研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
Nakasone T, Hara T, Yoshino N, Honda M.	Update on HIV/AIDS in Japan, 2003.	Y. Lu and M. Essex	AIDS in ASIA.	Kluwer Academic Publishers	London	2004	73-81
Matsuo K, Puthavathana P, Promkhatkaew D, Balachandra K, Ruxrungtham K, Hamano T, Sutthent R, Sittisombut N, Butraporn R, Sriwanthana B, Boonlong J, Izumi Y, Yamazaki S, Yamamoto N, Warachit P, Honda M.	Japan's Collaboration with Thailand in the Development of HIV/AIDS Vaccine.	Y. Lu and M. Essex	AIDS in ASIA.	Kluwer Academic Publishers	London	2004	561-569
Miyazawa, M., E. Kajiwara, N. Tabata, T. Ogawa, T. Yuasa, and H. Matsumura.	Pathogenicity of autoantibodies reactive with the endogenous retroviral envelope glycoprotein gp70.	K. Conrad, M. P. Buchmann, E. K. L.Chan, M. J. Fritzler, R. L. Humbel, U. Sack, and Y. Shoenfeld	From Animal Models to Human Genetics: Research on the Induction and Pathogenicity of Autoantibodies	Pabst Science Publishers	Lengerich	2004	85-96

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Matano T, Kobayashi M, Igarashi H, Takeda A, Nakamura H, Kano M, Sugimoto C, Mori K, Iida A, Hirata T, Hasegawa M, Yuasa T, Miyazawa M, Takahashi Y, Yasunami M, Kimura A, O'Connor DH, Watkins DI, Nagai Y.	Cytotoxic T lymphocyte-based control of simian immunodeficiency virus replication in a preclinical AIDS vaccine trial.	J Exp Med	199	1709-1718	2004
Lun WH, Takeda A, Nakamura H, Kano M, Mori K, Sata T, Nagai Y, Matano T.	Loss of virus-specific $CD4^+$ T cells with increases in viral loads in the chronic phase after vaccine-based partial control of primary simian immunodeficiency virus replication in macaques.	J Gen Virol	85	1955-1963	2004
Kato M, Igarashi H, Takeda A, Horie S, Higashihara E, Matano T.	Stimulation of virus-specific T cell responses by dendritic cell vaccination in the chronic phase of simian AIDS models.	Jpn J Infect Dis	57	220-223	2004

Kato M, Igarashi H, Takeda A, Sasaki Y, Nakamura H, Kano M, Sata T, Iida A, Hasegawa M, Horie S, Higashihara E, Nagai Y, Matano T.	Induction of Gag-specific T-cell responses by therapeutic immunization with a Gag-expressing Sendai virus vector in macaques chronically infected with simian-human immunodeficiency virus.	Vaccine		in press	
Villinger, F., Miller, R., Mori, K., Mayne, A.E., Bostik, P., Sundstrom, J.B., Sugimoto, C. and Ansari, A.A.	IL-15 is superior to IL-2 in the generation of long-lived antigen specific memory CD4 and CD8 T cells in rhesus macaques.	Vaccine	22	3510-3521	2004
Ansari, A.A., Mayne, A.E., Onlamoon, N., Pattanapanyasat, K., Mori, K. and Villinger, F	Use of recombinant cytokines for optimized induction of antiviral immunity against SIV in the nonhuman primate model of human AIDS.	Immunol. Res.	29	1-18	2004
Gzyl J, Bolesta E, Wierzbicki A, Kmieciak D, Naito T, Kaneko Y, Honda M, Komuro K, Kozbor D.	Effect of partial and complete variable loop deletions of the Human Immunodeficiency virus type 1 envelope glycoprotein and the breadth of gp160-specific immune responses.	Virology	318	493-506	2004
Hamano, T, Sawanpanyalert P, Yanai H, Piyaworawong S, Hara T, Sapsutthipas S, Phromjai J, Yamazaki S, Yamamoto N, Warachit P, Honda M, Matsuo K.	Determination of HIV-1 CRF01_AE gag p17 and env-V3 consensus sequences for HIV/AIDS vaccine design.	AIDS Res. Hum. Retroviruses	20	337-340	2004
Dewan Z, Watanabe M, Terashima K, Aoki M, Sata T, Honda M, Ito M, Yamaoka S, Watanabe T, Horie R, Yamamoto N.	Prompt tumor formation and maintenance of constitutive NF-κB activity of multiple myeloma cells in NOD/SCID γ^{cnull} mice.	Cancer Sci	95	564-568	2004
Someya K, Xin K Q, Matsuo K, Okuda K, Yamamoto N, Honda M.	A consecutive prime-boost vaccination of mice with simian immunodeficiency virus (SIV) gag/pol DNA and recombinant vaccinia virus strain DIs elicits effective anti-SIV immunity.	J. Virol.	78	9842-9853	2004
Takeda S, Shiosaki K, Kaneda Y, Nakasatomi T, Yoshizaki H, Someya K, Konno Y, Eda Y, Kino Y, Yamamoto N, Honda M.	Hemagglutinating virus of Japan (HVJ) protein is efficient for induction of CD4+ T-cell response by a hepatitis B core particle-based HIV vaccine.	Clin. Immunol	112	92-105	2004
Yamakami, K, Honda M, Takei M, Ami Y, Nakasone T, Kitamura N, Nishinarita S, Sawada S, Horie T.	Early bone marrow hematopoietic defect in SHIV C2/1-infected macaques and relevance to advance of disease.	J. Virol.	78	10906-10910	2004

Yoshino N, Lu FXS, Fujihashi K, Hagiwara Y, Kataoka K, Lu D, Hirst L, Honda M, van Ginkel FW, Takeda Y, Miller CJ, Kiyono H, McGhee JR.	A Novel Adjuvant for Mucosal Immunity to HIV-1 gp120 in Nonhuman Primates.	J. Immunol.	173	6850－6857	2004
Someya K, Cecilia D, Ami Y, Nakasone T, Matsuo K, Burda S, Yamamoto H, Yoshino N, Kaizu M, Ando S, Okuda K, Zolla-Pazner S, Yamazaki S, Yamamoto N, Honda M.	Vaccination of rhesus macaques with recombinant Mycobacterium bovis bacillus Calmette-Guéin Env V3 elicits neutralizing antibody-mediated protection against simian-human immunodeficiency virus with a homologous but not a heterologous V3 motif.	J. Virol.	79	1452-1462	2005
Kanari, Y., M. Clerici, H. Abe, H. Kawabata, D. Trabattoni, S. Lo Caputo, F. Mazzotta, H. Fujisawa, A. Niwa, C. Ishihara, Y. A. Takei, and M. Miyazawa	Genotypes at chromosome 22q12-13 are associated with HIV-1-exposed but uninfected status in Italians.	AIDS		in press	2005
Miyazawa, M.	Host genes that influence immune and non-immune resistance mechanisms against retrovrial infections.	Recent Res. Devel. Virol.	6	105-118	2004
Sugahara, D., S. Tsuji-Kawahara, and M. Miyazawa	Identification of a protective $CD4^+$ T-cell epitope in $p15^{gag}$ of Friend murine leukemia virus and role of the MA protein targeting to the plasma membrane in immunogenicity.	J. Virol.	78	6322-6334	2004
Tahara H., N. Iwanami, N. Tabata, H. Matsumura, T. Matsuura, T. Kurita, and M. Miyazawa	Both T and non-T cells with proliferating potentials are effective in inducing suppression of allograft responses by alloantigen-specific intravenous presensitization combined with suboptimal doses of 15-deoxyspergualin.	Transplant. Immunol.	13	25-32	2004
Takamura,S., Niikura,M., Li,T.C., Takeda,N., Kusagawa,S., Takebe,Y., Miyamura,T., and Yasutomi,Y.	DNA vaccine -encapsulated virus-like particles derivedfrom an orally transmissible virus stimulates mucosal and systemic immune responses by oral administration.	Gene Ther.	11	628-635	2004
保富康宏.	ウイルス用中空粒子（VLP）を用いた経口ワクチン.	臨床とウイルス	32	362-371	2004
Nguyen KL, Llano M, Akari H, Miyagi M, Poeschla EM, Strebel K, Bour S	Codon optimization of the HIV-1 vpu and vif genes stabilizes their messenger RNA and allows for highly efficient Rev- independent expression	Virology	319	163-175	2004

Akari H, Fujita M, Kao S, Khan MA, Shehu-Xhilaga M, Adachi A, Strebel K	High level expression of Human immunodeficiency virus type 1 Vif inhibits viral infectivity by modulating proteolytic processing of Gag precursor at the p2/NC processing site	Journal of Biological Chemistry	279	12355-12362	2004
Fujita M, Akari H, Sakurai A, Yoshida A, Chiba T, Tanaka K, Strebel K, Adachi A	Expression of the HIV-1 accessory protein Vif is controlled uniquely to be low and optimal by proteasome-degradation	Microbes and Infection	6	791-798	2004

IV. 研究成果の刊行物・別刷

Cytotoxic T Lymphocyte–based Control of Simian Immunodeficiency Virus Replication in a Preclinical AIDS Vaccine Trial

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Abstract

Recently, encouraging AIDS vaccine trials in macaques have implicated cytotoxic T lymphocytes (CTLs) in the control of the simian human immunodeficiency virus SHIV89.6P that induces acute $CD4^+$ T cell depletion. However, none of these vaccine regimens have been successful in the containment of replication of the pathogenic simian immunodeficiency viruses (SIVs) that induce chronic disease progression. Indeed, it has remained unclear if vaccine-induced CTL can control SIV replication. Here, we show evidence suggesting that vaccine-induced CTLs control SIVmac239 replication in rhesus macaques. Eight macaques vaccinated with DNA-prime/Gag-expressing Sendai virus vector boost were challenged intravenously with SIVmac239. Five of the vaccinees controlled viral replication and had undetectable plasma viremia after 5 wk of infection. CTLs from all of these five macaques rapidly selected for escape mutations in Gag, indicating that vaccine-induced CTLs successfully contained replication of the challenge virus. Interestingly, analysis of the escape variant selected in three vaccinees that share a major histocompatibility complex class I haplotype revealed that the escape variant virus was at a replicative disadvantage compared with SIVmac239. These findings suggested that the vaccine-induced CTLs had "crippled" the challenge virus. Our results indicate that vaccine induction of highly effective CTLs can result in the containment of replication of a highly pathogenic immunodeficiency virus.

Key words: $CD8^+$ T lymphocytes • selection • MHC • SIV • Sendai virus

Introduction

Virus-specific $CD8^+$ CTL responses are critical for the control of immunodeficiency virus infections. The importance of CTLs in the control has been indicated by several clinical correlations in HIV-1–infected humans (1–3) and $CD8^+$ T cell depletion experiments in macaque AIDS

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Abbreviations used in this paper: aa, amino acid(s); B-LCL, B lymphoblastoid cell line; DGGE, denaturing gradient gel electrophoresis; L, leucine; nt, nucleotide; RSCA, reference strand–mediated conformation analysis; S, serine; SeV, Sendai virus; SHIV, simian HIV; SIV, simian immunodeficiency virus; VSV-G, vesicular stomatitis virus G; Vv, vaccinia virus.

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models (4–6). Therefore, recent vaccine approaches have focused on eliciting CTL responses (7, 8). However, HIV-1–infected individuals often have high plasma virus concentrations despite the presence of high frequencies of CTLs (9) and it has remained unclear if HIV-1 replication can be contained by vaccine-elicited CTL responses.

DNA vaccines, recombinant viral vector–based vaccines, and their combinations are promising delivery methods for AIDS vaccine because of their potential for inducing CTL responses. Recently, encouraging trials of these vaccines in macaques have implicated vaccine-induced CTLs in the control of the simian HIV (SHIV)89.6P that induces acute $CD4^+$ T cell depletion (10–14). However, most of these vaccine regimens used Env as an immunogen and it is likely that Env-specific antibodies played a role in control of this chimeric virus. Additionally, it has been suggested that SHIV89.6P may not be an appropriate challenge virus (15) and none of these vaccine regimens have been successful in the containment of the more realistic challenge of the pathogenic simian immunodeficiency viruses (SIVs) smE660, mac251, or mac239 (16–19). Thus, it is quite important to know if vaccine induction of CTL responses can lead to the containment of replication of these SIVs that induce chronic disease progression.

We previously developed a DNA-prime/Gag-expressing Sendai virus (SeV) vector boost vaccine system and showed its potential for efficiently inducing Gag-specific cellular immune responses (13, 20). In the preclinical trial, all the vaccinated macaques controlled viremia and were protected from acute AIDS progression after SHIV challenge (13, 21). In this study, we examined if CTL induction by our vaccine system can result in the containment of SIVmac239 replication.

Materials and Methods

Animals. Male rhesus macaques (*Macaca mulatta*) originally from southeastern Asia (Myanmar) were maintained in accordance with the Guideline for Laboratory Animals of National Institute of Infectious Diseases. These macaques were tested negative for SeV, SIV, and simian retrovirus type D before use. Blood collection, vaccination, and virus challenge were performed under ketamine anesthesia.

Vaccination and Challenge. An *env*- and *nef*-deleted SHIV DNA, SIVGP1, was constructed from an infectious $SHIV_{MD14YE}$ clone DNA as described previously (13, 22). The DNA is deleted with a gene fragment encoding Env surface protein (SU; nucleotide [nt] 6211 to nt 7726 in $HIV\text{-}1_{DH12}$; these sequence data are available from GenBank/EMBL/DDBJ under accession no. AF069140), the 3′ portion of the *env* gene (nt 8628 to nt 8764 in $HIV\text{-}1_{DH12}$), and the 5′ quarter of the *nef* gene (nt 9333 to nt 9481 in SIVmac239; GenBank/EMBL/DDBJ accession no. M33262). From SIVGP1 DNA, the 5′ long terminal repeat region was replaced with a CMV promoter with immediate early enhancer and the 3′ portion containing the remaining *nef* and the 3′ long terminal repeat was replaced with Simian virus 40 poly A to obtain CMV-SHIVdEN DNA. Therefore, the CMV-SHIVdEN DNA has SIV-derived *gag*, *pol*, *vif*, *vpx*, and partial *vpr* sequences and HIV-1–derived partial *vpr*, *tat*, *rev*, and partial env (nt 7726 to nt 8628 containing the second exon of *tat*, the second exon of *rev*, and RRE) sequences. At DNA vaccination, animals received 5 mg CMV-SHIVdEN DNA intramuscularly. We used two kinds of SeV vectors, a transmissible one (SeV-Gag) and an F-deleted nontransmissible one (F[−]SeV-Gag), for the boost. Recombinant SeV-Gag and F(−)SeV-Gag were constructed and recovered as described previously (20, 23, 24). 6 wk after the DNA prime, animals received 10^8 cell-infectious units of SeV-Gag or 6×10^9 cell-infectious units of F(−)SeV-Gag intranasally as a boost. Four macaques (V1, V2, V3, and V4) were vaccinated with DNA-prime/SeV-Gag-boost, and the other four (V5, V6, V7, and V8) were vaccinated with DNA-prime/F(−)SeV-Gag-boost. 13 wk after the boost, animals were challenged intravenously with 1,000 $TCID_{50}$ (50% tissue culture–infective dose) of SIVmac239 (25). An SIVmac239 molecular clone DNA, pBRmac239, was provided by T. Kodama (University of Pittsburg, Pittsburgh, PA) and R.C. Desrosiers (New England Primate Research Center, Southborough, MA), and the virus obtained from COS1 cells transfected with pBRmac239 was propagated on rhesus macaque PBMCs to prepare the SIVmac239 challenge stock.

Flow Cytometric Analysis of Virus-specific IFN-γ Induction. We measured virus-specific T cell levels by flow cytometric analysis of IFN-γ induction after specific stimulation as described previously (13). In brief, PBMCs were cocultured with autologous herpesvirus papio-immortalized B lymphoblastoid cell lines (B-LCLs; reference 26) infected with a vaccinia virus (Vv) vector (27) for nonspecific Vv control stimulation and B-LCLs infected with a Vv vector expressing SIVmac239 Gag for Gag-specific Vv Gag stimulation, respectively. Intracellular IFN-γ staining was performed by using CytofixCytoperm kit (Becton Dickinson) according to the manufacturer's instructions. FITC-conjugated anti–human CD4, peridinin chlorophyll protein–conjugated anti–human CD8, allophycocyanin-conjugated anti–human CD3, and anti–human PE-conjugated IFN-γ antibodies (Becton Dickinson) were used. Gag-specific T cell levels were calculated by subtracting the IFN-γ^+ T cell frequencies after nonspecific Vv control stimulation from those after Gag-specific Vv Gag stimulation. Alternatively, for measurement of SIV-specific T cell levels, lymphocytes were cocultured with B-LCLs infected with a vesicular stomatitis virus G (VSV-G)–pseudotyped murine leukemia virus for nonspecific stimulation and B-LCLs infected with a VSV-G–pseudotyped SIVGP1 for SIV-specific stimulation, respectively. In the case of examining peptide-specific T cell levels, B-LCLs were pulsed with each peptide (at a final concentration of 1 μM) or peptide mixture (final concentration of each peptide was 1–10 μM) for peptide-specific stimulation or incubated without peptide for nonspecific stimulation. The peptides, including a panel of 117 overlapping peptides (15–17 amino acids [aa] in length and overlapping by 10 to 12 aa) spanning the entire SIVmac239 Gag sequence, were purchased from Sigma Genosys Japan. Specific T cell levels <100 cells per million PBMCs were considered negative, those between 100 and 200 borderline, and those >200 positive. Gag-specific T cells were undetectable before the vaccination in all of the vaccinees and before the challenge in all of the naive controls.

Quantitation of Plasma Viral Loads. Plasma RNA was extracted using High Pure Viral RNA kit (Roche Diagnostics). For quantitation of plasma SIV RNA levels, serial fivefold dilutions of RNA samples were amplified in quadruplicate by RT and nested PCR using SIV *gag*-specific primers to determine the end point as described previously (22). For preparing the RNA standard, we first set up the method for quantitation of SHIV RNA copy

number by using HIV-1 *vpu*-specific primers and an HIV-1 standard quantitated by Amplicor HIV-1 Monitor (Roche Diagnostics). By using this method, we prepared an SHIV standard for the present assay. The lower limit of detection in this assay is $\sim 4 \times 10^2$ copies/ml. The plasma viral loads at several time points were confirmed by real time PCR (28).

Sequencing. Plasma RNA was extracted using High Pure Viral RNA kit or RNA extraction system in Amplicor HIV-1 Monitor. The fragment spanning from nt 1231 to nt 2958 in SIVmac239 containing all of the *gag* region was amplified from plasma RNA by nested RT-PCR. In case of the plasma with low viral loads (<2,000 copies/ml), 8–16 tubes of nested RT-PCR amplifications were performed for each plasma to avoid obtaining only unrepresentative clones. The PCR products were sequenced using dye terminator chemistry and an automated DNA sequencer (Applied Biosystems). Alternatively, the PCR products were subcloned into a plasmid DNA by using the TOPO cloning system (Invitrogen) and sequenced.

Isolation of Mamu-A/B cDNA Clones. Total cellular RNA was used to synthesize oligo(dT)-primed cDNA with reverse transcriptase (Superscript II; Invitrogen). Full-length cDNAs of *Mamu-A* and *Mamu-B* were amplified by PCR with locus-specific primer pairs (*Mamu-A* forward: 5′-ATGGCGCCCCGAACCC-TCCTCCTG-3′, *Mamu-A* reverse: 5′-TCACACTTTACAAG-CCGTGAGAGA-3′; *Mamu-B* forward: 5′-ATGGCGCCCC-GAACCCTCCTCCTG-3′, *Mamu-B* reverse: 5′-TCAAGCC-GTGAGAGACACATC-3′) and cloned in pGEM-T Easy vector (Promega). The integrity of the clones was verified by reference strand–mediated conformation analysis (RSCA; 29) as the following and then sequenced.

Determination of Mamu MHC-I Haplotype. Locus-specific RT-PCR products were subjected to second round PCR to obtain 725-bp-long DNA fragments encoding Mamu-A/B extracellular domains using *Mamu-A/B* universal forward (5A: 5′-ATGGCGCCCCGAACCCTC-3′) and reverse (4R: 5′-CCAGGTCAGTGTGATCTCCG-3′) primers. The product was analyzed by RSCA conformation analysis essentially as described previously (31). In brief, the second round PCR products and "a reference strand," a fragment derived from the same PCR condition except for using 5′ Cy5-labeled forward primer and a certain cloned DNA template (its sequence is available upon request), were mixed together in a reaction tube, heat denatured, and then cooled down to form heteroduplex DNA. The mobility of heteroduplex DNA molecules in 6% nondenaturing Long Ranger gel (BioWhittaker Molecular Applications) was measured by ALF express II automated sequencing apparatus (Amersham Biosciences). Fluorescence electropherograms showed multiple peak patterns corresponding to multiple, different kinds of sequences expressed in individual macaques. The identity of each peak was determined by comparison of its mobility with those of heteroduplexes derived from parallel PCR using *Mamu-A/B* cDNA clones as templates. Alleles that were shared by a breeder macaque and subset of his sons were thought to be transmitted together and assigned to a single haplotype. The number of expressed alleles on one MHC-I haplotype ranged from one *A* and two *B* alleles to no less than three *A* and five *B* alleles.

Typing of MHC-II (Mamu-DRB and Mamu-DQA). MHC-II alleles and haplotype compositions of macaques were analyzed by sequencing of cloned cDNA and denaturing gradient gel electrophoresis (DGGE; reference 30). Total RNA was extracted from B-LCLs and cDNA was generated by using SuperScript II reverse transcriptase. The entire DRB cDNA and the DQA exon 2 fragments were amplified by PCR using the following primer sets designed to hybridize with the conserved monomorphic regions: 5′-CGCGAATTCTCAGCTCAGGAGTCC-3′ and 5′-GCGG-GATCCATGGTGTGTCTG-3′ for DRB, and 5′-CGCGAAT-TCGGTAGCAGCGGTAGAGT-3′ and 5′-GCGGGATCCGT-GTAAACTTGTACCAGTT-3′ for DQA. The PCR products were subcloned into pUC19 and sequenced. When more than four clones with an identical sequence were obtained for an allele, the allele was considered to be expressed in the animal (see Table I).

The number of DRB alleles expressed in macaques and their haplotype relationships were analyzed by comparing the patterns of DGGE and by cloning and sequencing of the DNA extracted from each band in the gel. For DGGE analyses, the DRB exon 2 fragment was amplified by PCR using the forward (5′-CACTG-GCTTTGGCTGGGGACAC-3′) and the GC-clamped reverse (5′-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCGC-CCCCGCCCGCAGGATACACAGTCACCTTAG-3′) primers. DGGE was performed in 6% polyacrylamide gel containing a gradient of 36–50% of the denaturant mixture (7 M urea and 40% formamide) at 100 V at 60°C for 2.5 h in a DCode system (Bio-Rad Laboratories). DNA eluted from each separate band was subcloned into a plasmid by using the TOPO cloning system and sequenced.

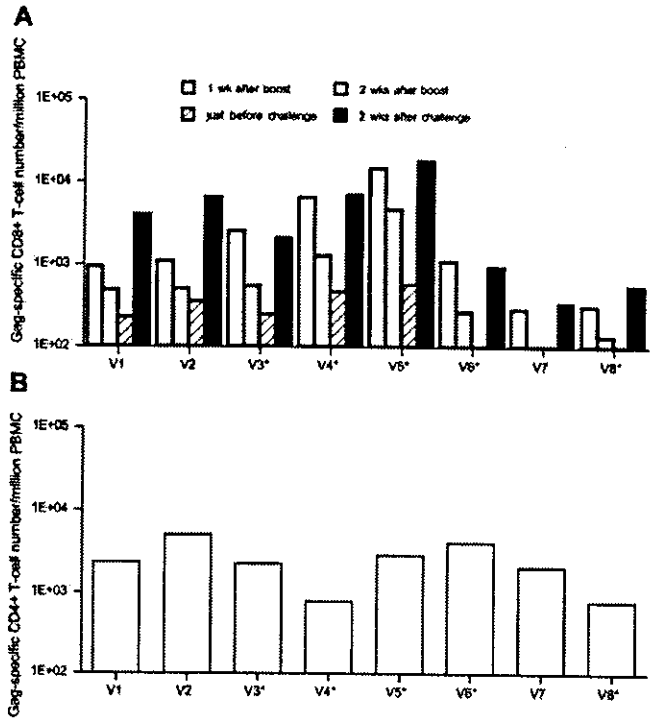


Figure 1. Gag-specific T cell frequencies in vaccinated macaques. Macaques V1, V2, V3, and V4 were boosted with a replication-competent SeV-Gag, whereas macaques V5, V6, V7, and V8 were boosted with a replication-defective F(−)SeV-Gag. *, macaques that controlled SIV replication after challenge. (A) Gag-specific $CD8^+$ T cell frequencies per million PBMCs. The frequencies at week 7 after vaccination (1 wk after boost), at week 8 after vaccination (2 wk after boost), at week 19 after vaccination (just before challenge), and at week 2 after challenge (2 wk after challenge) are shown. (B) Gag-specific $CD4^+$ T cell frequencies per million PBMCs at week 7 after vaccination (1 wk after boost). The frequencies were calculated by subtracting the IFN-γ^+ T cell frequencies after nonspecific Vv control stimulation from those after Gag-specific Vv Gag stimulation. The background IFN-γ^+ T cell frequencies after nonspecific stimulation were $<2.0 \times 10^2$.

Results

Gag-specific T Cell Induction after SeV-Gag-Boost. Our extremely simple vaccine protocol consisted of a single prime with DNA followed by a single boost with a recombinant SeV vector expressing SIVmac239 Gag 6 wk after the prime. Eight rhesus macaques (V1, V2, V3, V4, V5, V6, V7, and V8) were vaccinated with the prime/boost, and four naive controls (N1, N2, N3, and N4) received no vaccination before an intravenous SIVmac239 challenge.

We measured virus-specific T cell levels in the vaccinated macaques by flow cytometric detection of antigen-specific IFN-γ induction. SIV- and Gag-specific T cell responses were examined in PBMCs at weeks 2 and 6 after the DNA vaccination, respectively, but no responses to either SIV or Gag were detectable in any of the vaccinated macaques (not depicted). After the SeV boost, however, we found induction of Gag-specific $CD8^+$ T cells in all of the vaccinees (Fig. 1 A). The levels differed among the macaques, with five (V1, V2, V3, V4, and V5) maintaining detectable levels of Gag-specific $CD8^+$ T cells until challenge. The SeV boost also induced Gag-specific $CD4^+$ T cells in all eight vaccinees (Fig. 1 B).

Control of SIVmac239 Replication in Five of Eight Vaccinees. These vaccinated macaques were challenged intravenously with 1,000 $TCID_{50}$ of SIVmac239 at week 19 after the DNA prime (13 wk after the SeV boost). The unvaccinated control macaques had high peak viremia ($>10^7$ SIV RNA copies/ml plasma) on day 10 after challenge and maintained relatively high plasma viral concentrations (10^4–10^6 SIV RNA copies/ml plasma; Fig. 2). Three of them showed gradual loss of percent CD4 in peripheral T lymphocytes. In contrast, five vaccinated macaques (V3, V4, V5, V6, and V8) controlled replication of this highly pathogenic challenge virus. In these macaques, plasma viremia became undetectable after week 5 and peripheral $CD4^+$ T cells were maintained. The other three vaccinees (V1, V2, and V7) failed to control virus replication and

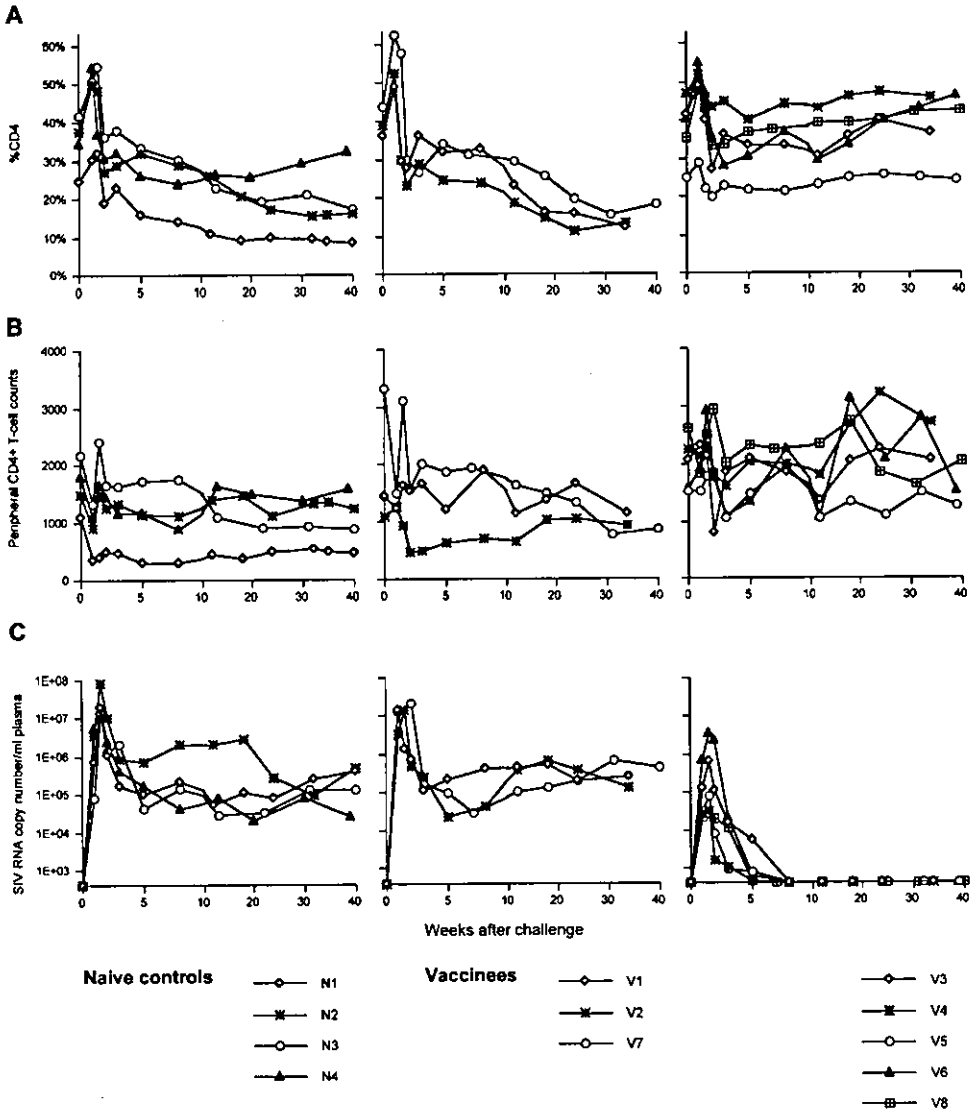
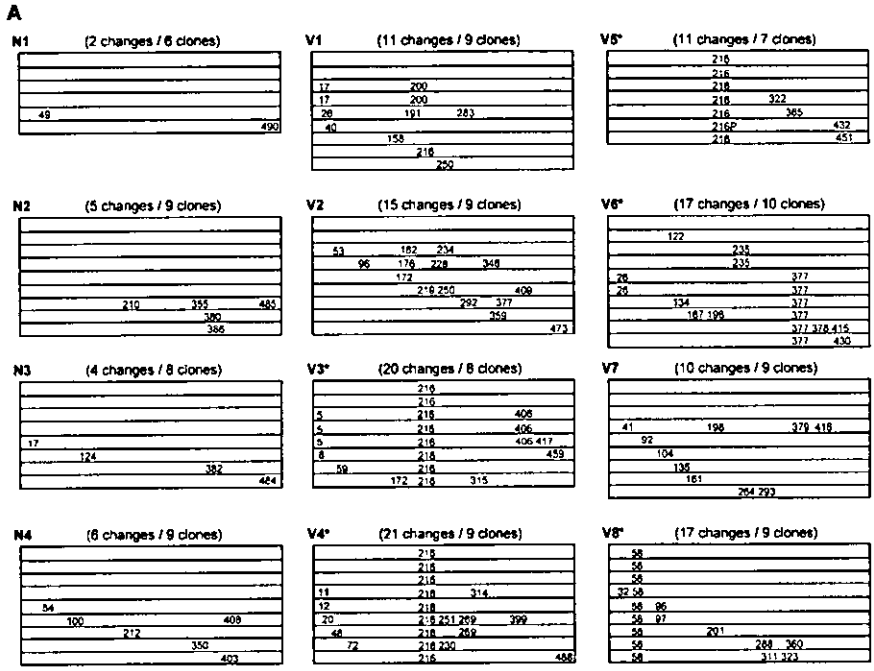


Figure 2. Changes in peripheral $CD4^+$ T cell levels and plasma viral loads after SIVmac239 challenge. (A) Percents of $CD4^+$ T cells in peripheral blood. (B) $CD4^+$ T cell counts in peripheral blood. (C) Plasma viral loads (SIV RNA copy number/ml). The left panels show the naive controls (N1, N2, N3, and N4), the middle panels show the vaccinees that failed to control SIV replication (V1, V2, and V7), and the right panels show the vaccinees that controlled SIV replication (V3, V4, V5, V6, and V8). The portion until week 10 after challenge is enlarged.



B

Animal	CTL escape mutant	Frequencies of the mutant		
		week 2	week 3	week 5
V3	mutation at the 216th aa	1/9	5/9	8/8
V4	mutation at the 216th aa	0/8	9/9	9/9
V5	mutation at the 216th aa	0/7	3/9	7/7
V6	mutation at the 377th aa	ND	1/10	6/10
V8	mutation at the 58th aa	ND	0/9	9/9

Figure 3. Mutations in SIV *gag*. (A) Schematic representation of the positions of aa changes in SIV Gag in each macaque after challenge. 6–10 clones of plasmids carrying the whole *gag* region amplified from plasma RNA at week 5 after challenge were obtained from each macaque and sequenced. Each lane represents the whole *gag* sequence derived from each clone and the positions of aa changes detected are indicated. Total number of aa changes and number of clones sequenced are shown in the parentheses. All the changes at aa 58 were glutamine to lysine, and all at aa 377 were isoleucine to threonine. All the changes at aa 216 other than the one indicated as 216P were L to S. The 216P represents L to P change at aa 216. (B) Frequencies of the CTL escape mutants at weeks 2, 3, and 5 in the vaccinees that controlled SIV replication. The ratio of the number of the clones with the escape mutation to the number of the sequenced clones is shown.

showed gradual loss of percent CD4 in peripheral T lymphocytes similar to the naive control animals. One of them (macaque V2) was killed at week 42 because of dyspnea, loss of body weight, and loss of peripheral $CD4^+$ T cells (4.4%, 97 cells/μl at week 42). Autopsy revealed that this animal developed AIDS with *Pneumocystis carinii* pneumonia.

At week 2 after challenge, we detected anamnestic Gag-specific $CD8^+$ T cell responses in all of the vaccinated macaques, indicating efficient secondary responses during the acute phase of infection (Fig. 1 A). These levels varied from macaque to macaque. Macaque V5 showed the highest level of Gag-specific $CD8^+$ T cells and macaque V7 showed the lowest. No significant difference in the levels was observed between the macaques that controlled viral replication and those that did not. The magnitude of the total prechallenge Gag-specific $CD8^+$ T cell or $CD4^+$ T cell responses did not appear to correlate with the level of control. We examined plasma-neutralizing activities against SIVmac239 as described previously (31), but found no neutralizing activities in any of the controls or the vaccinees at weeks 5 or 12 after challenge (not depicted), indicating that neutralizing antibodies were not essential for the control of SIV replication observed in this experiment.

Rapid Selection of CTL Escape Variants in the Vaccinees That Controlled SIVmac239 Replication. To determine whether vaccine-induced Gag-specific T cell responses exerted a selective pressure on the virus, we sequenced the SIV *gag* region in the viral genomes obtained from plasma RNA at week 5 after challenge (Fig. 3 A). The numbers of aa changes per clone in the vaccinated macaques were significantly higher than those in the unvaccinated (mean: unvaccinated, 0.51; vaccinated, 1.75; $P = 0.0006$ by t test). This may reflect the immune pressure by vaccine-induced Gag-specific T cell responses. Interestingly, all of the macaques that controlled SIVmac239 replication (V3, V4, V5, V6, and V8), but not those unable to control the virus, showed consistent aa changes in Gag (Fig. 3 A). Among them, three macaques (V3, V4, and V5) had a common aa change, leucine (L) to serine (S) at the 216th aa in Gag. We then examined peptide-specific T cell responses after the SeV boost and found, in these three macaques but not in the other vaccinees, efficient expansion of $CD8^+$ T cells specific for an epitope ($Gag_{206-216}$; IINEEAADWDL) spanning from the 206th to the 216th aa in SIVmac239 Gag. Interestingly, these three macaques showed no or diminished recognition of the mutant peptide, IINEEAADWDS ($Gag_{206-216}$L216S; Fig. 4 A), indicating that this mutant likely represents an escape variant. Sequence analysis of vi-

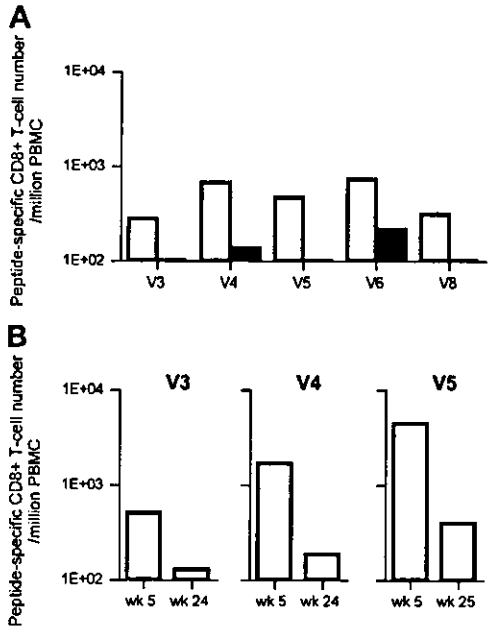
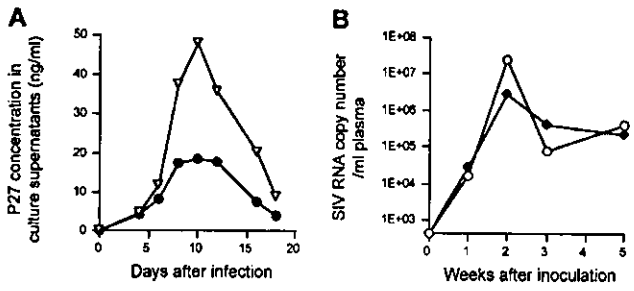


Figure 4. Peptide-specific T cell frequencies in the vaccinees that controlled SIV replications. (A) Comparison between the epitope peptide–specific and the variant peptide–specific $CD8^+$ T cell responses. PBMCs at week 10 after vaccination in macaque V3, at week 10 after vaccination in V4, at week 15 after vaccination in V5, at week 3 after challenge in V6, and at week 3 after challenge in V8 were used. The open bars indicate the levels of $CD8^+$ T cells specific for $Gag_{206-216}$ peptide in V3, V4, and V5, $Gag_{367-381}$ peptide in V6, and Gag_{50-65} peptide in V8, respectively. The solid bars indicate the levels of $CD8^+$ T cells specific for $Gag_{206-216}$L216S peptide in V3, V4, and V5, $Gag_{367-381}$I377T peptide in V6, and Gag_{50-65}Q58K peptide in V8, respectively. (B) $Gag_{206-216}$-specific $CD8^+$ T cell levels in macaques V3, V4, and V5 after challenge. The background IFN-γ^+ $CD8^+$ T cell frequencies after nonspecific stimulation were $<1.0 \times 10^2$.

ral genomes from the three macaques that made responses to this epitope at weeks 2 and 3 revealed that this CTL escape mutant became dominant around week 3 after challenge (Fig. 3 B). Thus, in these three macaques with high levels of $Gag_{206-216}$-specific $CD8^+$ T cells, the wild-type challenge virus disappeared quickly and only the CTL escape mutant was detectable in plasma at week 5. These three macaques had high levels of $Gag_{206-216}$-specific $CD8^+$ T cells 3 wk after challenge. However, these levels were considerably reduced in the chronic phase (Fig. 4 B).

We further examined epitope-specific $CD8^+$ T cell responses in the other two macaques that controlled viral replication. In macaque V6, a mutation leading to a change at the 377th aa (isoleucine to threonine) was observed at week 5 (Fig. 3 A). Analysis of peptide-specific responses revealed that this macaque had a high level of $CD8^+$ T cells specific for a 15-mer peptide corresponding to aa 367–381 in SIVmac239 Gag ($Gag_{367-381}$) at week 3 after challenge. Stimulation by the mutant $Gag_{367-381}$ peptide with the substitution ($Gag_{367-381}$I377T) induced IFN-γ^+ $CD8^+$ T cells, but their frequency was lower than that after stimulation by the wild-type $Gag_{367-381}$ peptide (Fig. 4 A). Additionally, viruses from macaque V8 had a mutation leading to a change at the 58th aa (glutamine to lysine; Fig. 3 A). In this animal, $CD8^+$ T cell responses specific for a 16-mer peptide corresponding to aa 50–65 in SIVmac239 Gag (Gag_{50-65}) were observed at week 3 after challenge. Stimulation by the mutant Gag_{50-65} peptide with the substitution (Gag_{50-65}Q58K) failed to induce IFN-γ^+ $CD8^+$ T cells (Fig. 4 A). Each of these mutants became dominant at approximately week 5 after challenge in the corresponding macaque (Fig. 3 B).

Among the 12 macaques used in the challenge experiment, 8 macaques (2 naive controls and 6 vaccinees) descended from a single male, macaque R90-120 (its sons: N2, V2, and V3; its grandsons: N3, V4, V5, V6, and V7; Table I). Analysis of MHC-I *Mamu-A* and *Mamu-B* alleles indicated that four macaques of the eight R90-120 descendants, N2, V3, V4, and V5, share an MHC-I haplotype (90–120-Ia) derived from macaque R90-120. Analysis of MHC-II also suggested that these macaques possibly share an MHC-II haplotype derived from macaque R90-120. Among these macaques possessing the 90–120-Ia haplotype, three (V3, V4, and V5) were vaccinees that controlled SIV replication with high levels of $Gag_{206-216}$-spe-



C

Animal	Sequencing	Frequencies of the L216S mutant			
		week 1	week 2	week 3	week 5
M1	direct	++	+	-	-
	clones	ND	3/10	0/10	ND
M2	direct	++	-	-	-
	clones	ND	0/10	0/10	ND

Figure 5. Comparison of replication efficiencies between the wild-type SIVmac239 and the escape variant SIVmac239G216S. (A) Replication kinetics of SIVmac239 (▽) and SIVmac239G216S (●) in macaque PBMCs. MT4 cells were transfected with pBRmac239 and pBRmac239G216S to obtain SIVmac239 and SIVmac239G216S, respectively. PBMCs were infected with the viruses at a multiplicity of infection of 0.0002 and concentrations of SIV Gag p27 in their culture supernatants were measured by ELISA (Beckman Coulter). A representative result from three independent experiments is shown. (B) Plasma viral loads (SIV RNA copy number/ml) in macaques M1 (○) and M2 (◆) after inoculation with both of the wild-type SIVmac239 molecular clone DNA and the mutant SIVmac239G216S molecular clone DNA. (C) Frequencies of the mutant viral genome in plasma in the macaques inoculated with both of the wild-type SIVmac239 molecular clone DNA and the mutant SIVmac239G216S molecular clone DNA. In case of direct sequencing of the PCR products (indicated by direct), ++ indicates detection of both the wild-type and the mutant at comparable levels, + indicates detection of the wild-type predominantly and the mutant slightly, and − indicates detection of the wild-type only. In case of sequencing clones (indicated by clones), the ratio of the number of the mutant clones to the number of the sequenced clones is shown.

Table I. *MHC-I and MHC-II Alleles of Macaques Used in This Study*

Animal[a]	Father	MHC-I *Mamu-A & B* RSCA pattern[b]	MHC-II *Mamu-DRB & DQA* alleles[c]
Naive control			
N1	R90-088	*1	*DRB1(Z26148), DRB*W502, DQA1*03(M76230)*
			*DRB(AB112040), DRB*W2603, DRB*W402, DQA1*0502*
N2	R90-120	90-120-Ia	*DRB1*1007, DRB1(Z26137), DQA1*03(M76228)*
			*DRB(Z26165), DRB(AB112039), DRB(AB112043), DQA1*06(MM76195)*
N3	R94-027[d]	*2	*DRB1*0316, DRB*W2507, DQA1*01(M76202)*
			*DRB*W2104, DRB*W2603, DRB*W606, DQA1*0502*
N4	R90-010	ND	*DRB*W2104, DRB*W2603, DQA1*0502*
			*DRB*0321, DRB*0323, DRB*W606, DQA1*05(M76227)*
Vaccinee			
V1	R90-088	*1	*DRB1(Z26148), DRB*W502, DQA1*03(M76230)*
			*DRB*W2505, DRB(AB112046), DRB(AB124813), DQA(AB124814)*
V2	R20-120	90-120-Ib	*DRB*W2002, DRB*W2501, DQA1*0502*
			*DRB1(Z26148), DRB*W502, DQA1*03(M76230)*
V3	R90-120	90-120-Ia	*DRB1*1007, DRB1(Z26137), DQA1*03(M76228)*
			*DRB1(Z26148), DRB*W502, DQA1*03(M76230)*
V4	R94-027	90-120-Ia	*DRB1*1007, DRB1(Z26137), DQA1*03(M76228)*
			*DRB*W2505, DRB(AB112046), DRB(AB124813), DQA(AB124814)*
V5	R94-027	90-120-Ia	*DRB1*1007, DRB1(Z26137), DQA1*03(M76228)*
			DRB(AB112043), DRB(AB112047)
V6	R94-027	*2	*DRB1*0316, DRB*W2507, DQA1*01(M76202)*
			*DRB1(Z26148), DRB*W502, DQA1*03(M76230)*
V7	R94-027	*2	*DRB1*0316, DRB*W2507, DQA1*01(M76202)*
			*DRB*W2104, DRB*W2603, DRB*W606, DQA1*0502*
V8	R90-010	ND	*DRB*W2104, DRB*W2603, DQA1*0502*
			*DRB1*0316, DRB*W2507, DQA1*09(M76200)*
Breeder			
R90-088	unknown	*1	*DRB1(Z26148), DRB*W502, DQA1*03(M76230)*
R90-120	unknown	90-120-Ia	*DRB1*1007, DRB1(Z26137), DQA1*03(M76228)*
		90-120-Ib	*DRB*W2002, DRB*W2501, DQA1*0502*
R90-010	unknown	ND	*DRB*W2104, DRB*W2603, DQA1*0502*
			*DRB1*0316, DRB*2507, DQA1*01(M76202)*

[a]The underlined macaques showed control of SIV replication.
[b]MHC-I *Mamu-A* and *Mamu-B* alleles and haplotype compositions of macaques were examined by RSCA and sequencing of cloned cDNA. The haplotype 90-120-Ia derived from macaque R90-120 consists of three *Mamu-A* alleles (*Mamu-A120-1*, *Mamu-A120-4*, and *Mamu-A120-5*) and four *Mamu-B* alleles (*Mamu-B120-1*, *Mamu-B120-6*, *Mamu-B120-8*, and *Mamu-B120-9*). The haplotype 90-120-Ib derived from macaque R90-120 consists of two *Mamu-A* alleles (*Mamu-A120-2* and *Mamu-A120-3* [= *Mamu-A*05*]) and five *Mamu-B* alleles (*Mamu-B120-2*, *Mamu-B120-3*, *Mamu-B120-4*, *Mamu-B120-5* [= *Mamu-B*36*], and *Mamu-B120-7*). Macaques N1 and V1 shared an RSCA pattern of a haplotype derived from R90-088 (*1). Macaques N3, V6, and V7 shared an RSCA pattern of a haplotype not derived from R90-120 (*2).
[c]MHC-II *Mamu-DRB* and *Mamu-DQA* alleles were analyzed by DGGE and sequencing of cDNA. The determined alleles are shown. Each number in parentheses indicates the corresponding accession number for the nt sequence of the allele that has not yet been designated.
[d]The father of macaque R94-027 is macaque R90-120.

cific $CD8^+$ T cell responses. The remaining one (naive control macaque N2) showed a detectable level of $Gag_{206-216}$-specific $CD8^+$ T cell responses at week 3 after challenge, although the level was low (2.5×10^2 cells/million PBMCs). These results strongly suggest that the $Gag_{206-216}$ epitope is restricted by an MHC-I molecule derived from the 90–120-Ia haplotype.

Diminished Replicative Ability of the CTL Escape Variant SIV. We then explored the hypothesis that the escape mutation selected by the vaccine-induced $Gag_{206-216}$-spe-

cific CTL resulted in a loss of viral fitness. We constructed a molecular clone of the escape mutant SIV, referred to as SIVmac239G216S, with a mutation resulting in the L to S substitution at the 216th aa in Gag. The mutant SIV was replication competent in vitro but showed lower levels of proliferation kinetics in PBMC culture compared with the wild-type SIVmac239 (Fig. 5 A). To compare the SIVmac239G216S replication kinetics with the wild-type in macaques, two macaques (M1 and M2, neither of them descended from macaque R90-120) were coinoculated intramuscularly with 5 mg of the SIVmac239 molecular clone DNA (pBRmac239) and 5 mg of the SIVmac239G216S molecular clone DNA (pBRmac239G216S; Fig. 5, B and C). Both viral genomes were detected at comparable levels in plasma from both of the macaques at week 1 after the inoculation. After that, however, the mutant SIVmac239G216S disappeared and the wild-type SIVmac239 became dominant. Neither of the macaques showed $Gag_{206-216}$-specific $CD8^+$ T cell responses at week 3 (not depicted). These results indicate that the L to S change at the 216th aa in Gag is disadvantageous for SIV replication in the absence of $Gag_{206-216}$-specific $CD8^+$ T cell responses in macaques.

Discussion

In this study, we present evidence indicating that vaccine-induced CTLs control SIVmac239 replication in rhesus macaques. Each of the macaques that controlled viral replication had a mutation in Gag leading to an aa change in a CTL epitope by week 5 after challenge, reflecting strong CTL-induced selective pressure. This finding lends support to the notion that epitope-specific CTL responses played a central role in the control of replication of the SIVmac239 challenge virus because it was difficult to detect the challenge virus at week 5 after challenge.

Among the 12 macaques used in the challenge experiment, 8 macaques descended from macaque R90-120 and 4 of them shared an MHC-I haplotype, 90–120-Ia. Among the four, not the naive (N2) but the three vaccinees (V3, V4, and V5) controlled SIV replication and selected for the same $Gag_{206-216}$-specific CTL escape variant with L to S change at the 216th aa in Gag. Therefore, we examined the reproducibly selected escape variant SIVmac239G216S intensively and found that in the absence of $Gag_{206-216}$-specific $CD8^+$ T cell responses, its replication efficiency is diminished compared with the wild-type SIVmac239 in vivo as well as in vitro. The rapid selection of the escape variant with lower viral fitness in the vaccinees with $Gag_{206-216}$-specific CTLs indicates that the vaccine-induced CTLs exerted strong immune pressure leading to clearance of the wild-type SIVmac239.

The emergence of escape variants depends on the balance between CTL-induced immune pressure and viral fitness costs (32). Viral escape from CTLs during the acute phase of natural immunodeficiency virus infections has been observed in Tat, Nef, Vpr, and Env (33–36). Escape variants with mutations in the structural protein Gag have been also reported (37), but it has been shown that they mostly diminish viral fitness and require multiple additional compensatory mutations to restore their replicative competence (38–41). Indeed, the $Gag_{206-216}$-specific CTL escape variant selected in macaques V3, V4, and V5 diminished viral replication. Therefore, our results suggest that the vaccine-induced CTLs were crucial to the rapid containment of replication of the challenge virus and selected for the virus with diminished replicative ability. Without compensatory mutations, the crippled virus might be easily controlled by the immune system.

The macaques used in our challenge experiment were non-Indian rhesus and the setpoint plasma viral loads in the naive control group might be lower than those usually observed in SIVmac239-infected Indian rhesus. However, the viral loads are higher than those typically observed in untreated humans infected with HIV-1 and equivalent to viral loads seen in SIVsmE660-infected Indian rhesus (16, 42). Indeed, all of the naive animals failed to control the virus replication after SIVmac239 challenge, indicating that CTLs are unable to contain and clear the virus in natural SIVmac239 infections of our non-Indian rhesus macaques. Thus, this study provides clear evidence demonstrating that vaccine induction of effective CTLs that can cripple the virus can result in the containment of replication of a neutralization-resistant, highly pathogenic immunodeficiency virus that is unable to be contained in the natural chronic course of infections. In conclusion, our results show that vaccine-induced CTLs can control SIVmac239 replication and indicate that induction of highly effective CTLs might be critical for the vaccine-based containment of immunodeficiency virus replication.

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References

1. Koup, R.A., J.T. Safrit, Y. Cao, C.A. Andrews, G. McLeod, W. Borkowsky, C. Farthing, and D.D. Ho. 1994. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J. Virol.* 68:4650–4655.
2. Borrow, P., H. Lewicki, B.H. Hahn, G,M. Shaw, and M.B. Oldstone. 1994. Virus-specific $CD8^+$ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. *J. Virol.* 68:6103–6110.
3. Ogg, G.S., X. Jin, S. Bonhoeffer, P.R. Dunbar, M.A.

Nowak, S. Monard, J.P. Segal, Y. Cao, S.L. Rowland-Jones, V. Cerundolo, et al. 1998. Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA. *Science.* 279:2103–2106.
4. Matano, T., R. Shibata, C. Siemon, M. Connors, H.C. Lane, and M.A. Martin. 1998. Administration of an anti-CD8 monoclonal antibody interferes with the clearance of chimeric simian/human immunodeficiency virus during primary infections of rhesus macaques. *J. Virol.* 72:164–169.
5. Schmitz, J.E., M.J. Kuroda, S. Santra, V.G. Sasseville, M.A. Simon, M.A. Lifton, P. Racz, K. Tenner-Racz, M. Dalesandro, B.J. Scallon, et al. 1999. Control of viremia in simian immunodeficiency virus infection by $CD8^+$ lymphocytes. *Science.* 283:857–860.
6. Jin, X., D.E. Bauer, S.E. Tuttleton, S. Lewin, A. Gettie, J. Blanchard, C.E. Irwin, J.T. Safrit, J. Mittler, L. Weinberger, et al. 1999. Dramatic rise in plasma viremia after $CD8^+$ T cell depletion in simian immunodeficiency virus–infected macaques. *J. Exp. Med.* 189:991–998.
7. Walker, B.D., and B.T. Korber. 2001. Immune control of HIV: the obstacles of HLA and viral diversity. *Nat. Immunol.* 2:473–475.
8. MaMichael, A.J., and T. Hanke. 2003. HIV vaccines 1983–2003. *Nat. Med.* 9:874–880.
9. Gea-Banacloche, J.C., S.A. Migueles, L. Martino, W.L. Shupert, A.C. McNeil, M.S. Sabbaghian, L. Ehler, C. Prussin, R. Stevens, L. Lambert, et al. 2000. Maintenance of large numbers of virus-specific $CD8^+$ T cells in HIV-infected progressors and long-term nonprogressors. *J. Immunol.* 165: 1082–1092.
10. Barouch, D.H., S. Santra, J.E. Schmitz, M.J. Kuroda, T.-M. Fu, W. Wagner, M. Bilska, A. Craiu, X.X. Zheng, G.R. Krivulka, et al. 2000. Control of viremia and prevention of clinical AIDS in rhesus monkeys by cytokine-augmented DNA vaccination. *Science.* 290:486–492.
11. Amara, R.R., F. Villinger, J.D. Altman, S.L. Lydy, S.P. O'Neil, S.I. Staprans, D.C. Montefiori, Y. Xu, J.G. Herndon, L.S. Wyatt, et al. 2001. Control of a mucosal challenge and prevention of AIDS in rhesus macaques by a multiprotein DNA/MVA vaccine. *Science.* 292:69–74.
12. Rose, N.F., P.A. Marx, A. Luckay, D.F. Nixon, W.J. Moretto, S.M. Donahoe, D. Montefiori, A. Roberts, L. Buonocore, and J.K. Rose. 2001. An effective AIDS vaccine based on live attenuated vesicular stomatitis virus recombinants. *Cell.* 106:539–549.
13. Matano, T., M. Kano, H. Nakamura, A. Takeda, and Y. Nagai. 2001. Rapid appearance of secondary immune responses and protection from acute CD4 depletion after a highly pathogenic immunodeficiency virus challenge in macaques vaccinated with a DNA-prime/Sendai viral vector-boost regimen. *J. Virol.* 75:11891–11896.
14. Shiver, J.W., T.M. Fu, L. Chen, D.R. Casimiro, M.E. Davies, R.K. Evans, Z.Q. Zhang, A.J. Simon, W.L. Trigona, S.A. Dubey, et al. 2002. Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. *Nature.* 415:331–335.
15. Feinberg, M.B., and J.P. Moore. 2002. AIDS vaccine models: challenging challenge viruses. *Nat. Med.* 8:207–210.
16. Egan, M.A., W.A. Charini, M.J. Kuroda, J.E. Schmitz, P. Racz, K. Tenner-Racz, K. Manson, M. Wyand, M.A. Lifton, C.E. Nickerson, et al. 2000. Simian immunodeficiency virus (SIV) gag DNA-vaccinated rhesus monkeys develop secondary cytotoxic T-lymphocyte responses and control viral replication after pathogenic SIV infection. *J. Virol.* 74: 7485–7495.
17. Allen, T.M., L. Mortara, B.R. Mothe, M. Liebl, P. Jing, B. Calore, M. Piekarczyk, R. Ruddersdorf, D.H. O'Connor, X. Wang, et al. 2002. Tat-vaccinated macaques do not control simian immunodeficiency virus SIVmac239 replication. *J. Virol.* 76:4108–4112.
18. Horton, H., T.U. Vogel, D.K. Carter, K. Vielhuber, D.H. Fuller, T. Shipley, J.T. Fuller, K.J. Kunstman, G. Sutter, D.C. Montefiori, et al. 2002. Immunization of rhesus macaques with a DNA prime/modified vaccinia virus Ankara boost regimen induces broad simian immunodeficiency virus (SIV)-specific T-cell responses and reduces initial viral replication but does not prevent disease progression following challenge with pathogenic SIVmac239. *J. Virol.* 76:7187–7202.
19. Allen, T.M., P. Jing, B. Calore, H. Horton, D.H. O'Connor, T. Hanke, M. Piekarczyk, R. Ruddersdorf, B.R. Mothe, C. Emerson, et al. 2002. Effects of cytotoxic T lymphocytes (CTL) directed against a single simian immunodeficiency virus (SIV) Gag CTL epitope on the course of SIVmac239 infection. *J. Virol.* 76:10507–10511.
20. Kano, M., T. Matano, A. Kato, H. Nakamura, A. Takeda, Y. Suzaki, Y. Ami, K. Terao, and Y. Nagai. 2002. Primary replication of a recombinant Sendai viral vector in macaques. *J. Gen. Virol.* 83:1377–1386.
21. Takeda, A., H. Igarashi, H. Nakamura, M. Kano, A. Iida, T. Hirata, M. Hasegawa, Y. Nagai, and T. Matano. 2003. Protective efficacy of an AIDS vaccine, a single DNA-prime followed by a single booster with a recombinant replication-defective Sendai virus vector, in a macaque AIDS model. *J. Virol.* 77:9710–9715.
22. Shibata, R., F. Maldarelli, C. Siemon, T. Matano, M. Parta, G. Miller, T. Fredrickson, and M.A. Martin. 1997. Infection and pathogenicity of chimeric simian-human immunodeficiency viruses in macaques: determinants of high virus loads and CD4 cell killing. *J. Infect. Dis.* 176:362–373.
23. Kato, A., Y. Sakai, T. Shioda, T. Kondo, M. Nakanishi, and Y. Nagai. 1996. Initiation of Sendai virus multiplication from transfected cDNA or RNA with negative or positive sense. *Genes Cells.* 1:569–579.
24. Li, H.O., Y.F. Zhu, M. Asakawa, H. Kuma, T. Hirata, Y. Ueda, Y.S. Lee, M. Fukumura, A. Iida, A. Kato, et al. 2000. A cytoplasmic RNA vector derived from nontransmissible Sendai virus with efficient gene transfer and expression. *J. Virol.* 74:6564–6569.
25. Kestler, H., D.J. Ringler, K. Mori, D.L. Panicali, P.K. Sehgal, M.D. Daniel, and R.C. Desrosiers. 1991. Importance of the nef gene for maintenance of high virus loads and for development of AIDS. *Cell.* 65:651–662.
26. Voss, G., S. Nick, C. Stahl-Hennig, K. Ritter, and G. Hunsmann. 1992. Generation of macaque B lymphoblastoid cell lines with simian Epstein-Barr-like viruses: transformation procedure, characterization of the cell lines and occurrence of simian foamy virus. *J. Virol. Methods.* 39:185–195.
27. Mackett, M., G.L. Smith, and B. Moss. 1982. Vaccinia virus: a selectable eukaryotic cloning and expression vector. *Proc. Natl. Acad. Sci. USA.* 79:7415–7419.
28. Mori, K., Y. Yasutomi, S. Ohgimoto, T. Nakasone, S. Takamura, T. Shioda, and Y. Nagai. 2001. Quintuple deglycosylation mutant of simian immunodeficiency virus SIVmac239 in rhesus macaques: robust primary replication, tightly contained chronic infection, and elicitation of potent immunity against the parental wild-type strain. *J. Virol.* 75:4023–4028.

29. Arguello, J.R., A.M. Little, A.L. Pay, D. Gallardo, I. Rojas, S.G. Marsh, J.M. Goldman, and J.A. Madrigal. 1998. Mutation detection and typing of polymorphic loci through double-strand conformation analysis. *Nat. Genet.* 18:192–194.
30. Knapp, L.A., L.F. Cadavid, M.E. Eberle, S.J. Knechtle, R.E. Bontrop, and D.I. Watkins. 1997. Identification of new *Manu-DRB* alleles using DGGE and direct sequencing. *Immunogenetics.* 45:171–179.
31. Shibata, R., C. Siemon, S.C. Czajak, R.C. Desrosiers, and M.A. Martin. 1997. Live, attenuated simian immunodeficiency virus vaccines elicit potent resistance against a challenge with a human immunodeficiency virus type 1 chimeric virus. *J. Virol.* 71:8141–8148.
32. Yang, O.O., P.T.N. Sarkis, A. Ali, J.D. Harlow, C. Brander, S.A. Kalams, and B.D. Walker. 2003. Determinant of HIV-1 mutational escape from cytotoxic T lymphocytes. *J. Exp. Med.* 197:1365–1375.
33. Price, D.A., P.J. Goulder, P. Klenerman, A.K. Sewell, P.J. Easterbrook, M. Troop, C.R. Bangham, and R.E. Phillips. 1997. Positive selection of HIV-1 cytotoxic T lymphocyte escape variants during primary infection. *Proc. Natl. Acad. Sci. USA.* 94:1890–1895.
34. Borrow, P., H. Lewicki, X. Wei, M.S. Horwitz, N. Peffer, H. Meyers, J.A. Nelson, J.E. Gairin, B.H. Hahn, M.B. Oldstone, et al. 1997. Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTL) during primary infection demonstrated by rapid selection of CTL escape virus. *Nat. Med.* 3:205–211.
35. Allen, T.M., D.H. O'Connor, P. Jing, J.L. Dzuris, B.R. Mothe, T.U. Vogel, E. Dunphy, M.E. Liebl, C. Emerson, N. Wilson, et al. 2000. Tat-specific cytotoxic T lymphocytes select for SIV escape variants during resolution of primary viraemia. *Nature.* 407:386–390.
36. O'Connor, D.H., T.M. Allen, T.U. Vogel, P. Jing, I.P. DeSouza, E. Dodds, E.J. Dunphy, C. Melsaether, B. Mothe, H. Yamamoto, et al. 2002. Acute phase cytotoxic T lymphocyte escape is a hallmark of simian immunodeficiency virus infection. *Nat. Med.* 8:493–499.
37. Leslie, A.J., K.J. Pfafferott, P. Chetty, R. Draenert, M.M. Addo, M. Feeney, Y. Tang, E.C. Holmes, T. Allen, J.G. Prado, et al. 2004. HIV evolution: CTL escape mutation and reversion after transmission. *Nat. Med.* 10:282–289.
38. Kelleher, A.D., C. Long, E.C. Holmes, R.L. Allen, J. Wilson, C. Conlon, C. Workman, S. Shaunak, K. Olson, P. Goulder, et al. 2001. Clustered mutations in HIV-1 gag are consistently required for escape from HLA-B27–restricted cytotoxic T lymphocyte responses. *J. Exp. Med.* 193:375–386.
39. Peyerl, F.W., D.H. Barouch, W.W. Yeh, H.S. Bazick, J. Kunstman, K.J. Kunstman, S.M. Wolinsky, and N.L. Letvin. 2003. Simian-human immunodeficiency virus escape from cytotoxic T-lymphocyte recognition at a structurally constrained epitope. *J. Virol.* 77:12572–12578.
40. Friedrich, T.C., C.A. Frye, L.J. Yant, D.H. O'Connor, N.A. Kriewaldt, M. Benson, L. Vojnov, E.J. Dodds, C. Cullen, R. Rudersdorf, et al. 2004. Extra-epitopic compensatory substitutions partially restore fitness to simian immunodeficiency virus variants that escape from an immunodominant cytotoxic T-lymphocyte response. *J. Virol.* 78:2581–2585.
41. Friedrich, T.C., E.J. Dodds, L.J. Yant, L. Vojnov, R. Rudersdorf, C. Cullen, D.T. Evans, R.C. Desrosiers, B.R. Mothe, J. Sidney, et al. 2004. Reversion of CTL escape-variant immunodeficiency viruses in vivo. *Nat. Med.* 10:275–281.
42. Ling, B., R.S. Veazey, A. Luckay, C. Penedo, K. Xu, J.D. Lifson, and P.A. Marx. 2002. SIV(mac) pathogenesis in rhesus macaques of Chinese and Indian origin compared with primary HIV infections in humans. *AIDS.* 16:1489–1496.

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Loss of virus-specific CD4$^+$ T cells with increases in viral loads in the chronic phase after vaccine-based partial control of primary simian immunodeficiency virus replication in macaques

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Virus-specific cellular immune responses play an important role in the control of immunodeficiency virus replication. However, preclinical trials of vaccines that induce virus-specific cellular immune responses have failed to contain simian immunodeficiency virus (SIV) replication in macaques. A defective provirus DNA vaccine system that efficiently induces virus-specific CD8$^+$ T-cell responses has previously been developed. The vaccinated macaques showed reduced viral loads, but failed to contain SIVmac239 replication. In this study, macaques that showed partial control of SIV replication were followed up to see if or how they lost this control in the chronic phase. Two of them showed increased viral loads about 4 or 8 months after challenge and finally developed AIDS. Analysis of SIV-specific T-cell levels by detection of SIV-specific gamma interferon (IFN-γ) production revealed that these two macaques maintained SIV-specific CD8$^+$ T cells, even after loss of control, but lost SIV-specific CD4$^+$ T cells when plasma viral loads increased. The remaining macaque kept viral loads at low levels and maintained SIV-specific CD4$^+$ T cells, as well as CD8$^+$ T cells, for more than 3 years. Additional analysis using macaques vaccinated with a Gag-expressing Sendai virus vector also found loss of viraemia control, with loss of SIV-specific CD4$^+$ T cells in the chronic phase of SIV infection. Thus, SIV-specific CD4$^+$ T cells that were able to produce IFN-γ in response to SIV antigens were preserved by the vaccine-based partial control of primary SIV replication, but were lost with abrogation of control in the chronic phase.

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INTRODUCTION

Cellular immune responses play a critical role in the control of immunodeficiency virus infections (Brander & Walker, 1999; Seder & Hill, 2000). The importance of virus-specific CD8$^+$ T cells, especially cytotoxic T lymphocytes (CTLs), in this control has been indicated in human immunodeficiency virus type 1 (HIV-1)-infected individuals (Borrow *et al.*, 1994; Koup *et al.*, 1994; Ogg *et al.*, 1998) and in macaque AIDS models (Matano *et al.*, 1998; Jin *et al.*, 1999; Schmitz *et al.*, 1999). Therefore, CTL-based vaccine strategies may be promising for the development of AIDS vaccine candidates.

AIDS vaccine strategies have been evaluated in macaque models by using simian immunodeficiency viruses (SIV) or simian–human immunodeficiency viruses (SHIV) (Nathanson *et al.*, 1999). Macaques infected with a pathogenic SIV strain, SIVmac239 (Kestler *et al.*, 1990), generally show chronic clinical courses in the development of AIDS, whereas infections with a pathogenic SHIV strain, SHIV89.6P (Karlsson *et al.*, 1997), induce acute CD4$^+$ T-cell depletion in a few weeks, leading to the acute onset of AIDS in macaques. Recently, in the latter model, several groups have developed vaccine strategies that induced high levels of virus-specific CTLs, leading to containment of SHIV89.6P replication (Barouch *et al.*, 2000; Amara *et al.*, 2001; Matano *et al.*, 2001; Rose *et al.*, 2001; Shiver *et al.*, 2002). However, it has been suggested that SIV infection models may reflect HIV-1 infections in humans more closely, whereas no preclinical vaccine trials successfully contained SIV replication in rhesus macaques (Feinberg & Moore, 2002; Horton *et al.*, 2002).

We previously developed a DNA vaccine system by using FMSIV (Matano *et al.*, 2000), which is a chimeric SHIV with ecotropic Friend murine leukaemia virus (FMLV) *env* in place of SHIV *env*, in combination with the FMLV receptor, mCAT1 (Albritton *et al.*, 1989), which is not normally expressed in primate cells. Vaccination of macaques with both FMSIV proviral DNA and mCAT1 expression plasmid DNA allowed mCAT1-dependent FMSIV replication and efficiently induced SIV-specific $CD8^+$ T-cell responses (Matano *et al.*, 2000; Takeda *et al.*, 2000). After intravenous SIVmac239 challenge, the vaccinated macaques showed low plasma viral loads during the early phase of infection, although they failed to contain SIV replication.

Further, we established a Sendai virus (SeV) vector-based vaccine system that efficiently induced virus-specific $CD8^+$ T-cell responses (Kano *et al.*, 2002). Intranasal immunization of macaques with a recombinant SeV vector expressing SIV Gag (SeV–Gag) elicited Gag-specific $CD8^+$ T-cell responses, leading to marked reduction in set point plasma viral loads after intravenous SIVmac239 challenge (Kano *et al.*, 2000).

This study is a longitudinal analysis of those vaccinated macaques that showed partial control of primary SIVmac239 replication after challenge. Analysis revealed that some of them failed to keep plasma viral loads at low levels. Analysis of SIV-specific T-cell levels by detection of SIV-specific gamma interferon (IFN-γ) production revealed that the increases in viral loads in the chronic phase were accompanied by loss of SIV-specific $CD4^+$ T cells, but occurred in the presence of SIV-specific $CD8^+$ T cells.

METHODS

DNA and SeV vectors. DNA of an infectious SHIV clone, $SHIV_{MD14YE}$ (Shibata *et al.*, 1997), was provided by M. A. Martin. The gene fragment encoding the Env surface protein of $SHIV_{MD14YE}$ was removed and replaced with an FMLV *env* fragment (Koch *et al.*, 1983) to obtain infectious FMSIV clone DNA, as described previously (Matano *et al.*, 2000). The 3′ portion of the *env* gene and the 5′ quarter of the *nef* gene were deleted in the FMSIV DNA. Therefore, the FMSIV DNA has SIV-derived long terminal repeat, *gag*, *pol*, *vif*, *vpx* and partial *vpr* sequences, HIV-1-derived partial *vpr*, *tat*, *rev* and partial *env* (containing the second exon of *tat*, the second exon of *rev*, and RRE) sequences and FMLV-derived *env* sequences. A plasmid expressing mCAT1, pJET (Albritton *et al.*, 1989), was provided by J. M. Cunningham. An *env*- and *nef*-deleted SHIV proviral DNA, SIVGP1 DNA, was obtained by removing the whole FMLV *env* region from the FMSIV DNA (Matano *et al.*, 2001). An infectious SIVmac239 clone DNA, pBRmac239, was provided by T. Kodama and R. C. Desrosiers (Kestler *et al.*, 1990). The plasmid pVSV-G, which expresses vesicular stomatitis virus G protein (VSV-G), was purchased from Clontech. A recombinant SeV vector expressing SIV Gag (SeV–Gag) was prepared as described previously (Kato *et al.*, 1996; Kano *et al.*, 2002).

Animal experiments. All Indian rhesus macaques (*Macaca mulatta*) and cynomolgus macaques (*Macaca fascicularis*) used in this study were male and were maintained in accordance with the Guidelines for Laboratory Animals of the National Institute of Infectious Diseases. These macaques tested negative for SeV and SIV before use. Blood collection, lymph node (LN) biopsy, vaccination and virus challenge were performed under ketamine anaesthesia. The DNA vaccine experiment using rhesus macaques was performed as described previously (Matano *et al.*, 2000). Rhesus macaques #20, #21 and #18 received FMSIV DNA and pJET (100 or 200 μg of each DNA intramuscularly and 5 or 10 μg of each DNA by gene gun) five times at weeks 0, 1, 2, 6 and 12 after the initial vaccination. Rhesus macaque #17 received 200 μg FMSIV DNA intramuscularly and 10 μg FMSIV DNA by gene gun five times at weeks 0, 1, 2, 6 and 12 as a control. Rhesus macaque #22 was a naive control. These macaques were challenged intravenously with 100 $TCID_{50}$ (50 percent tissue culture infective dose) of SIVmac239 12 weeks after the last vaccination. Macaque #21 was observed until week 218 after challenge and other rhesus macaques were observed until their death. The SeV–Gag vaccine experiment using cynomolgus macaques was performed as described previously (Kano *et al.*, 2000). Cynomolgus macaques Cy01 and Cy62 were immunized intranasally with 10^8 cell infectious units (CIU) of SeV–Gag three times at weeks 0, 4 and 14 after the initial vaccination and challenged intravenously with 100 $TCID_{50}$ of SIVmac239 8 weeks after the last vaccination. These macaques were observed until week 60. Diagnosis of AIDS was based on clinical signs, such as diarrhoea and loss of body weight, and histological signs, such as lymphocyte depletion and lymphoma.

Quantification of plasma viral loads. Plasma RNA was extracted by using a High Pure Viral RNA kit (Roche). For quantification of plasma SIV RNA levels, serial five-fold dilutions of RNA samples were amplified in quadruplicate by reverse transcription and nested PCR using SIV *gag*-specific primers to determine the end point as described previously (Shibata *et al.*, 1997). For preparing the RNA standard, we first set up the method for quantification of SHIV RNA copy number by using HIV-1 *vpu*-specific primers and an HIV-1 standard, which was quantified by an Amplicor HIV-1 monitor (Roche). By using this method, we prepared an SHIV standard for the present assay. At several time-points, RNA copy number was reassessed by real-time PCR using the LightCycler system (Roche) with SIV *gag*-specific primers (GTAGTATGGGCAGCAAATGA and TGTTCCTGTTTCCACCACTA) and probes (GCATTCACGCAGA-AGAGAAAGTGAAACA and ACTGAGGAAGCAAAACAGATAGT-GCAGAGA).

Flow cytometric analysis of SIV-specific IFN-γ induction. We measured frequencies of SIV-specific T cells by flow cytometric analysis of intracellular IFN-γ induction after SIV-specific stimulation, as described previously (Matano *et al.*, 2001). In brief, COS-1 cells were cotransfected with SIVGP1 and pVSV-G to obtain a pseudotyped SIV bearing VSV-G, SIVGP1(VSV-G). Alternatively, another pseudotyped SIV, SIV239(VSV-G), was obtained by cotransfection of COS-1 cells with pBRmac239 and pVSV-G. Peripheral blood mononuclear cells (PBMCs) were prepared by density-gradient centrifugation using Ficoll-Paque PLUS (Amersham Biosciences) and frozen until use. For SIV-specific stimulation, PBMCs were co-cultured with autologous herpesvirus papio-immortalized B lymphoblastoid cells (BLCs) (Voss *et al.*, 1992) that were infected with SIVGP1(VSV-G) or SIV239(VSV-G). Stimulation with SIVGP1(VSV-G)-infected BLCs was expected to stimulate SIV Gag-, Pol-, Vif- and Vpx-specific T cells and is referred to as Env-independent SIV-specific or SIVGagPol-specific stimulation. On the other hand, stimulation with SIV239(VSV-G) was expected to stimulate all T cells that were reactive to SIV antigens and is referred to as SIVGagPolEnv-specific stimulation. For non-specific stimulation, a VSV-G-pseudotyped murine leukaemia virus (MLV), MLVGP(VSV-G), was used instead of SIVGP1(VSV-G) or SIV239(VSV-G). After co-culture in the presence of GolgiStop (monensin) (Becton Dickinson) for 6 h, intracellular IFN-γ staining was performed by using a Cytofix–Cytoperm kit (Becton Dickinson).

FITC-conjugated anti-human CD4, peridinin chlorophyll protein-conjugated anti-human CD8, allophycocyanin-conjugated anti-human CD3, FITC-conjugated anti-human CD45RA and phycoerythrin-conjugated anti-human IFN-γ antibodies (Becton Dickinson) were used. Stained samples were collected by FACSCalibur and analysed by using CellQuest software (Becton Dickinson). SIV-specific T-cell levels were calculated by subtracting the IFN-γ^+ T-cell frequencies after non-specific stimulation from those after SIV-specific stimulation. The frequencies of CD4$^+$ IFN-γ^+ T cells in CD4$^+$ T cells or those of CD8$^+$ IFN-γ^+ T cells in CD8$^+$ T cells after non-specific stimulation were $<0{\cdot}05$ %.

RESULTS

Follow-up of DNA-vaccinated macaques after SIV challenge

In our previous study (Matano *et al.*, 2000), two rhesus macaques (#20 and #21) that had been immunized with our DNA vaccine system using FMSIV and mCAT1 DNA were challenged intravenously with SIVmac239; both of them showed reduced plasma viral loads, compared to the control rhesus macaques (#22 and #17) in the early phase of infection. In addition, rhesus macaque #18 was subjected to the same FMSIV and mCAT1 DNA vaccine and SIVmac239 challenge protocol and also showed partial control of primary SIV replication with low plasma viral loads, $<2\times10^3$ copies ml^{-1}, at the set point. We followed up these macaques in the chronic phase of SIV infection in this study.

The unvaccinated macaque, #22, failed to control viraemia after challenge and showed acute onset of AIDS, as described previously (Matano *et al.*, 2000). The animal lost its body weight with severe diarrhoea and maintained high plasma viral loads, $>1\times10^6$ RNA copies ml^{-1}, until its death at week 17. Macaque #17, which was vaccinated with FMSIV DNA alone, also failed to control viraemia, with high set point plasma viral loads of $>1\times10^5$ RNA copies ml^{-1}, as described previously (Matano *et al.*, 2000), and showed disease progression with loss of body weight until euthanasia at week 45 (data not shown). The autopsy revealed malignant lymphoma and *Pneumocystis carinii* pneumonia.

Three macaques that were vaccinated with FMSIV and mCAT1 DNA showed partial control of primary SIV replication, but two of them lost this control and developed AIDS 2 or 3 years after challenge. In macaque #18 (Fig. 1), plasma viral loads were kept at low levels ($<2\times10^3$ copies ml^{-1}) until week 24 after challenge, but began to increase after that. The animal maintained high viral loads, about 1×10^6 RNA copies ml^{-1}, after week 36, began to lose body weight after week 54 and died at week 81. In macaque #20 (Fig. 2), plasma viral loads were kept at low levels, about 1×10^4 RNA copies ml^{-1}, until week 12, but began to increase after that. Peripheral CD4$^+$ T-cell counts decreased gradually after week 15. The animal remained alive with a high viral load for more than

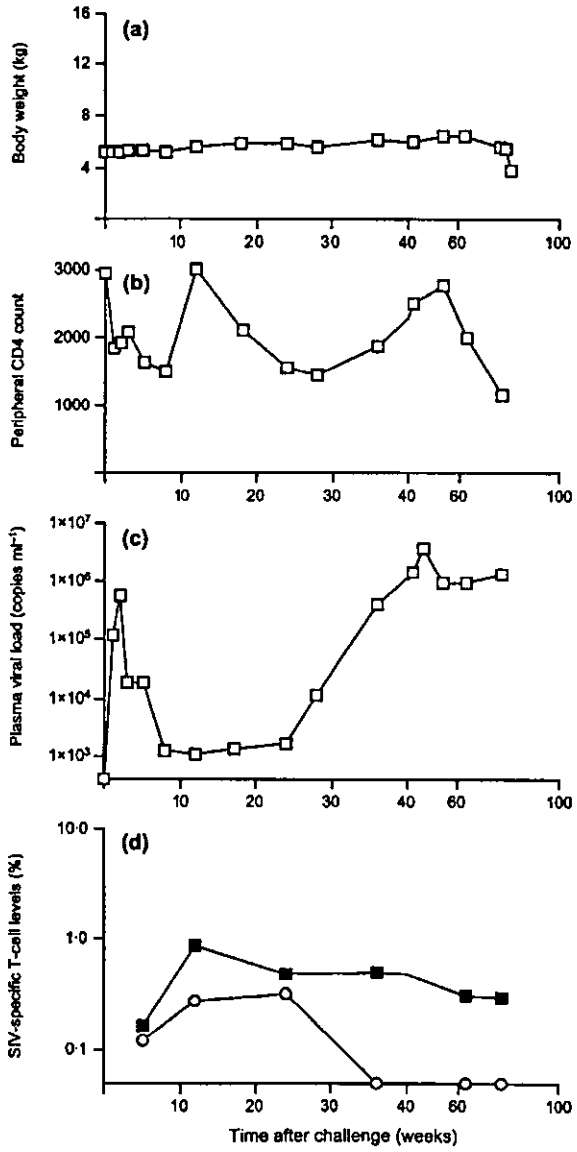

Fig. 1. Follow-up of macaque #18 after SIV challenge. (a) Body weight (kg); (b) peripheral CD4$^+$ T-cell count (μl^{-1}); (c) plasma viral load (SIV RNA copy number ml^{-1}); (d) SIV-specific T-cell level, measured by assessing frequencies of IFN-γ producing cells after SIVGagPol-specific stimulation. Proportions of SIVGagPol-specific CD4$^+$ T-cell number in the total CD4$^+$ T-cell number (○) and of SIVGagPol-specific CD8$^+$ T-cell number in the total CD8$^+$ T-cell number (■) are shown.

2 years, but finally developed AIDS and was euthanized at week 136. On the other hand, macaque #21 showed sustained control of SIV replication without disease for more than 3 years (Fig. 3). Plasma viral loads were below or just above the detectable level from week 31 to week

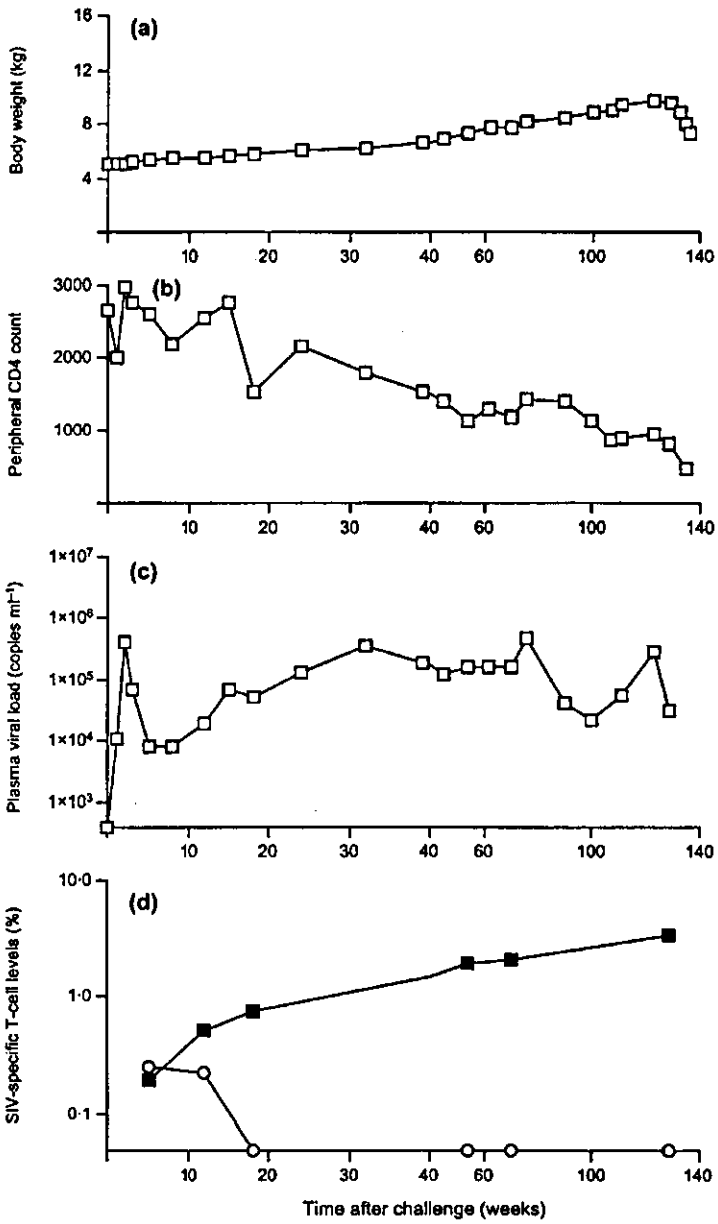

Fig. 2. Follow-up of macaque #20 after SIV challenge. Legend as for Fig. 1.

139 and then began to increase gradually, but were still $<2 \times 10^4$ RNA copies ml^{-1}, even at week 218.

SIV-specific T-cell levels in DNA-vaccinated macaques after SIV challenge

The FMSIV DNA used in the vaccine has SIVmac239 Gag-, Pol-, Vif- and Vpx-coding regions. To detect T cells that were specific for the FMSIV-derived SIV antigens, we previously developed the SIVGagPol-specific stimulation method (see Methods). We measured SIV-specific T-cell levels by using this method in the present study. In addition, we examined IFN-γ induction after SIVGagPolEnv-specific stimulation (see Methods) at several time-points to detect all T cells that were reactive to SIV antigens, including Env.

We first examined SIV-specific T-cell levels at week 12 after challenge in both control macaques that failed to control viraemia (data not shown). SIV-specific $CD8^+$ T cells were undetectable in macaque #22, but were detected in macaque #17. However, SIV-specific $CD4^+$ T cells were undetectable in both macaques.

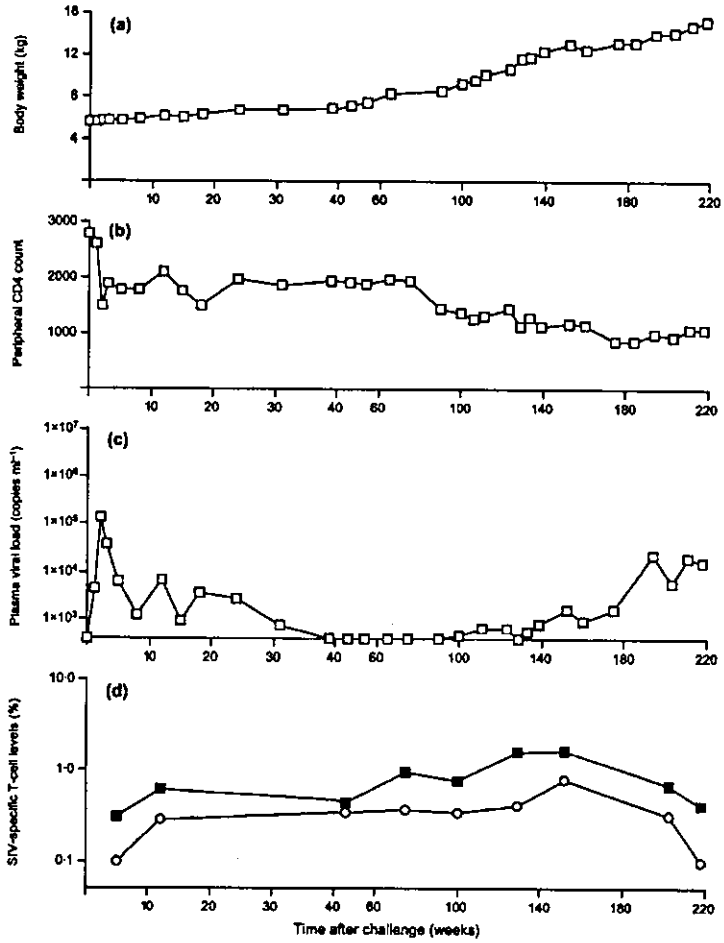

Fig. 3. Follow-up of macaque #21 after SIV challenge. Legend as for Fig. 1.

In contrast, SIV-specific $CD4^+$ T cells, as well as $CD8^+$ T cells, were detected in the early phase in all three vaccinated macaques, which showed low set point viral loads. In macaque #18, SIV-specific $CD4^+$ T cells and $CD8^+$ T cells were detected at week 5 and their levels increased at week 12. Levels of both were maintained at week 24, when the animal still kept viral loads at low levels, but SIV-specific $CD4^+$ T cells were lost suddenly at week 36, when plasma viral loads increased (Fig. 1). In contrast, SIV-specific $CD8^+$ T cells were maintained after that until death.

In macaque #20, SIV-specific $CD4^+$ T cells and $CD8^+$ T cells were both maintained until week 12, but SIV-specific $CD4^+$ T cells were lost at week 18, when plasma viral loads increased to $>1 \times 10^5$ RNA copies ml^{-1} (Fig. 2). In contrast, SIV-specific $CD8^+$ T cells were maintained after that and their levels increased until death. Not only SIVGagPol-specific $CD4^+$ T cells, but also SIVGagPolEnv-specific $CD4^+$ T cells were undetectable after loss of the partial control in macaques #18 and #20 (#18, at weeks 42 and 77; #20, at weeks 32, 62 and 90).

In macaque #21, which showed sustained control, SIV-specific $CD4^+$ T cells, as well as $CD8^+$ T cells, were detected in the early phase and were maintained for more than 3 years (Fig. 3). After this time, the animal showed gradual increases in plasma viral loads and decreases in SIV-specific $CD4^+$ T-cell levels. However, these cells were still detectable at week 218.

Specific $CD8^+$ T cells with IFN-γ-producing function can be divided into $CD45RA^-$ and $CD45RA^+$ subpopulations; the latter has been suggested to be more differentiated (Hamann *et al.*, 1997; Sallusto *et al.*, 1999). We examined whether the $CD45RA^+$ subpopulation in SIV-specific $CD8^+$ T cells was maintained in macaques #20 and #21 (Fig. 4). In macaque #21, about 30 % of SIV-specific $CD8^+$ T cells were $CD45RA^+$ at week 12 and this proportion was almost constant, even in the late phase, indicating maintenance of SIV-specific $CD8^+$ $CD45RA^+$ T cells. Macaque #20 showed a higher percentage of the $CD45RA^+$ subpopulation in SIV-specific $CD8^+$ T cells, compared to macaque #21, at week 12. The fraction decreased, but was still >30 % at week 39. Although levels of SIV-specific