml at 1 WPI and remained at a high level (Fig. 6B).

All of the monkeys in the SHIV-TNF group were more resistant to the challenge than the control monkeys. The circulating $CD4^+$ T cell counts were maintained in all SHIV-TNF-infected monkeys (Fig. 6A). The peak plasma virus loads in the SHIV-TNF group (10^5 and 10^6 copies/ml) were two orders of magnitude lower than those of the naive SHIV-C2/1 KS661-inoculated monkeys (Fig. 6B). Interestingly, the plasma virus loads in two of the three SHIV-TNF-inoculated monkeys (MM343 and MM351) rapidly declined to below the detection limit at 4 weeks post-challenge (WPC). The peak plasma viral load of one of the SHIV-NI-infected monkeys (MM349; 10^7 copies/ml) was similar to that of the control monkeys. The circulating $CD4^+$ T cell count in MM349, an SHIV-NI-infected monkey, remained within the normal range in spite of a transient but intense viremia in the plasma (Fig. 6A). These results show that all SHIV-TNF-inoculated monkeys were partially protected from challenge, with a pathogenic SHIV having a heterologous HIV-1 Env. Moreover, some SHIV-TNF-infected monkeys may have immediately eliminated the heterologous challenge virus in the plasma.

Discussion

The aim of this study was to clarify the role of TNF-α against HIV-1-related virus infection in an SHIV-macaque model. For this purpose, we have genetically engineered an SHIV to express the TNF-α gene so that, when viral transcription occurs, TNF-α will also be produced locally. We compared the in vivo properties of macaques infected with SHIV-TNF with those of its parental SHIV-NI. Although the number of animals examined was small, some virologic and immunologic parameters in macaques infected with SHIV-TNF and with SHIV-NI were different between the groups.

In a previous in vitro study, SHIV-TNF was found to replicate better than SHIV-NI does in macaque PBMCs (Haga et al., 2002). In vivo, however, the plasma viral loads were about the same in the two groups during acute infection. The plasma viral loads in the SHIV-TNF-inoculated monkeys dramatically declined to undetectable levels after the acute phase of the infection, while the viral loads of the parental SHIV-NI-inoculated monkey declined gradually. The spread of the virus was suppressed in the animals infected with SHIV-TNF. Suppression of the virus may have been due to cell deaths in the lymph nodes from the SHIV-TNF-inoculated monkeys. An efficient induction of cell death, through the TNF/TNF-receptor (TNFR) pathway, could restrict virus replication by eliminating virus-infected cells (Herbein and O'Brien, 2000). HIV-1-infected lymphocytes treated with TNF-α underwent apoptosis resulting in a decreased HIV-1 replication, suggesting that cell death helps to inhibit the spread of HIV-1 (Lazdins et al., 1997). Insertion of the TNF-α gene in the SHIV induced a rapid cell death at the site where virus replicates, with a subsequent reduction of the spread of the virus.

A cytokine-expressing SIV was observed to lose the cytokine gene within several weeks in vivo (Gundlach et al., 1997). In our study, the proviral DNA isolated from PBMCs of macaques inoculated with SHIV-TNF during the viremia retained full-length TNF-α inserts. Due to the limited and local nature of viral replication, we could not detect TNF-α in the plasma of animals inoculated with SHIV-TNF. However, the TNF-α gene inserted in SHIV-TNF was stable in vivo during the viremia.

In vitro studies have suggested that pretreatment of macrophages with TNF-α helps to protect them against HIV-

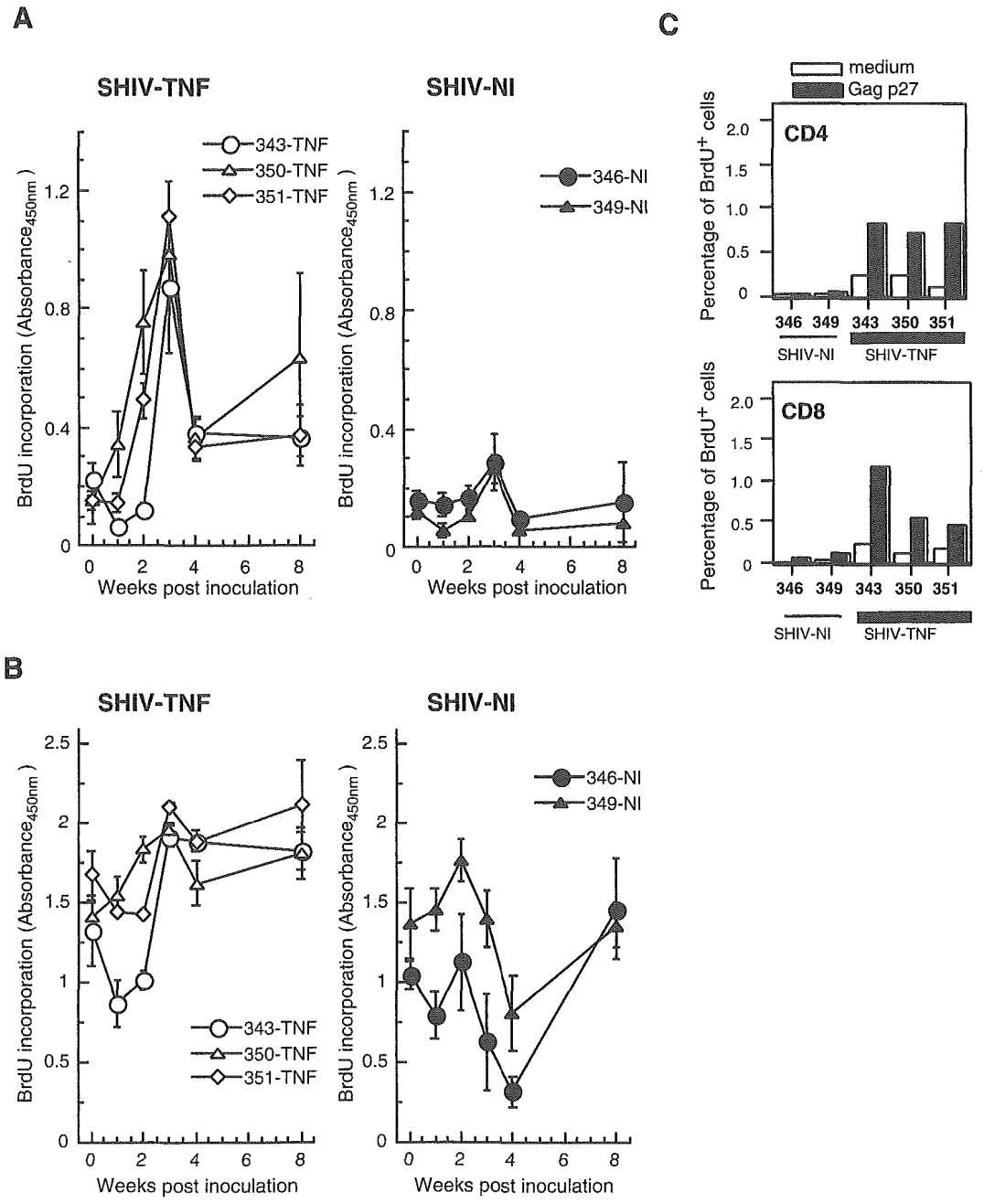

Fig. 5. T cell proliferation in SHIV-TNF- and SHIV-NI-inoculated rhesus monkeys in response to an SHIV-specific antigen (SIV Gag; A and C) or a mitogen (ConA; B). (A, B) Lymphocytes proliferation, as measured by BrdU uptake, in response to SIV Gag (A) and ConA (B). Data are expressed as the mean of triplicate determinations ± SEM. (C) Percentage of the SIV Gag-specific proliferated cells (solid bars) and unstimulated cells (open bars) at 6 WPI that were $CD4^+$ or $CD8^+$ cells, as measured by flow cytometry.

1. This is largely because TNF-α induces the secretion of CC-chemokines such as macrophage inflammatory protein-1 alpha (MIP-1α), beta (MIP-1β), and RANTES (Herbein and Gordon, 1997; Lane et al., 1999). In this experiment, an increase in RANTES production was observed in the SHIV-TNF-inoculated monkeys. At the peak, the plasma level of RANTES was three times higher in the SHIV-TNF-infected monkeys than in the SHIV-NI-infected monkeys. RANTES is known to suppress a CC-chemokine receptor 5 (R5)-tropic HIV-1 in vitro (Alkhatib et al., 1996; Cocchi et al., 1995), but not a CXCR4 (X4)-tropic virus including HIV-1 NL432, the source of the *env* gene of SHIV-TNF. Therefore, the high RANTES production probably did not limit the replication of SHIV-TNF in this study. Many cohort studies have suggested that an increase of RANTES inhibits the development of AIDS (Aukrust et al., 1998; Garzino-Demo et al., 1999). Furthermore, RANTES regulates cell migration to the site of inflammation. The appropriate expression of RANTES can improve the host immune response against HIV-1. Together, our result suggests that TNF-α helps to induce an effective immune response against HIV-1 by mediating the expression of the CC-chemokine RANTES.

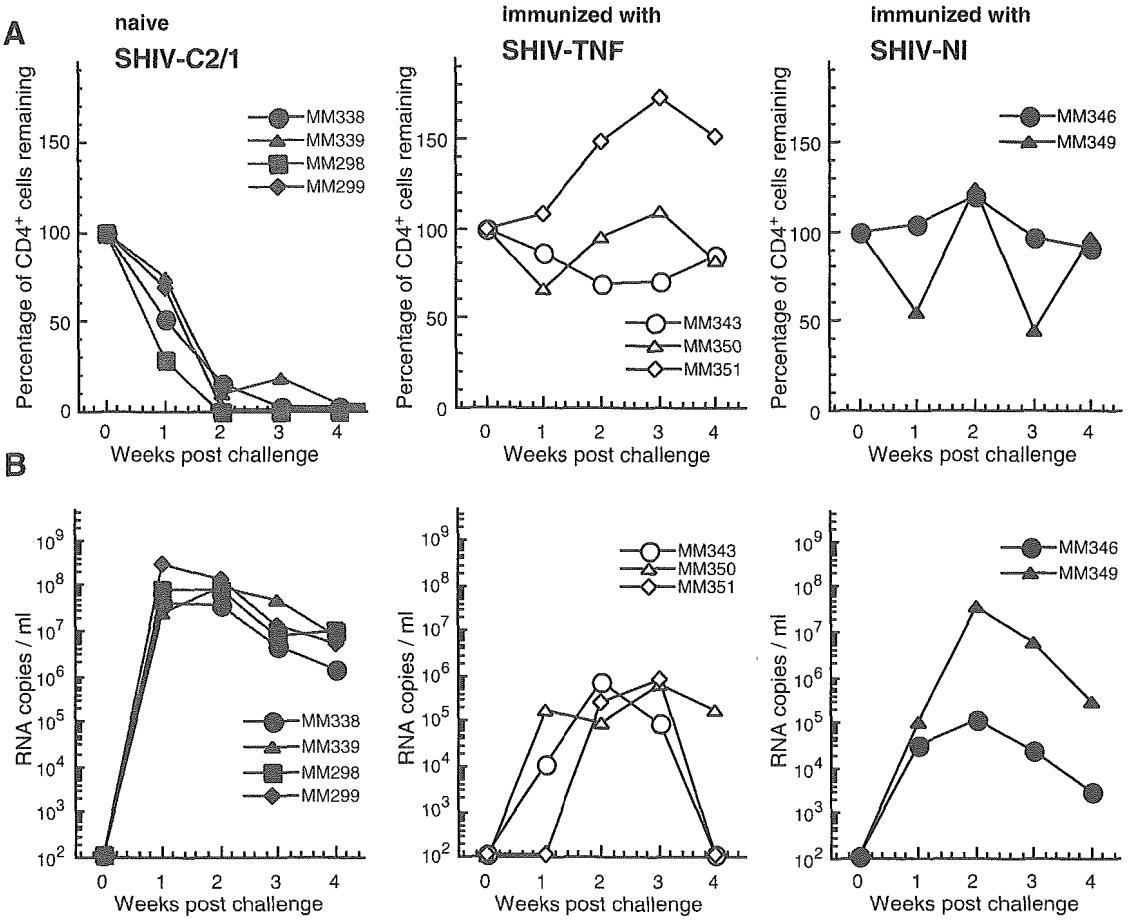

Fig. 6. Change in the number of circulating $CD4^+$ T cells (A) and SHIV-C2/1-specific viral RNA loads (B) of the SHIV-NI- and SHIV-TNF-inoculated monkeys after SHIV-C2/1 challenge. Values are expressed as a percentage of the pre-challenge values of each monkey. Plasma viral RNA loads after the heterologous pathogenic SHIV (SHIV-C2/1) challenge were measured by RT-PCR. The detection limit of this assay was 1.2×10^2 copies/ml. Left panels show results for naive SHIV-C2/1-inoculated monkeys (MM338, MM339, MM298, and MM299).

Another finding of the present study is that the inoculation of SHIV-TNF induced a remarkable antigen-specific T cell proliferation at the early stage of infection. In human, TNF-α enhances mitogen- and antigen-induced T cell proliferation (Tartaglia et al., 1993; Yokota et al., 1988). TNF-α produced by HIV-1-infected macrophages has been reported to stimulate bystander T cell proliferation (Godard and Chermann, 1998). Our results suggest that the co-expression of viral antigen and TNF-α stimulates antigen-specific T cell proliferation. Antigen-specific T cell proliferation is important for the control of AIDS pathogenesis. Proliferation of HIV-1 p24-specific $CD4^+$ T cells, which has been associated with control of acute infection and delayed disease progression, has been shown to inhibit the replication of HIV-1 (Rosenberg et al., 1997). Therefore, TNF-α appears to induce effective antigen-specific immune responses of lymphocytes, which may help to prevent the progression of disease.

The proliferative responses to a mitogen (ConA) were low or absent in parental SHIV-NI-inoculated monkeys during the period from 3 WPI to 4 WPI. Mitogen-stimulated proliferation of lymphocytes was also found to be inhibited during the acute stage of HIV-1 infection (Cooper et al., 1988; Pedersen et al., 1990). A transient impairment of proliferative response to mitogens has been correlated with host immune suppression. A decrease in the level of TNF-α in a virus infection was found to suppress mitogen- and antigen-stimulated lymphocyte proliferation (Griffin et al., 1994), and mitogen- and antigen-stimulated lymphocyte proliferation was restored by addition of exogenous TNF-α (Kasahara et al., 2003). In the present study, the SHIV-TNF-infected macaques showed a continuous proliferative response to ConA during the observation period. These results suggest that co-expression of TNF-α by a genetically engineered virus sustains the virus-induced immune suppression.

Most studies of the relationship between TNF-α and HIV-1 infection have focused on patients at the AIDS stage. TNF-α has been implicated in the progression of AIDS because of the possibilities that TNF-α enhances HIV-1 replication (Folks et al., 1989; Poli et al., 1990) and induces apoptosis of lymphocytes (Badley et al., 1997; Herbein et al., 1998). In this study, SHIV-TNF replicated transiently in inoculated monkeys, and the number of peripheral $CD4^+$ lymphocytes remained within the normal level in all SHIV-TNF-inoculated monkeys, despite the induction of cell death in inguinal lymph nodes. Although we cannot exclude the possibility that TNF-α contributes to disease progression in the late stage of

infections, the SHIV-TNF-inoculated macaques did not develop AIDS-like disease during the experimental period.

At the early stage of HIV-1 infection, expression of TNF-α also increased in some individuals (von Sydow et al., 1991), which was considered to contribute to the inflammatory response seen in acute HIV-1 infection. The present results demonstrate that SHIV-TNF induced the expression of RANTES, antigen-specific T cell proliferation, and rapid cell death with a subsequent reduction of plasma viral loads at the early stage of infection. The appearance of TNF-α activates the virus-specific immune response, which contributes to viral clearance. Therefore, at the early stage of HIV-1 infection, we propose that TNF-α plays a role in the induction of an effective immune response, rather than in the progression of the disease.

The immune responses induced by an attenuated virus can increase the immunity to a pathogenic virus. We previously reported that macaque monkeys immunized with the parental SHIV-NI were partially protected from a challenge with a heterologous pathogenic SHIV (Enose et al., 2002; Ui et al., 1999). In the present study, the monkeys that received the inoculation of SHIV-TNF were partially protected from a challenge with a heterologous pathogenic SHIV-C2/1. The immune response induced by the inoculation of SHIV-TNF helped to suppress the spread of a heterologous pathogenic SHIV. It is noteworthy that the challenge virus load rapidly declined to below the detection limit in some SHIV-TNF-inoculated monkeys.

In general, the immunogenicity of live-attenuated vaccines tends to increase with increasing virulence (Johnson and Desrosiers, 1998). Therefore, in attenuating a live virus, there is a trade-off between safety and immunogenicity. A good way to overcome this problem is to genetically engineer a virus to co-express an anti-viral agent such as a cytokine adjuvant. The expression of an inserted cytokine gene from a genetically engineered vaccine virus provides adjuvant effects locally at the site of virus replication. Several studies have demonstrated that insertion of a cytokine in a gene-deleted live-attenuated SIVs could boost the immunogenicity of the viruses and enhance its protection ability (Giavedoni et al., 1997; Stahl-Hennig et al., 2003). For the immune adjuvant effect of TNF-α, a combination of cytokines including TNF-α enhanced cellular immune responses resulting in a more effective immune-modulating effect against HIV-1 in the rodent model (Ahlers et al., 1997, 2003). In this study, SHIV-TNF induced antiviral immune responses in the virus-inoculated monkeys that subsequently rapidly cleared the virus. The rapid viral clearance and enhanced immunogenicity may improve the safety of a live-attenuated virus vaccine candidate because these responses will reduce the risks of residual pathogenicity, reversion to a pathogenic virus, and recombination of the vaccine virus with the challenge virus (Baba et al., 1999; Gundlach et al., 2000). These results raise the possibility that a genetically engineered anti-viral agent, such as TNF-α, can improve the safety and immunogenicity of an attenuated live virus vaccine.

In conclusion, we showed that SHIV-TNF induces (i) rapid clearance of virus after the acute viremic stage, (ii) cell death in peripheral lymph nodes without depletion of circulating $CD4^+$ T cells, (iii) up-regulation of the plasma level of the chemokine RANTES, (iv) antigen-specific proliferation of lymphocytes, and (v) protection against a pathogenic SHIV-C2/1 challenge. These findings suggest that TNF-α contributes to the induction of an effective immune response at the early stage of HIV-1 infection and that a genetically engineered virus to co-express the TNF-α gene can be useful for studying the immune-modulating effect of TNF-α in HIV-1-related infections.

Materials and methods

Viruses and animals

SHIV-NI and SHIV-TNF were constructed from SHIV-NM3rN as previously described (Fig. 1A) (Haga et al., 2002). A heterologous pathogenic SHIV-C2/1 KS661 was used for the challenge virus (Fig. 1B). SHIV-C2/1 KS661 is a molecular clone derived from SHIV-C2/1 and causes rapid $CD4^+$ T cell depletion (GenBank accession number AF217181) (Shinohara et al., 1999). The *env* gene of SHIV-C2/1 was derived from HIV-1 89.6 and is antigenically different from the *env* gene of SHIV-NI and SHIV-TNF. Virus stocks were prepared with the $CD4^+$ human T cell line M8166 (a subclone of C8166) as described elsewhere (Haga et al., 2002). Five female rhesus monkeys (*Macaca mulatta*) were maintained in accordance with regulations approved by the Institutional Animal Care and Use Committee of the Institute for Virus Research, Kyoto University, and were housed in single cages within a biosafety level 3 facility. Blood samples were collected under anesthesia with ketamine hydrochloride.

Inoculation of rhesus monkeys with SHIV and sample collection

To investigate the in vivo properties of SHIV-TNF and SHIV-NI, three rhesus monkeys (MM343, MM350, and MM351) were inoculated intravenously with 10^5 $TCID_{50}$ of SHIV-TNF, and two monkeys (MM346 and MM349) were inoculated with 10^5 $TCID_{50}$ of SHIV-NI. Blood samples were phenotypically characterized on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) and were separated into plasma and PBMCs with lymphocyte separation solution (Nacalai Tesque, Inc. Kyoto, Japan). Inguinal lymph nodes were obtained from these animals by biopsy at 2 and 4 WPI and before infection. Single-cell suspensions were prepared from the inguinal lymph nodes with a 100 μm nylon cell strainer (Becton Dickinson). Part of the inguinal lymph nodes was fixed in 4% paraformaldehyde and embedded in paraffin for histopathologic analysis. To evaluate the protective effects provided by the SHIV-TNF or SHIV-NI inoculation, all monkeys were challenged intravenously with 10^5 $TCID_{50}$ of SHIV-C2/1 KS661 at 8 WPI.

Plasma viral RNA loads

Plasma viral RNA loads were determined by quantitative RT-PCR (Enose et al., 2002; Suryanarayana et al., 1998). The

plasma viral loads of SHIV-NI, SHIV-TNF, and the challenge virus were differentially evaluated with primer pairs specific to SHIV-NM3rN and SHIV-C2/1, respectively (Enose et al., 2004). These reactions were performed with a Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA) and analyzed using the manufacturer's software. The viral RNA in the plasma samples was quantified based on the copy number of the standard samples.

Isolation of proviral DNA

Proviral DNA was extracted from 1×10^6 PBMCs and amplified by PCR with primers specific for the *nef* region. Cellular DNAs were extracted from PBMCs of the inoculated monkeys using DNeasy tissue kits (QIAGEN, Hilden, Germany) according to the manufacturer's recommendations. The proviral DNA fragments covering the inserted TNF-α gene in SHIV-TNF were amplified from each cellular DNA by PCR as previously described (Haga et al., 2000). The lengths of DNA fragments generated by this reaction were 847 bp for the SHIV containing the intact TNF-α gene and 154 bp for SHIV-NI.

Determination of TNF-α and RANTES

Plasma samples were thawed and centrifuged at $10,000 \times g$ for 10 min at 4 °C. TNF-α levels in the plasma were determined by ELISA using a Quantikine Human TNF-α ELISA kit (R&D Systems, Inc., Minneapolis, MN) and a Monkey TNF-α ELISA kit (Biosource International, Inc., Camarillo, CA). Plasma RANTES concentration was measured using a human RANTES ELISA kit (R&D Systems), which is known to cross-react with rhesus RANTES (Kwofie et al., 2000).

Lymphocyte analysis

Blood samples and inguinal lymph node suspensions were stained with combinations of the following monoclonal antibodies (MAbs): anti-human CD4 (NU-TH/I, Nichirei, Tokyo, Japan), anti-human CD8 (Leu-2a, Becton Dickinson), anti-monkey CD3 (FN18, Biosource International), anti-human CD20 (Leu-16, Becton Dickinson), anti-human CD29 (4B4; Beckman Coulter, Miami, FL), anti-human CD14 (RMO52; Beckman Coulter), anti-human CCR5 (3A9; Pharmingen, San Diego, CA), anti-human CXCR4 (12G5; Pharmingen), and anti-Ki67 antigen (Ki-67, DAKO, Glostrup, Denmark). Ten thousand events per sample were acquired by a FACScan flow cytometer, and data were analyzed by CellQuest software (Becton Dickinson) and FlowJo software (TreeStar, San Carlos, CA). Absolute lymphocyte counts in the blood were determined with an automated blood cell counter (F-820; Sysmex, Kobe, Japan).

Analysis of cell death

The percentages of lymphocytes undergoing cell death were determined by Annexin V assay and TUNEL assay. Mononuclear cells (1×10^6) from the blood and lymph nodes were cultured for 24 h ex vivo in 24-well culture plates in complete RPMI medium (RPMI 1640 with 10% heat-inactivated fetal calf serum) per milliliter at 37 °C. In the Annexin V assay, a MEBCYTO apoptosis kit (MBL, Nagoya, Japan) was used according to the manufacturer's instructions. As another assay of cell death, fragmentation of chromosomal DNA was detected with a TUNEL assay. An APO-BrdU kit (Pharmingen) was used according to the manufacturer's instructions. The cells were then analyzed by flow cytometry.

Proliferation assays

Lymphocyte proliferation was measured by incorporation of BrdU into the stimulated lymphocytes. PBMCs (2×10^5) were cultured in a 96-well plate in complete RPMI medium. The recombinant viral proteins of SIV Gag (SIVmac251 p27; 5.0 μg/ml; Advanced Biotechnologies, Inc., Columbia, MD) were used for antigen-specific stimulation. Concanavalin A (ConA; 0.5 μg/ml; Sigma, St. Louis, MO) was used for polyclonal stimulation. The plates were incubated for 72 h at 37 °C. After the incubation, the cells were cultured for another 24 h in the presence of BrdU. Then, lymphocyte proliferation was measured using a cell proliferation ELISA kit (Roche Diagnostics, Basel, Switzerland) and a BrdU-Flow kit (Pharmingen) following the manufacturer's recommendations. In the ELISA, the absorbance of the incorporated BrdU was measured by a microplate reader at 450 nm. The experiment was performed in triplicate. In another cell proliferation assay, to characterize the lymphocyte subsets in the proliferated cells, cells were stained for surface markers with MAb CD4-PE (Nichirei) and CD8-PerCP (Becton Dickinson). The cells were stained with MAb BrdU-FITC after fixation and permeabilization and then analyzed by flow cytometry. Proliferation responses to the antigens were considered positive if the stimulation index (SI = titers for antigen-stimulated wells / titers for control wells without antigens) exceeded 2.0 (Ahmed et al., 2002).

Determination of anti-SHIV antibody titers

Anti-SHIV antibody titers in the plasma of the monkeys were determined using a commercial particle agglutination test kit (Genedia HIV-1/2, Fujirebio Inc., Tokyo, Japan). The samples were serially two-fold diluted and assayed following the manufacturer's recommendations. The end-point titer was determined as the highest dilution to give a positive result.

Statistical analysis

Differences between the groups of plasma viral RNA loads, plasma RANTES level, and anti-SHIV antibody titers in the plasma were compared by statistical analysis using the Mann–Whitney U test; P values <0.05 were considered statistically significant.

Acknowledgments

We thank Drs. H. Kato and H. Nagatomo for their valuable comments and suggestions, T. Kazama and K. Tamaru for technical assistance with the analyses, Drs. K. Sakai, K. Shinohara, and E. Takahashi for kindly providing SHIV-C2/1 KS661, and Dr. J. Raymond for editing the manuscript. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education and Science, Japan, a Health Sciences Research Grant from the Ministry of Health, Labour and Welfare, Japan, and a Research Grant on Health Sciences focusing on Drug innovation from the Japan Health Science Foundation.

References

Ahlers, J.D., Dunlop, N., Alling, D.W., Nara, P.L., Berzofsky, J.A., 1997. Cytokine-in-adjuvant steering of the immune response phenotype to HIV-1 vaccine constructs: granulocyte–macrophage colony-stimulating factor and TNF-alpha synergize with IL-12 to enhance induction of cytotoxic T lymphocytes. J. Immunol. 158, 3947–3958.

Ahlers, J.D., Belyakov, I.M., Berzofsky, J.A., 2003. Cytokine, chemokine, and costimulatory molecule modulation to enhance efficacy of HIV vaccines. Curr. Mol. Med. 3, 285–301.

Ahmed, R.K., Makitalo, B., Karlen, K., Nilsson, C., Biberfeld, G., Thorstensson, R., 2002. Spontaneous production of RANTES and antigen-specific IFN-gamma production in macaques vaccinated with SHIV-4 correlates with protection against SIVsm challenge. Clin. Exp. Immunol. 129, 11–18.

Alkhatib, G., Combadiere, C., Broder, C.C., Feng, Y., Kennedy, P.E., Murphy, P.M., Berger, E.A., 1996. CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1. Science 272, 1955–1958.

Aukrust, P., Liabakk, N.B., Muller, F., Lien, E., Espevik, T., Froland, S.S., 1994. Serum levels of tumor necrosis factor-alpha (TNF alpha) and soluble TNF receptors in human immunodeficiency virus type 1 infection—Correlations to clinical, immunologic, and virologic parameters. J. Infect. Dis. 169, 420–424.

Aukrust, P., Muller, F., Froland, S.S., 1998. Circulating levels of RANTES in human immunodeficiency virus type 1 infection: effect of potent antiretroviral therapy. J. Infect. Dis. 177, 1091–1096.

Baba, T.W., Liska, V., Khimani, A.H., Ray, N.B., Dailey, P.J., Penninck, D., Bronson, R., Greene, M.F., McClure, H.M., Martin, L.N., Ruprecht, R.M., 1999. Live attenuated, multiply deleted simian immunodeficiency virus causes AIDS in infant and adult macaques. Nat. Med. 5, 194–203.

Badley, A.D., Dockrell, D., Simpson, M., Schut, R., Lynch, D.H., Leibson, P., Paya, C.V., 1997. Macrophage-dependent apoptosis of $CD4^+$ T lymphocytes from HIV-infected individuals is mediated by FasL and tumor necrosis factor. J. Exp. Med. 185, 55–64.

Cocchi, F., DeVico, A.L., Garzino-Demo, A., Arya, S.K., Gallo, R.C., Lusso, P., 1995. Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by $CD8^+$ T cells. Science 270, 1811–1815.

Cooper, D.A., Tindall, B., Wilson, E.J., Imrie, A.A., Penny, R., 1988. Characterization of T lymphocyte responses during primary infection with human immunodeficiency virus. J. Infect. Dis. 157, 889–896.

Enose, Y., Ui, M., Miyake, A., Suzuki, H., Uesaka, H., Kuwata, T., Kunisawa, J., Kiyono, H., Takahashi, H., Miura, T., Hayami, M., 2002. Protection by intranasal immunization of a nef-deleted, nonpathogenic SHIV against intravaginal challenge with a heterologous pathogenic SHIV. Virology 298, 306–316.

Enose, Y., Kita, M., Yamamoto, T., Suzuki, H., Miyake, A., Horiuchi, R., Ibuki, K., Kaneyasu, K., Kuwata, T., Takahashi, E., Sakai, K., Shinohara, K., Miura, T., Hayami, M., 2004. Protective effects of nef-deleted SHIV or that having IFN-gamma against disease induced with a pathogenic virus early after vaccination. Arch. Virol. 149, 1705–1720.

Fauci, A.S., 1993. Multifactorial nature of human immunodeficiency virus disease: implications for therapy. Science 262, 1011–1018.

Fauci, A.S., 1996. Host factors and the pathogenesis of HIV-induced disease. Nature 384, 529–534.

Folks, T.M., Clouse, K.A., Justement, J., Rabson, A., Duh, E., Kehrl, J.H., Fauci, A.S., 1989. Tumor necrosis factor alpha induces expression of human immunodeficiency virus in a chronically infected T-cell clone. Proc. Natl. Acad. Sci. U.S.A. 86, 2365–2368.

Garzino-Demo, A., Moss, R.B., Margolick, J.B., Cleghorn, F., Sill, A., Blattner, W.A., Cocchi, F., Carlo, D.J., DeVico, A.L., Gallo, R.C., 1999. Spontaneous and antigen-induced production of HIV-inhibitory beta-chemokines are associated with AIDS-free status. Proc. Natl. Acad. Sci. U.S.A. 96, 11986–11991.

Giavedoni, L., Ahmad, S., Jones, L., Yilma, T., 1997. Expression of gamma interferon by simian immunodeficiency virus increases attenuation and reduces postchallenge virus load in vaccinated rhesus macaques. J. Virol. 71, 866–872.

Godard, C.M., Chermann, J.C., 1998. Stimulation of bystander T-cell proliferation by tumor necrosis factor produced by HIV-1-infected macrophages. J. Hum. Virol. 1, 257–266.

Gougeon, M.L., Lecoeur, H., Dulioust, A., Enouf, M.G., Crouvoiser, M., Goujard, C., Debord, T., Montagnier, L., 1996. Programmed cell death in peripheral lymphocytes from HIV-infected persons: increased susceptibility to apoptosis of CD4 and CD8 T cells correlates with lymphocyte activation and with disease progression. J. Immunol. 156, 3509–3520.

Griffin, D.E., Ward, B.J., Esolen, L.M., 1994. Pathogenesis of measles virus infection: an hypothesis for altered immune responses. J. Infect. Dis. 170, S24–S31.

Gundlach, B.R., Linhart, H., Dittmer, U., Sopper, S., Reiprich, S., Fuchs, D., Fleckenstein, B., Hunsmann, G., Stahl-Hennig, C., Uberla, K., 1997. Construction, replication, and immunogenic properties of a simian immunodeficiency virus expressing interleukin-2. J. Virol. 71, 2225–2232.

Gundlach, B.R., Lewis, M.G., Sopper, S., Schnell, T., Sodroski, J., Stahl-Hennig, C., Uberla, K., 2000. Evidence for recombination of live, attenuated immunodeficiency virus vaccine with challenge virus to a more virulent strain. J. Virol. 74, 3537–3542.

Haga, T., Kuwata, T., Kozyrev, I., Kwofie, T.B., Hayami, M., Miura, T., 2000. Construction of an SIV/HIV type 1 chimeric virus with the human interleukin 6 gene and its production of interleukin 6 in monkey and human cells. AIDS Res. Hum. Retrovir. 16, 577–582.

Haga, T., Shimizu, Y., Okoba, M., Kumabe, S., Goto, Y., Shinjo, T., Ichimura, H., Kuwata, T., Hayami, M., Miura, T., 2002. Construction and in vitro properties of chimeric simian and human immunodeficiency virus with the human TNF-alpha gene. Microbiol. Immunol. 46, 849–855.

Herbein, G., Gordon, S., 1997. 55- and 75-kilodalton tumor necrosis factor receptors mediate distinct actions in regard to human immunodeficiency virus type 1 replication in primary human macrophages. J. Virol. 71, 4150–4156.

Herbein, G., O'Brien, W.A., 2000. Tumor necrosis factor (TNF)-alpha and TNF receptors in viral pathogenesis. Proc. Soc. Exp. Biol. Med. 223, 241–257.

Herbein, G., Mahlknecht, U., Batliwalla, F., Gregersen, P., Pappas, T., Butler, J., O'Brien, W.A., Verdin, E., 1998. Apoptosis of $CD8^+$ T cells is mediated by macrophages through interaction of HIV gp120 with chemokine receptor CXCR4. Nature 395, 189–194.

Iida, T., Ichimura, H., Shimada, T., Ibuki, K., Ui, M., Tamaru, K., Kuwata, T., Yonehara, S., Imanishi, J., Hayami, M., 2000. Role of apoptosis induction in both peripheral lymph nodes and thymus in progressive loss of $CD4^+$ cells in SHIV-infected macaques. AIDS Res. Hum. Retrovir. 16, 9–18.

Johnson, R.P., Desrosiers, R.C., 1998. Protective immunity induced by live attenuated simian immunodeficiency virus. Curr. Opin. Immunol. 10, 436–443.

Kasahara, S., Ando, K., Saito, K., Sekikawa, K., Ito, H., Ishikawa, T., Ohnishi, H., Seishima, M., Kakumu, S., Moriwaki, H., 2003. Lack of tumor necrosis factor alpha induces impaired proliferation of hepatitis B virus-specific cytotoxic T lymphocytes. J. Virol. 77, 2469–2476.

Kozyrev, I.L., Miura, T., Takemura, T., Kuwata, T., Ui, M., Ibuki, K., Iida, T., Hayami, M., 2002. Co-expression of interleukin-5 influences

replication of simian/human immunodeficiency viruses in vivo. J. Gen. Virol. 83, 1183–1188.

Kuwata, T., Igarashi, T., Ido, E., Jin, M., Mizuno, A., Chen, J., Hayami, M., 1995. Construction of human immunodeficiency virus 1/simian immunodeficiency virus strain mac chimeric viruses having vpr and/or nef of different parental origins and their in vitro and in vivo replication. J. Gen.Virol. 76, 2181–2191.

Kwofie, T.B., Haga, T., Iida, T., Hayami, M., Miura, T., 2000. Plasma levels of the chemokine RANTES in macaque monkeys infected with pathogenic and non-pathogenic SIV/HIV-1 chimeric viruses at an early stage of infection. J. Vet. Med. Sci. 62, 1311–1312.

Lane, B.R., Markovitz, D.M., Woodford, N.L., Rochford, R., Strieter, R.M., Coffey, M.J., 1999. TNF-alpha inhibits HIV-1 replication in peripheral blood monocytes and alveolar macrophages by inducing the production of RANTES and decreasing C-C chemokine receptor 5 (CCR5) expression. J. Immunol. 163, 3653–3661.

Lazdins, J.K., Grell, M., Walker, M.R., Woods-Cook, K., Scheurich, P., Pfizenmaier, K., 1997. Membrane tumor necrosis factor (TNF) induced cooperative signaling of TNFR60 and TNFR80 favors induction of cell death rather than virus production in HIV-infected T cells. J. Exp. Med. 185, 81–90.

Matsuyama, T., Kobayashi, N., Yamamoto, N., 1991. Cytokines and HIV infection: is AIDS a tumor necrosis factor disease? AIDS 5, 1405–1417.

Navikas, V., Link, J., Persson, C., Olsson, T., Hojeberg, B., Ljungdahl, A., Link, H., Wahren, B., 1995. Increased mRNA expression of IL-6, IL-10, TNF-alpha, and perforin in blood mononuclear cells in human HIV infection. J. Acquired Immune Defic. Syndr. Hum. Retrovirol. 9, 484–489.

Pedersen, C., Dickmeiss, E., Gaub, J., Ryder, L.P., Platz, P., Lindhardt, B.O., Lundgren, J.D., 1990. T-cell subset alterations and lymphocyte responsiveness to mitogens and antigen during severe primary infection with HIV: a case series of seven consecutive HIV seroconverters. AIDS 4, 523–526.

Poli, G., Kinter, A., Justement, J.S., Kehrl, J.H., Bressler, P., Stanley, S., Fauci, A.S., 1990. Tumor necrosis factor alpha functions in an autocrine manner in the induction of human immunodeficiency virus expression. Proc. Natl. Acad. Sci. U.S.A. 87, 782–785.

Reinberger, S., Spring, M., Nisslein, T., Stahl-Hennig, C., Hunsmann, G., Dittmer, U., 1999. Kinetics of lymphocyte apoptosis in macaques infected with different simian immunodeficiency viruses or simian/human immunodeficiency hybrid viruses. Clin. Immunol. 90, 141–146.

Rosenberg, E.S., Billingsley, J.M., Caliendo, A.M., Boswell, S.L., Sax, P.E., Kalams, S.A., Walker, B.D., 1997. Vigorous HIV-1-specific $CD4^+$ T cell responses associated with control of viremia. Science 278, 1447–1450.

Shinohara, K., Sakai, K., Ando, S., Ami, Y., Yoshino, N., Takahashi, E., Someya, K., Suzaki, Y., Nakasone, T., Sasaki, Y., Kaizu, M., Lu, Y., Honda, M., 1999. A highly pathogenic simian/human immunodeficiency virus with genetic changes in cynomolgus monkey. J. Gen. Virol. 80, 1231–1240.

Stahl-Hennig, C., Gundlach, B.R., Dittmer, U., ten Haaft, P., Heeney, J., Zou, W., Emilie, D., Sopper, S., Uberla, K., 2003. Replication, immunogenicity, and protective properties of live-attenuated simian immunodeficiency viruses expressing interleukin-4 or interferon-gamma. Virology 305, 473–485.

Suryanarayana, K., Wiltrout, T.A., Vasquez, G.M., Hirsch, V.M., Lifson, J.D., 1998. Plasma SIV RNA viral load determination by real-time quantification of product generation in reverse transcriptase-polymerase chain reaction. AIDS Res. Hum. Retrovir. 14, 183–189.

Tartaglia, L.A., Goeddel, D.V., Reynolds, C., Figari, I.S., Weber, R.F., Fendly, B.M., Palladino Jr., M.A., 1993. Stimulation of human T-cell proliferation by specific activation of the 75-kDa tumor necrosis factor receptor. J. Immunol. 151, 4637–4641.

Ui, M., Kuwata, T., Igarashi, T., Ibuki, K., Miyazaki, Y., Kozyrev, I.L., Enose, Y., Shimada, T., Uesaka, H., Yamamoto, H., Miura, T., Hayami, M., 1999. Protection of macaques against a SHIV with a homologous HIV-1 Env and a pathogenic SHIV-89.6P with a heterologous Env by vaccination with multiple gene-deleted SHIVs. Virology 265, 252–263.

von Sydow, M., Sonnerborg, A., Gaines, H., Strannegard, O., 1991. Interferon-alpha and tumor necrosis factor-alpha in serum of patients in various stages of HIV-1 infection. AIDS Res. Hum. Retrovir. 7, 375–380.

Yokota, S., Geppert, T.D., Lipsky, P.E., 1988. Enhancement of antigen- and mitogen-induced human T lymphocyte proliferation by tumor necrosis factor-alpha. J. Immunol. 140, 531–536.

Zheng, L., Fisher, G., Miller, R.E., Peschon, J., Lynch, D.H., Lenardo, M.J., 1995. Induction of apoptosis in mature T cells by tumour necrosis factor. Nature 377, 348–351.

Microbiol. Immunol., 49(12), 1083–1094, 2005

Editor-Communicated Paper

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

Kentaro Kaneyasu[1], Masakazu Kita[2], Sadayuki Ohkura[1], Toshiro Yamamoto[2], Kentaro Ibuki[1], Yoshimi Enose[1], Akihiko Sato[3], Makoto Kodama[3], Tomoyuki Miura[1], and Masanori Hayami*[,1]

[1]Institute for Virus Research, Kyoto University, Kyoto, Kyoto 606–8507, Japan, [2]Department of Microbiology, Kyoto Prefectural University of Medicine, Kyoto, Kyoto 602–8566, Japan, and [3]Discovery Research Laboratories, Shionogi & Co., Ltd., Osaka, Osaka 566–0022, Japan

Communicated by Dr. Hidechika Okada: Received September 29, 2005. Accepted October 11, 2005

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

*Address correspondence to Dr. Masanori Hayami, Laboratory of Primate Model, Experimental Research Center for Infectious Disease, Institute for Virus Research, Kyoto University, 53 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto, Kyoto 606–8507, Japan. Fax: +81–75–761–9335. E-mail: mhayami@virus.kyoto-u.ac.jp

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-