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
Yasutomi Y.	Chimeric recombinant hepatitis E virus–like particles presenting foreign epitopes as a novel vector of vaccine by oral administration.	Holland CR & Miyamura T	Structure-based viral replication.	World Scientific Publishing	USA	2006	in press

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
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	23	3166-3173	2005
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Ⅳ.　研究成果の刊行物・別刷

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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

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Abstract

Recent prophylactic vaccine trials inducing virus-specific $CD8^+$ T-cell responses have shown control of primary infections of a pathogenic simian-human immunodeficiency virus (SHIV) in macaques. In the chronic phase, therapeutic immunization replenishing virus-specific $CD8^+$ T-cells is likely to contribute to sustained control of virus replication. In this study, we have administered a recombinant Sendai virus (SeV) vector into five rhesus macaques that had received prophylactic vaccinations and had controlled SHIV replication for more than 1 year after challenge. Our results indicate that virus-specific $CD8^+$ T-cell responses can be expanded and broadened by therapeutic immunization with SeV vectors in the chronic phase after prophylactic vaccine-based control of primary immunodeficiency virus infections.

Keywords: AIDS; Sendai virus; Therapeutic vaccine

1. Introduction

Virus-specific $CD8^+$ cytotoxic T lymphocyte (CTL) responses play a central role in the control of immunodeficiency virus infections. The importance of CTL in the control has been indicated not only in the acute phase but also in the chronic phase of infections by several clinical correlations in human immunodeficiency virus type 1 (HIV-1)-infected humans [1–3] and $CD8^+$ T-cell-depletion experiments in macaque AIDS models [4–6]. Therefore, AIDS vaccine studies have been making efforts to develop methods efficiently inducing virus-specific $CD8^+$ T-cell responses.

Recombinant viral vectors can be a promising tool for AIDS vaccines because of their potential for inducing virus-specific $CD8^+$ T-cell responses. Recently, preclinical trials of prophylactic vaccines using recombinant viral vectors have shown control of primary infections of a pathogenic simian-human immunodeficiency virus (SHIV) that induces acute $CD4^+$ T-cell depletion in macaques [7–10]. These vaccinated macaques have contained the challenge virus leading to reduction in plasma viral loads to be undetectable at the setpoint and maintained peripheral $CD4^+$ T-cell counts, although they have failed to eliminate the virus and shown detectable levels of proviral DNA in lymphocytes in the chronic phase

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[11]. $CD8^+$ T-cell depletion by anti-CD8 monoclonal antibody treatment in these macaques in the chronic phase after prophylactic vaccine-based control of primary SHIV infection has shown a rise in plasma viral loads [12]. Additionally, loss of the control due to appearance of a CTL escape mutant has been observed in the chronic phase in a macaque that had controlled primary SHIV infection [13]. Thus, virus-specific $CD8^+$ T-cell responses have been indicated to play a central role in maintaining the control of virus replication in the chronic phase and therapeutic immunization replenishing virus-specific $CD8^+$ T-cell responses are likely to contribute to the sustained control.

We previously developed a prophylactic DNA vaccine system that uses FMSIV [14], which is a chimeric SHIV with ecotropic Friend murine leukemia virus (FMLV) *env* in place of SHIV *env*, in combination with the FMLV receptor, mCAT1 [15], which is not normally expressed in primate cells. Vaccination of macaques with both of the FMSIV proviral DNA and an mCAT1-expression plasmid DNA induced mCAT1-dependent FMSIV replication leading to efficient elicitation of virus-specific $CD8^+$ T-cell responses. We also established a prophylactic Sendai virus (SeV) vector-based vaccine system [16–19]. Not only the replication-competent (transmissible) but also the replication-defective (non-transmissible) SeV vector showed the potential for efficiently inducing virus-specific $CD8^+$ T-cell responses [20,21]. Additionally, combination of the DNA vaccine and the SeV vector vaccine, DNA-prime/SeV-boost, elicited extremely high levels of virus-specific $CD8^+$ T-cell responses [8]. Preclinical trials of these prophylactic vaccine systems showed control of replication of a pathogenic SHIV89.6PD and prevented macaques from acute AIDS progression [8,21].

In this study, we have examined if the SeV vector can be used for therapeutic immunization to induce virus-specific $CD8^+$ T-cell responses in the chronic phase. We considered Gag as a promising vaccine-antigen candidate to avoid CTL escape because it has been indicated that Gag CTL escape variants mostly diminish viral fitness and require multiple additional compensatory mutations to restore their replicative competence [22]. We administered a Gag-expressing SeV (SeV-Gag) vector into those macaques that had controlled SHIV replication for more than 1 year after challenge and analyzed Gag-specific T-cell responses.

2. Materials and methods

2.1. Animals

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

Macaques used in this study were previously subjected to prophylactic vaccination and challenge experiments (Table 1) [8,23]. In brief, macaques R011 and R012 received four times FMSIV plus mCAT1 DNA vaccinations and single intranasal SeV-Gag booster, whereas macaques R003 and R006 were boosted intranasally with a recombinant SeV expressing HIV-1 Tat (SeV-Tat) after the DNA vaccinations. Macaque R022 received the DNA vaccinations only. An infectious FMSIV clone DNA obtained by replacing the gene fragment encoding Env surface protein of $SHIV_{MD14YE}$ [24] with an FMLV *env* fragment [25] has simian immunodeficiency virus-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 [14]. At each DNA vaccination, animals received 800 μg of individual DNA intramuscularly and 10 μg of individual DNA by gene gun. At the booster, animals received 1×10^8 cell infectious units (CIU) of replication-competent F(+)SeV-Tat or F(+)SeV-Gag. Animals were challenged intravenously with 10 TCID50 (50% tissue culture infective doses) of SHIV89.6PD [26].

2.2. Therapeutic immunization

We used two kinds of SeV vectors expressing SIVmac239 gag, a replication-competent one (F[+]SeV-gag) and a replication-defective F-deleted one (F[−]SeV-gag), for therapeutic immunization. Recombinant F(+)SeV-Gag and F(−)SeV-Gag were prepared as described previously [16,19,20]. Animals received 1×10^8 CIU of F(+)SeV-Gag (macaques R003 and R006) or 6×10^9 CIU of F(−)SeV-

Table 1
Vaccination and challenge protocol in macaques

Macaques	Prophylactic vaccination	Challenge	Therapeutic vaccination
R003	DNA and F(+)SeV-Tat	SHIV89.6PD	F(+)SeV-Gag at week 56
R006	DNA and F(+)SeV-Tat	SHIV89.6PD	F(+)SeV-Gag at week 56
R011	DNA and F(+)SeV-Gag	SHIV89.6PD	F(−)SeV-Gag at week 176
R012	DNA and F(+)SeV-Gag	SHIV89.6PD	F(−)SeV-Gag at week 176
R022	DNA	SHIV89.6PD	F(−)SeV-Gag at weeks 139 and 146

DNA vaccinations were performed four times at weeks 0, 0.5, 1, and 6 after the initial vaccination. SeV-Tat or SeV-Gag vaccination for booster was performed once at week 12 after the initial vaccination. Macaques R003, R006, R011, and R012 were challenged with SHIV89.6PD at week 26 after the initial vaccination, whereas macaque R022 at week 14. Therapeutic SeV-Gag vaccination was performed at indicated time points after challenge. F(+)SeV-Tat and F(+)SeV-Gag are replication-competent and F(−)SeV-Gag is replication-defective.

Gag (macaques R011, R012, and R022) intranasally for the immunization.

2.3. Detection of SeV RNA in lymph nodes (LN)

Lymphocytes were prepared from minced lymph nodes (LN) by using Ficoll-Paque Plus (Amersham Biosciences). RNA was isolated from 1×10^6 lymphocytes by using RNeasy Mini kit (Qiagen K.K.) and eluted by 50 μl of water. Ten microliters of RNA was subjected to reverse transcription and nested PCR (RT-PCR) using SeV NP-specific primers (ATGGCCGGGTTGTTGAG and GGGCTCTTGTTGACCATAGG for the first RT-PCR, and AGTCGGGAAGAGGTGCTG and CGTCTTCACAATGAATCCGTC for the second DNA PCR) for detection of SeV RNA.

2.4. Quantitation of plasma viral loads

Plasma RNA was extracted using high pure viral RNA kit (Roche Diagnostics). Serial 5-fold dilutions of RNA samples were amplified in quadruplicate by nested RT-PCR using SIV *gag*-specific primers (AGAAACTCCGTCTTGTCAGG and TGATAATCTGCATAGCCGC for the first RT-PCR, and GATTAGCAGAAAGCCTGTTGG and TGCAACCTTCTGACAGTGC for the second DNA PCR) to determine the end-point. Plasma SIV RNA levels were calculated according to the Reed–Muench method as described [24,27]. The lower limit of detection in this assay is about 4×10^2 copies/ml.

2.5. Quantitation of proviral DNA levels in peripheral blood mononuclear cells (PBMC)

Genomic DNA was extracted from PBMC by using DNeasy kit (Qiagen K.K.). For quantitation of proviral SIV DNA copy numbers in cell lysates, serial 5-fold dilutions of cell lysates were amplified in quadruplicate by nested DNA-PCR using SIV *gag*-specific primers to determine the end-point as described [24]. The lower limit of detection in this assay is about 5 copies/μg DNA.

2.6. Measurement of antigen-specific T-cell frequencies

We measured antigen-specific T-cell frequencies by flow-cytometric analysis of interferon-γ (IFN-γ) induction after specific stimulation as described previously [8]. In brief, PBMC were cocultured with autologous herpesvirus papio-immortalized B lymphoblastoid cell lines (B-LCL) [28] infected with a vaccinia virus (Vv) vector [29] for non-specific Vv-control-stimulation, B-LCL infected with a Vv vector expressing SIVmac239 Gag for Gag-specific Vv-Gag-stimulation, and B-LCL infected with SeV for SeV-specific stimulation, respectively. Intracellular IFN-γ staining was performed by using Cytofix-Cytoperm kit (BD Biosciences). Fluorescein isothiocyanate (FITC)-conjugated anti-human CD4, peridinin chlorophyll protein (PerCP)-conjugated anti-human CD8, allophycocyanin (APC)-conjugated anti-human CD3, and phycoerythrin (PE)-conjugated anti-human IFN-γ antibodies (BD Biosciences) were used. Gag-specific T-cell frequencies and SeV-specific T-cell frequencies were calculated by subtracting the IFN-γ^+ T-cell frequencies after non-specific Vv-control-stimulation from those after Gag-specific Vv-Gag-stimulation and those after SeV-specific stimulation, respectively. The background IFN-γ^+ T-cell frequencies after non-specific Vv-control-stimulation were less than 200 cells per million PBMC. Gag-specific T-cell frequencies (and SeV-specific T-cell frequencies) less than 100 cells per million PBMC were considered negative, those between 100 and 200 borderline, and those greater than 200 positive.

In case of examining peptide-specific T-cell frequencies, B-LCL were pulsed with peptide mixture (final concentration of each peptide, 1–10 μM) for peptide-specific stimulation or incubated without peptide for non-specific stimulation. A panel of 117 overlapping peptides (15–17 amino acid (aa) in length and overlapping by 10–12 aa) spanning the entire SIVmac239 Gag aa sequence were purchased from Sigma Genosys, Japan, and divided into 10 pools, each consisting of 11 or 12 peptides. The background IFN-γ^+ T-cell frequencies were less than 100 cells per million PBMC. Peptide-specific T-cell levels less than 100 cells per million PBMC were considered negative, and those greater than 100 positive.

3. Results

3.1. SeV-Gag immunization into macaques chronically infected with SHIV

In this study, we used five rhesus macaques that had received prophylactic vaccines and had controlled SHIV replication for more than 1 year after challenge (Table 1). All five macaques (R003, R006, R011, R012, and R022) had received DNA vaccinations; additionally, macaques R003 and R006 had been boosted with SeV-Tat whereas macaques R011 and R012 boosted with SeV-Gag as described previously [8,23]. They had been challenged with SHIV89.6PD 8 weeks (in R022) or 14 weeks (in R003, R006, R011, and R012) after the last vaccination. Plasma viral loads had been below the detectable levels after the setpoint and peripheral $CD4^+$ T-cell counts had been maintained until therapeutic immunization in all five macaques (data not shown). Macaques R003 and R006 received therapeutic immunization with replication-competent F(+)SeV-Gag at week 56 post-challenge, whereas macaques R011 and R012 with replication-defective F(−)SeV-Gag at week 176. Macaque R022 was immunized with F(−)SeV-Gag twice at weeks 139 and 146.

No macaques displayed apparent clinical symptoms after the therapeutic SeV-Gag immunization. No apparent pathological signs were observed by histological analysis of tissues obtained at autopsy from macaque R003 euthanized 1 week post-immunization (p.i.) (at week 57 after challenge),

Table 2
Detection of SeV RNA by nested RT-PCR

Macaques	Therapeutic vaccination	Autopsy	SeV RNA[a]		
			SM-LN	MC-LN	IG-LN
R003	At week 56	At week 57	Positive	Negative	Negative
R006	At week 56	At week 58	Negative	Negative	Negative
R011	At week 176	At week 181	Negative	Negative	Negative
R012	At week 176	At week 181	Positive	Negative	Negative
R022	At weeks 139 and 146	At week 147	Positive	Negative	Negative

[a] RNA was extracted from LN-derived lymphocytes and nested RT-PCR was performed for detection of SeV RNA. SM-LN, submandibular LN; MC-LN, mesenchymal LN; IG-LN, inguinal LN.

macaque R006 euthanized 2 weeks p.i. (at week 58), R011 or R012 euthanized 5 weeks p.i. (at week 181) or R022 euthanized 1 week after the second SeV-Gag immunization (at week 147). SeV RNA was detected by nested RT-PCR in the submandibular lymph node in three macaques (R003, R012, and R022) but undetectable in other two (R006 and R011) (Table 2). In the mesenchymal LN and the inguinal LN, however, SeV RNA was undetectable in all five macaques.

3.2. *Gag-specific T-cell responses after therapeutic SeV-Gag immunization*

To see the effect of therapeutic SeV-Gag immunization on Gag-specific T-cell responses, we measured Gag-specific T-cell frequencies in PBMC before and after the immunization by detection of Gag-specific IFN-γ induction. In all five macaques, Gag-specific $CD8^+$ T-cell levels were increased after SeV-Gag immunization (Fig. 1). The second SeV-Gag immunization at week 146, 7 weeks after the first immunization, also increased the levels in macaque R022.

In macaques R012 and R022, Gag-specific $CD8^+$ T-cell responses were not clearly detected before SeV-Gag immunization but appeared after that, indicating that new epitope-specific $CD8^+$ T-cell responses were induced by the immunization. In contrast, Gag-specific $CD8^+$ T-cells were detectable even before immunization and their levels were largely increased after that in macaques R003, R006, and R011. We then examined whether increases in their levels were only due to expansion of epitope-specific $CD8^+$ T-cells that had been detectable before immunization or new-epitope specific $CD8^+$ T-cells were induced by the immunization. Using a panel of overlapping peptides spanning the entire SIV Gag aa sequence, IFN-γ induction was assessed after stimulation with pools of peptides (Fig. 2). In macaque R003, analysis of PBMC at week 56 (just before immunization) detected $CD8^+$ T-cells specific for three pools of peptides, #4 (corresponding to the 155th–213th aa in SIV Gag), #5 (202nd–265th aa), and #10 (453rd–510th aa). At week 57, 1 week p.i., no significant changes were observed in #4-specific $CD8^+$ T-cell or #10-specific $CD8^+$ T-cell levels, but #5-specific $CD8^+$ T-cells expanded efficiently. Additionally, the immunization induced #7 (306th–364th aa)-specific $CD8^+$ T-cells that had been undetectable before immunization. In

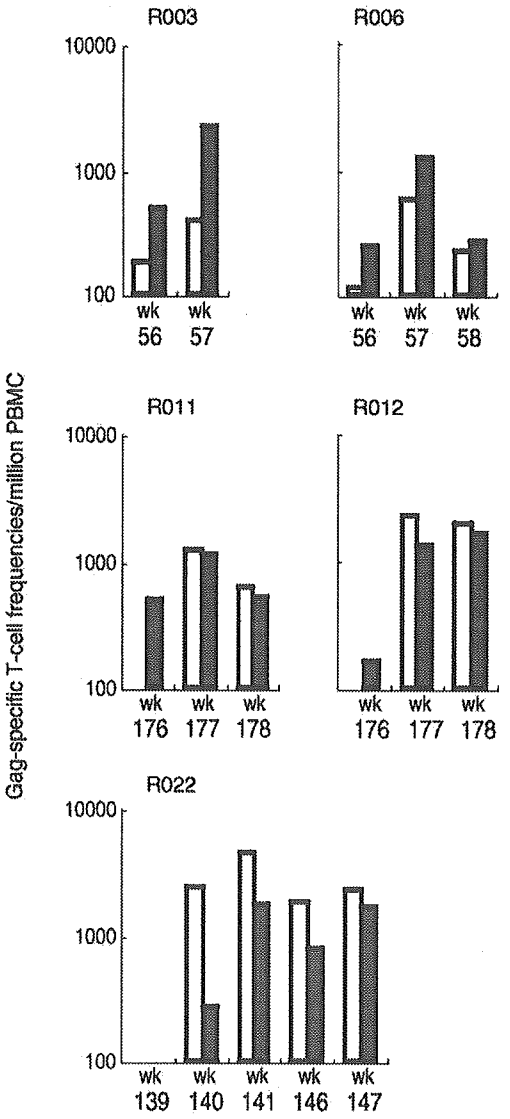

Fig. 1. Frequencies of Gag-specific $CD4^+$ T-cells (open bar) and $CD8^+$ T-cells (shaded bar) in PBMC before and after therapeutic SeV-Gag immunization. Macaques R003 and R006 were immunized at week 56 post-challenge, macaques R011 and R012 at week 176, and macaque R022 at weeks 139 and 146.

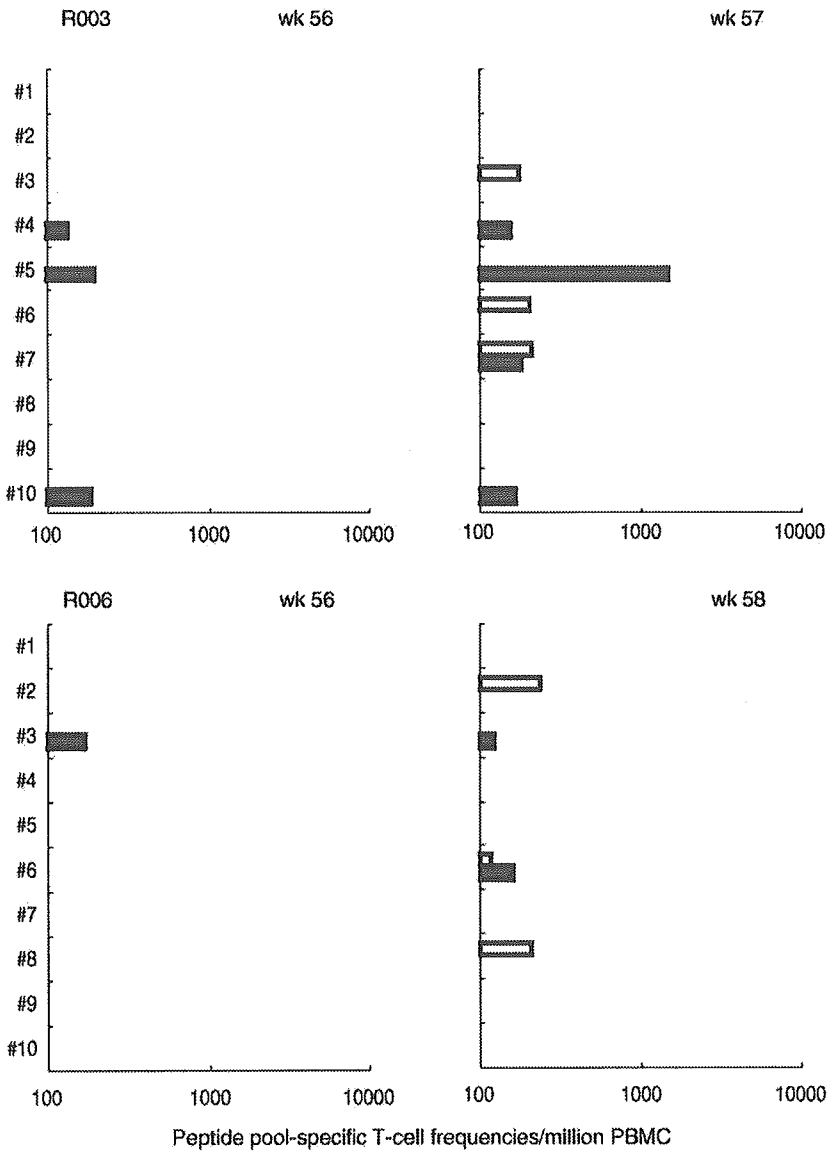

Fig. 2. Frequencies of $CD4^+$ T-cells (open bar) and $CD8^+$ T-cells (shaded bar) specific for pools of SIV Gag peptides in PBMC. A panel of overlapping peptides spanning the entire SIV Gag aa sequence were divided into 10 pools (each consisting of 11 or 12 peptides), #1 (corresponding to the 1st–65th aa in SIV Gag), #2 (55th–114th aa), #3 (104th–165th aa), #4 (155th–213th aa), #5 (202nd–265th aa), #6 (255th–316th aa), #7 (306th–364th aa), #8 (354th–416th aa), #9 (406th–464th aa), and #10 (453rd–510th aa), and used for the stimulation to detect peptide pool-specific T-cells.

macaque R006, #3 (104th–165th aa)-specific $CD8^+$ T-cell levels remained unchanged at week 58, 2 weeks p.i. The immunization, however, induced #6 (255th–316th aa)-specific $CD8^+$ T-cells that had been undetectable before immunization. Thus, the immunization induced new epitope-specific $CD8^+$ T-cells that had been undetectable before immunization in macaques R003 and R006 as well as in macaques R012 and R022, indicating that therapeutic SeV-Gag immunization not only expanded but also broadened Gag-specific $CD8^+$ T-cell responses. We failed to obtain enough PBMC samples for analysis of peptide-specific responses in macaque R011.

Therapeutic SeV-Gag immunization efficiently induced Gag-specific $CD4^+$ T-cell responses also although the responses were undetectable before immunization in all five macaques (Fig. 1). In macaques R003 and R006, several epitope-specific $CD4^+$ T-cells became detectable after immunization (Fig. 2).

3.3. SeV-specific T-cell responses after therapeutic SeV-Gag immunization

We also examined SeV-specific T-cell responses in macaques (Fig. 3). SeV-specific $CD8^+$ T-cells and $CD4^+$ T-cells both were undetectable just before immunization not only in macaque R022 that had been naive to SeV but also in other four macaques that had received a prophylactic vaccination with SeV vectors before challenge. In the latter, efficient induction of SeV-specific $CD8^+$ T-cell and $CD4^+$ T-

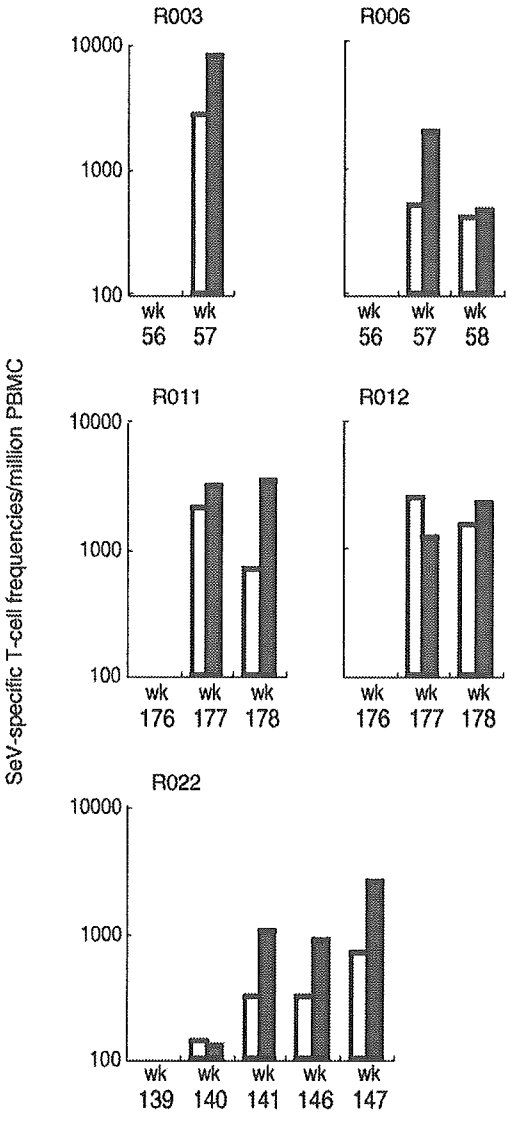

Fig. 3. Frequencies of SeV-specific $CD4^+$ T-cells (open bar) and $CD8^+$ T-cells (shaded bar) in PBMC before and after therapeutic SeV-Gag immunization.

cell responses both was observed 1 week p.i. In the former (R022), SeV-specific T-cell responses appeared with some delay and became apparent 2 weeks after the first immunization, whereas the second SeV-Gag immunization resulted in efficient expansion of SeV-specific T-cells in a week.

3.4. Viral loads after therapeutic SeV-Gag immunization

In all five macaques, plasma viremia remained undetectable after therapeutic SeV-Gag immunization. We then examined proviral DNA levels in PBMC in macaques R011, R012, and R022 (Fig. 4). These macaques kept proviral loads at low levels and we found no significant changes in their levels after immunization.

4. Discussion

The purpose of current therapeutic AIDS vaccines is to maintain HIV-1-specific CTL in the HIV-1-infected individuals who control viral replication and show little CTL responses, because CTL is crucial for sustained control in the chronic phase. The first object is HIV-1-infected individuals who control HIV-1 replication due to antiretroviral therapy, and therapeutic vaccines with recombinant viral vectors for replenishing CTL responses have been studied in HIV-1-infected individuals and SIV-infected macaques during antiretroviral treatment [30–32]. Further, HIV-1-infected individuals who show prophylactic vaccine-based control of HIV-1 replication can be an object of therapeutic vaccines, if an effective prophylactic AIDS vaccine is developed. Indeed, advances in recombinant viral vector technologies have contributed to progress in the development of CTL-based prophylactic AIDS vaccines, and recent studies in macaques have shown the importance of CTL maintenance for keeping prophylactic vaccine-based control of SHIV replication in the chronic phase [12]. Our study presents the first trial of therapeutic immunization into macaques that

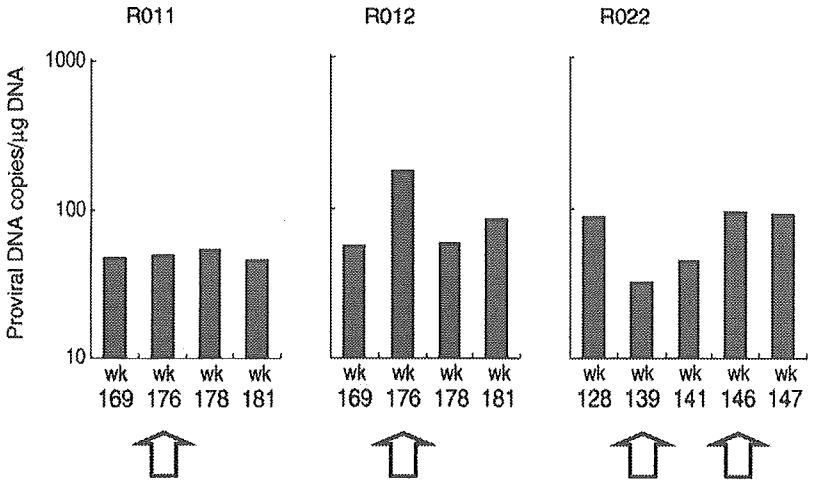

Fig. 4. SHIV proviral DNA copy numbers in PBMC before and after therapeutic SeV-Gag immunization. Arrows indicate the time-points of immunization.

have maintained prophylactic vaccine-based control of viral replication without antiretroviral help and suggests that the therapeutic SeV vector immunization can contribute to the maintenance of virus-specific CTL responses in the chronic phase.

The potential of the SeV vector as a prophylactic AIDS vaccine for inducing virus-specific T-cell responses have been studied, but its potential as a therapeutic vaccine has not yet been examined. The present study is the first report on its therapeutic administration in the chronic phase of immunodeficiency virus infections. Histological analysis revealed no pathological signs after immunization. The SeV vector distribution after therapeutic immunization was consistent with the previous analysis of prophylactic vaccination indicating that the vector was not disseminated but localized in the nasal mucosa and its primary LN [19]. These results support the notion that this vector can be safely used as a therapeutic vaccine.

Four of five macaques in this study had received a prophylactic SeV vaccination before SHIV challenge. SeV-specific T-cell responses that can interfere with the vector expression were undetectable before therapeutic SeV-Gag immunization but appeared rapidly after that. However, Gag-specific T-cell responses were efficiently induced in the presence of SeV-specific T-cell responses. Notably, SeV-Gag re-immunization only 7 weeks after the first immunization showed rapid expansion of SeV-specific T-cell responses but was able to augment Gag-specific T-cell responses although not so efficiently in macaque R022. These results suggest feasibility of SeV vector re-administration for induction of virus-specific T-cell responses.

In this study, Gag-specific $CD8^+$ T-cell responses were diminished in the chronic phase but augmented by therapeutic SeV-Gag immunization. Importantly, the immunization not only expanded but also broadened CTL responses. Broader responses may be advantageous for avoiding appearance of CTL escape mutants. A long-term follow-up study would be required to see if such CTL expansion and broadening by therapeutic immunization can contribute to sustained control of immunodeficiency virus replication.

Therapeutic SeV-Gag immunization elicited Gag-specific $CD4^+$ T-cell as well as Gag-specific $CD8^+$ T-cell responses in this study. It has been indicated that virus-specific $CD4^+$ T-cell as well as $CD8^+$ T-cell responses play an important role in the control of immunodeficiency virus infections [33–35]. Recent studies, however, have reported that HIV-1-infected patients with viremia frequently keep HIV-1-specific $CD4^+$ T-cells able to produce IFN-γ but do not have those able to proliferate and produce interleukin-2 in response to HIV-1 antigens, suggesting that the HIV-1-specific $CD4^+$ T-cell subpopulation able to produce IFN-γ may not contribute to the proliferative responses for the $CD4^+$ T-cell helper function [36,37]. Therefore, it has remained unclear if Gag-specific $CD4^+$ T-cell responses induced by SeV-Gag immunization can contribute to sustained control of virus replication. On the other hand, virus-specific $CD4^+$ T-cell induction may result in augmentation of virus replication because HIV-1 has been reported to preferentially infect HIV-1-specific specific $CD4^+$ T-cells [38]. However, no significant changes were observed in proviral loads after therapeutic SeV-Gag immunization in the present study.

In conclusion, we administered SeV-Gag as a therapeutic immunization into macaques that had maintained prophylactic vaccine-based control of SHIV replication for more than 1 year. Our results indicate that the therapeutic immunization can induce higher and broader virus-specific T-cell responses that may contribute to sustained control of immunodeficiency virus replication.

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Reversion In Vivo after Inoculation of a Molecular Proviral DNA Clone of Simian Immunodeficiency Virus with a Cytotoxic-T-Lymphocyte Escape Mutation

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Vaccine-based control of the replication of a simian immunodeficiency virus (SIV), SIVmac239, in macaques has recently been shown. In the process of the control, a mutant virus escaping from epitope-specific cytotoxic-T-lymphocyte (CTL) responses was rapidly selected and contained. In this study, we show that the wild-type virus appeared and became predominant in the absence of the epitope-specific CTL after inoculation of naive macaques with a molecular clone DNA of the CTL escape mutant SIV. This is the first report describing reversion in vivo from an inoculated, molecular proviral DNA clone of immunodeficiency virus with a CTL escape mutation.

Virus-specific $CD8^+$ cytotoxic-T-lymphocyte (CTL) responses are crucial for the control of immunodeficiency virus infection. The importance of CTL has been indicated by temporal association of CTL appearance with the resolution of primary viremia in human immunodeficiency virus type 1-infected humans (4, 13) and by monoclonal anti-CD8 antibody-mediated CD8 depletion experiments with macaque AIDS models (10, 15, 23). Therefore, AIDS vaccine studies have been making efforts to develop methods efficiently eliciting virus-specific CTL responses (18). However, viral escape from CTL recognition can lead to viral evasion from immune control and has frequently been observed in immunodeficiency virus infection (1, 5, 8, 19, 21, 22). Under strong immune pressure exerted by CTL, viruses are often forced to mutate, with viral fitness costs, to escape from the CTL responses (7, 9, 11, 17, 20, 24). Some CTL escape mutant viruses with lower viral fitness require additional compensatory mutations to restore their replicative competence (6, 11, 20). It is important to evaluate replicative ability of CTL escape mutants in vivo.

Recently, CTL-based control of replication of a pathogenic simian immunodeficiency virus (SIV), SIVmac239 (12), has been shown in a preclinical vaccine trial using non-Indian rhesus macaques (17). In that study, macaques vaccinated with a DNA priming followed by a Gag-expressing Sendai virus vector-booster were challenged intravenously with SIVmac239. Five of eight vaccinees controlled viral replication and had undetectable levels of plasma viremia after 5 weeks of infection. All of the five macaques showed rapid selection of CTL escape mutations in *gag*, indicating that vaccine-induced CTL contained replication of the wild-type challenge virus. Among the five, three vaccinees that share a major histocompatibility complex class I (MHC-I) haplotype, *90-120-Ia*, showed high levels of $Gag_{206\text{-}216}$ (IINEEAADWDL) epitope-specific CTL responses and rapid selection of a mutant escaping from the CTL. The replicative ability of the virus with the CTL escape mutation, Gag216S, leading to a substitution from leucine (L) to serine (S) at the 216th amino acid (aa) in Gag was diminished compared to the wild type. In the present study, we have observed replication of this mutant SIV, SIVmac239Gag216S, in the absence of $Gag_{206\text{-}216}$-specific CTL responses after its inoculation into naive macaques. All the animal experiments in this study were performed in accordance with the guidelines for laboratory animals of the National Institute of Infectious Diseases.

First, two cynomolgus macaques (*Macaca fascicularis*), C99049 and C99058, were coinoculated intramuscularly with 5 mg of the wild-type SIVmac239 molecular clone DNA (pBRmac239) and 5 mg of the mutant SIVmac239Gag216S molecular clone DNA (pBRmac239Gag216S) (17). We extracted RNA from plasma and quantitated plasma SIV RNA levels as described previously (17); both that of the wild type and that of the mutant are detectable in this assay. In both of the animals, plasma viremia was observed after the inoculation (Fig. 1A). Both the wild type and mutant viral genomes were detected at comparable levels by sequencing of a *gag* gene fragment amplified by reverse transcription and nested PCR from plasma RNA at week 1, but the mutant was poorly detected and the wild type was dominant at weeks 2 and 3. We then subcloned the amplified fragments into plasmids for sequencing and counted the numbers of clones carrying the wild-type (Gag216L) or the mutant (Gag216S) sequence at the region encoding the 216th aa in Gag. It revealed that the wild-type SIV became dominant 2 or 3 weeks after the inoculation (Fig. 1B). This result indicates that the replicative ability of this CTL escape mutant virus was diminished compared to

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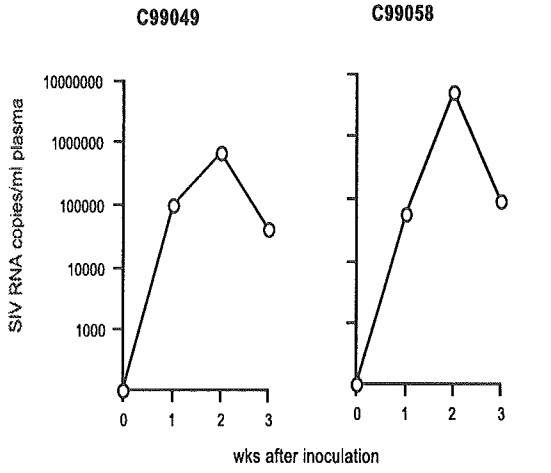


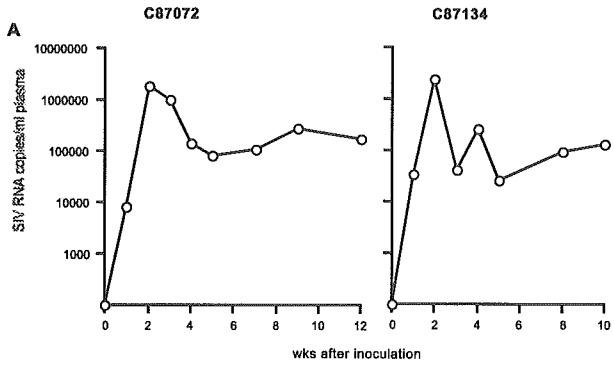
B

	wk 1	wk 2	wk 3	wk 1	wk 2	wk 3
wt clones	4	13	9	4	6	11
mt clones	6	3	1	6	2	1

C

	SM	Mes	Ing	SM	Mes	Ing
wt clones	11	9	10	11	11	6
mt clones	0	1	2	1	3	2

FIG. 1. Comparison of replication efficiencies in vivo of the wild-type SIVmac239 and the CTL escape mutant SIVmac239Gag216S. Macaques C99049 (left panels) and C99058 (right panels) were coinoculated with both wild-type and mutant SIV molecular clone DNAs. (A) Plasma viral loads after the inoculation. These indicate the sums of the wild-type and the mutant SIV RNA levels. The lower limit of detection in this assay is about 4×10^2 copies/ml. (B) Frequencies of the wild-type and mutant viral genomes in plasma. At each time point, a *gag* gene fragment was amplified by nested reverse transcription-PCR from plasma RNA and subcloned into plasmids for sequencing. The numbers of clones carrying the wild-type sequence (wt clones) and the mutant sequence (mt clones) at the region encoding the 216th aa in Gag are shown. (C) Frequencies of the wild-type and the mutant proviral genomes in the submandibular (SM) LN, the mesenchymal (Mes) LN, and the inguinal (Ing) LN. Genomic DNAs were extracted from LNs at euthanasia, and *gag* gene fragments amplified by nested PCR from the DNAs were subcloned into plasmids for sequencing.



B

	wk 4	wk 5	wk 7	wk 9	wk 4	wk 5	wk 8	wk 10
wt clones	0	1	9	11	0	1	3	12
mt clones	26	9	1	1	22	13	8	9

C

	SM	Mes	Ing	SM	Mes	Ing
wt clones	12	9	10	3	6	3
mt clones	0	2	0	10	4	8

FIG. 2. Reversion in vivo from the CTL escape mutant molecular clone. Macaques C87072 (left panels) and C87134 (right panels) were inoculated with the mutant SIV molecular clone DNA. (A) Plasma viral loads after the inoculation. (B) Frequencies of the wild-type and mutant viral genomes in plasma. See the legend for Fig. 1B. (C) Frequencies of the wild-type and the mutant proviral genomes in LNs. See the legend for Fig. 1C.

that of the wild type, confirming the previous results obtained with rhesus macaques (17). After euthanasia of macaques at week 3, we extracted genomic DNA, by using the DNeasy kit (QIAGEN K.K., Tokyo, Japan), from the submandibular lymph node (LN), the mesenchymal LN, and the inguinal LN and subcloned proviral *gag* gene fragments amplified by nested PCR from the DNA into plasmids for sequencing. The mutant Gag216S was detected in most of the LNs, but the wild-type sequence was dominant in the proviral genomes in all the LNs (Fig. 1C).

Second, two cynomolgus macaques, C87072 and C87134, were inoculated intramuscularly with 5 mg of the mutant SIVmac239Gag216S molecular clone DNA alone. Plasma viremia was maintained until euthanasia of macaques at week 12 (C87072) or week 10 (C87134) after the inoculation (Fig. 2A). We subcloned viral *gag* gene fragments amplified from plasma RNA and found the wild-type Gag216L sequence at week 5, although it was undetectable at week 4 in both of the animals (Fig. 2B). In macaque C87072, 9 of 10 viral *gag* clones showed the wild-type Gag216L sequence at week 7 (Fig. 2B), and most of the proviral *gag* clones were the wild type in the submandibular LN, the mesenchymal LN, and the inguinal LN at week 12 (Fig. 2C). In macaque C87134, the ratio of wild-type to total viral *gag* clones was 3/11 at week 8 but 12/21 at week 10 (Fig. 2B). The wild-type Gag216L sequence was detected but was not predominant in the proviral *gag* clones from the LNs at week 10 (Fig. 2C). These results indicate that the mutant SIVmac239Gag216S proliferated in all the LNs but was outgrown by the wild-type virus.

We further examined virus-specific $CD8^+$ T-cell responses in macaques by flow cytometric analysis of antigen-specific interferon-γ (IFN-γ) induction as described previously (16, 17). In brief, peripheral blood mononuclear cells (PBMCs) were cocultured with autologous herpesvirus papio-immortalized B lymphoblastoid cells infected with a vesicular stomatitis virus G-pseudotyped SIV for SIV-specific stimulation. Alternatively, PBMCs were cocultured with B lymphoblastoid cells pulsed with the $Gag_{206\text{-}216}$-epitope peptide for $Gag_{206\text{-}216}$-spe-

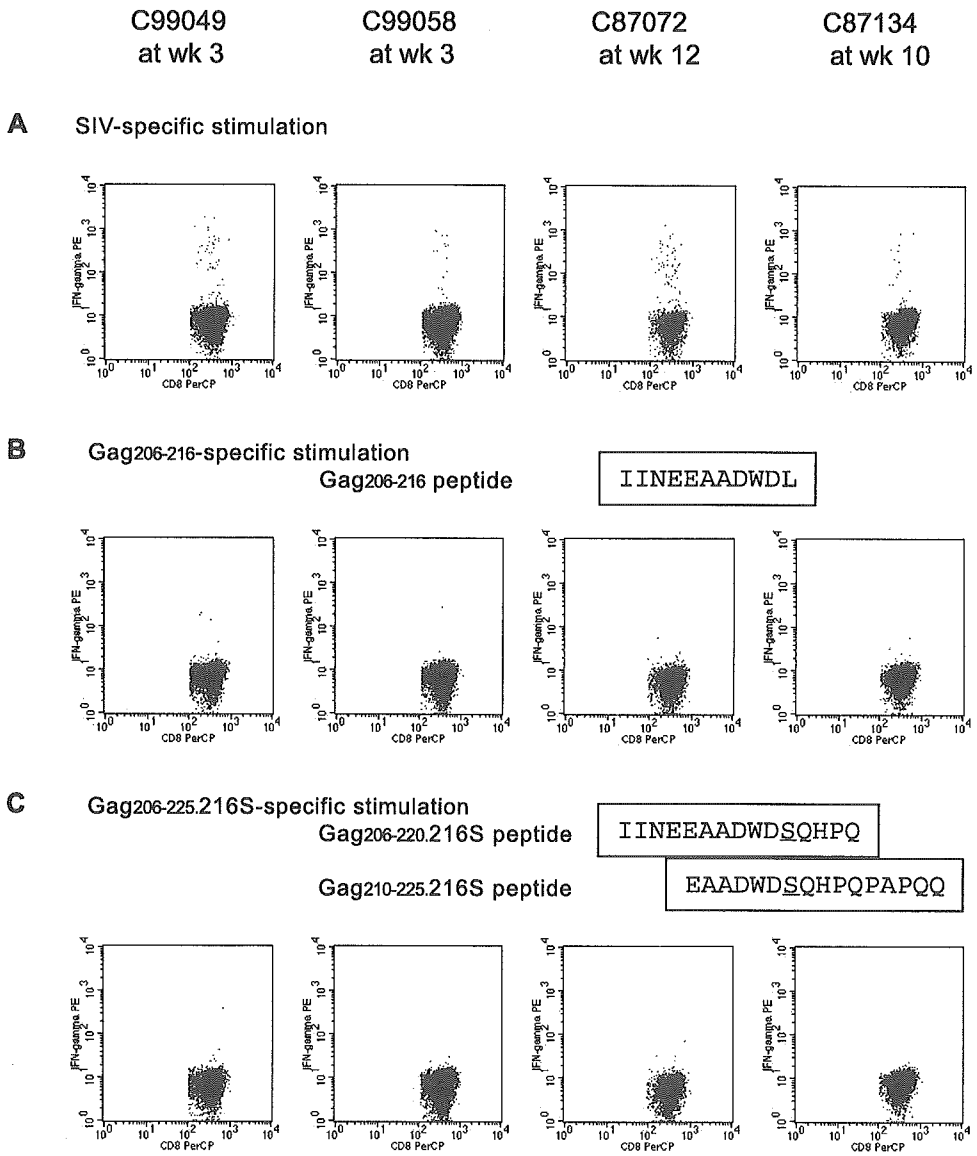


FIG. 3. SIV-specific $CD8^+$ T-cell responses in macaques. We examined IFN-γ induction after SIV-specific stimulation using vesicular stomatitis virus G-pseudotyped SIV (A), $Gag_{206-216}$-specific stimulation using $Gag_{206-216}$ peptide (B), or $Gag_{206-225}$.216S-specific stimulation using a mixture of $Gag_{206-220}$.216S and $Gag_{210-225}$.216S peptides (C). The aa sequences of $Gag_{206-216}$, $Gag_{206-220}$.216S, and $Gag_{210-225}$.216S peptides are indicated. Dot plots gated on $CD3^+CD8^+$ lymphocytes are shown. PE, phycoerythrin; PerCP, peridinin chlorophyll protein.

cific stimulation or a mixture of the peptides with a 216S mutation corresponding to the 206th through 220th aa and the 210th through 225th aa in Gag ($Gag_{206-220}$.216S and $Gag_{210-225}$.216S peptides, respectively) for $Gag_{206-225}$.216S-specific stimulation. PBMCs derived from macaques C99049 at week 3, C99058 at week 3, C87072 at week 12, and C87134 at week 10 were subjected to this assay (Fig. 3). SIV-specific $CD8^+$ T-cell responses were detected but $Gag_{206-216}$-specific IFN-γ induction was undetectable, confirming no $Gag_{206-216}$-specific CTL responses in any of four animals. $Gag_{206-225}$.216S-specific $CD8^+$ T-cell responses were also undetectable, indicating that the predominance of the wild-type virus in these four macaques was not due to immune pressure exerted by the mutant-specific CTL recognizing an epitope with the mutant Gag216S sequence.

In the previous study (17), the Gag216S mutant virus escaping from $Gag_{206-216}$-specific CTL was rapidly selected in the vaccinees possessing the MHC-I haplotype *90-120-Ia* after SIVmac239 challenge. However, the CTL escape mutant with lower viral fitness was rapidly contained and became undetectable in plasma after week 5 postchallenge. The present study shows that this mutant SIV, which was rapidly contained in the vaccinees in the previous study, can replicate and is unable to be rapidly contained in naive macaques, leading to the appearance of the wild-type virus in the absence of $Gag_{206-216}$-specific CTL responses. This suggests the requirement of additional

adaptive immune responses as well as $Gag_{206-216}$-specific CTLs for containment of this CTL escape mutant virus with lower viral fitness.

Viral adaptation by escape mutations under CTL pressure and reversion after transmission to MHC-I-mismatched hosts have been indicated in immunodeficiency virus infection (2, 3, 7, 14). It has recently been shown that reversion by de novo mutation can really occur after challenge of macaques with a cloned SIV with CTL escape mutations (7). In that study, preparation of the challenge virus stock from a molecular clone DNA of the mutant SIV required viral replication in vitro for more than a week. In the present study, to see the reversion by de novo mutation only in vivo by deleting the in vitro replication process for virus stock preparation, we directly inoculated macaques with a molecular clone DNA of the mutant SIV. Our results show that the reversion by de novo mutation can really occur and be detected in 5 weeks after inoculation of the mutant molecular clone DNA. Thus, this is the first report describing the reversion in vivo from an inoculated, molecular proviral DNA clone of immunodeficiency virus with a CTL escape mutation.

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Involvement of Multiple Epitope-Specific Cytotoxic T-Lymphocyte Responses in Vaccine-Based Control of Simian Immunodeficiency Virus Replication in Rhesus Macaques

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Cytotoxic T-lymphocyte (CTL) responses are crucial for the control of immunodeficiency virus replication. Possible involvement of a dominant single epitope-specific CTL in control of viral replication has recently been indicated in preclinical AIDS vaccine trials, but it has remained unclear if multiple epitope-specific CTLs can be involved in the vaccine-based control. Here, by following up five rhesus macaques that showed vaccine-based control of primary replication of a simian immunodeficiency virus, SIVmac239, we present evidence indicating involvement of multiple epitope-specific CTL responses in this control. Three macaques maintained control for more than 2 years without additional mutations in the provirus. However, in the other two that shared a major histocompatibility complex haplotype, viral mutations were accumulated in a similar order, leading to viral evasion from three epitope-specific CTL responses with viral fitness costs. Accumulation of these multiple escape mutations resulted in the reappearance of plasma viremia around week 60 after challenge. Our results implicate multiple epitope-specific CTL responses in control of immunodeficiency virus replication and furthermore suggest that sequential accumulation of multiple CTL escape mutations, if allowed, can result in viral evasion from this control.

Virus-specific cytotoxic T-lymphocyte (CTL) responses are crucial for the control of immunodeficiency virus infections. The importance of CTLs for control has been indicated by temporal association of CTL appearance with the resolution of primary viremia in human immunodeficiency virus type 1 (HIV-1)-infected humans (9, 24, 33) and by monoclonal anti-CD8 antibody-mediated CD8-depletion experiments in macaque AIDS models (18, 29, 38). Therefore, AIDS vaccine researchers have been making efforts to develop methods efficiently eliciting CTL responses (15, 30), and most of them have used multiple antigens for CTL induction (3, 8). However, it has remained unclear if multiple epitope-specific CTLs can really take part in vaccine-based control of viral replication.

Several preclinical trials of CTL-based AIDS vaccines in macaques have succeeded in the control of replication of a simian-human immunodeficiency virus, SHIV89.6P, that induces acute $CD4^+$ T-cell depletion (3, 8, 27, 37, 40). Unfortunately, most of these vaccine regimens have failed to contain the more realistic challenge of pathogenic simian immunodeficiency viruses (SIVs) that induce chronic disease progression (12, 17). Recently, however, CTL-based control of replication of a pathogenic SIV clone, SIVmac239, has been shown in a preclinical vaccine trial using Burmese rhesus macaques (28). In that study, macaques immunized with a DNA prime/Gag-expressing Sendai virus (SeV-Gag) vector-boost vaccine were challenged intravenously with SIVmac239. Five of eight vaccinees controlled viral replication and had undetectable levels of plasma viremia after 5 weeks of infection. All of the five macaques showed rapid selection of CTL escape mutations in *gag*, indicating that vaccine-induced CTLs were crucial for the containment of the wild-type, challenge virus. Of the five, three vaccinees that share a major histocompatibility complex class I (MHC-I) haplotype, *90-120-Ia*, showed high levels of $Gag_{206–216}$ (IINEEAADWDL) epitope-specific CTL and rapid selection of a mutant escaping from this CTL. The virus with the CTL escape mutation, GagL216S, leading to an alteration from leucine (L) to serine (S) at the 216th amino acid (aa) in Gag showed diminished replicative ability compared to the wild type. Inoculation of naive macaques with this mutant resulted in persistent viral replication and reversion in the absence of the $Gag_{206–216}$-specific CTL responses (23). These results have suggested that additional adaptive immune responses as well as $Gag_{206–216}$-specific CTLs are important for containment of this CTL escape mutant virus with lower viral fitness.

Viral escape from CTL recognition has been frequently observed in HIV-1 and SIV infections, and it may be critical for viral evasion from immune control (5, 6, 10, 15, 16, 32, 35, 36). Indeed, viral evasion from immune control with a single escape mutation from a dominant CTL has been reported in preclinical AIDS vaccine trials, indicating involvement of the single epitope-specific CTL in this control (5, 6). However, these reports have not made it clear whether multiple epitope-spe-

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cific CTLs can be involved in the vaccine-based control of immunodeficiency virus replication.

In the present study, we have followed, for more than 2 years, the five macaques that showed vaccine-based control of SIVmac239 replication. We have found that three of them maintained control of viral replication for more than 2 years while the other two lost control at approximately week 60 after challenge. Analysis of the latter two has revealed viral evasion from the vaccine-based control by accumulation of multiple CTL escape mutations, indicating involvement of multiple epitope-specific CTLs in this control.

MATERIALS AND METHODS

Animal experiments. Twelve male Burmese rhesus macaques (*Macaca mulatta*) used in our previous SIVmac239 challenge experiment (28) were followed up in the present study. These macaques were maintained in accordance with the guidelines for laboratory animals of the National Institute of Infectious Diseases. Blood collection, vaccination, and virus challenge were performed under ketamine anesthesia. Four of the macaques were naive whereas the other eight macaques received a DNA vaccine followed by a single boost with SeV-Gag before an intravenous SIVmac239 challenge. The DNA, CMV-SHIVdEN, used for the vaccination was constructed from an *env*- and *nef*-deleted $SHIV_{MD14YE}$ molecular clone DNA (39) and has the genes encoding SIVmac239 Gag, Pol, Vif, and Vpx; SIVmac239-$HIV\text{-}1_{DH12}$ chimeric Vpr; and $HIV\text{-}1_{DH12}$ Tat and Rev as described previously (28). At the DNA vaccination, animals received 5 mg of CMV-SHIVdEN DNA intramuscularly. Six weeks after the DNA prime, animals intranasally received a single boost with 1×10^8 cell infectious units of replication-competent SeV-Gag (V1, V2, V3, and V4) or 6×10^9 cell infectious units of F-deleted replication-defective F(−)SeV-Gag (19, 20, 26, 41). Thirteen weeks after the boost, animals were challenged intravenously with 1,000 50% tissue culture infective doses of SIVmac239 (22).

Quantitation of plasma viral loads. Plasma RNA was extracted using the High Pure viral RNA kit (Roche Diagnostics, Tokyo, Japan). Serial fivefold dilutions of RNA samples were amplified in quadruplicate by reverse transcription (RT) and nested PCR using SIV *gag*-specific primers (AGAAACTCCGTCTTGT CAGG and TGATAATCTGCATAGCCGC for the first RT-PCR and GATTA GCAGAAAGCCTGTTGG and TGCAACCTTCTGACAGTGC for the second DNA PCR) to determine the endpoint. Plasma SIV RNA levels were calculated according to the Reed-Muench method as described previously (28, 39). The lower limit of detection in this standard assay is about 4×10^2 copies/ml. For fivefold concentration of plasma, after centrifugation of 1 ml of plasma at 25,000 $\times g$ for 2 h, 0.8 ml of its supernatant was discarded and the remaining 0.2 ml was subjected to RNA extraction.

Sequencing. Fragments corresponding to nucleotides (nt) 1231 to 2958 (containing the entire *gag* region), nt 2827 to 3960, nt 3811 to 4970, nt 4829 to 5986, nt 5852 to 7000, nt 6843 to 7901, nt 7684 to 8831, nt 8677 to 9723, and nt 9499 to 10196 in the SIVmac239 genome (GenBank accession number M33262) were amplified by nested RT-PCR. Alternatively, genomic DNA was extracted from peripheral blood mononuclear cells (PBMCs) by using the DNeasy kit (QIAGEN K.K., Tokyo, Japan), and the *gag* fragment was amplified by nested PCR. The PCR products were sequenced using dye terminator chemistry and an automated DNA sequencer (Applied Biosystems, Tokyo, Japan). Alternatively, the PCR products were subcloned into plasmids by using the TOPO cloning system (Invitrogen, Tokyo, Japan) and sequenced.

Peptide-specific CTL responses. We measured virus-specific T-cell levels by flow cytometric analysis of gamma interferon (IFN-γ) induction after specific stimulation as described previously (28). In brief, PBMCs were cocultured with autologous herpesvirus papio-immortalized B-lymphoblastoid cell lines (B-LCL) (42) pulsed with 1 μM or indicated concentrations of peptides (Sigma Genosys, Ishikari, Japan) for peptide-specific stimulation or unpulsed B-LCL for nonspecific stimulation. Intracellular IFN-γ staining was performed by using the Cytofix-Cytoperm kit (Becton Dickinson, San Jose, California). Peridinin chlorophyll protein-conjugated anti-human CD8, allophycocyanin-conjugated anti-human CD3, and phycoerythrin-conjugated anti-human IFN-γ antibodies (Becton Dickinson) were used. Specific T-cell levels were calculated by subtracting the $IFN\text{-}\gamma^+$ T-cell frequencies after nonspecific stimulation from those after peptide-specific stimulation. Specific T-cell levels less than 100 cells per million PBMCs were considered negative.

Generation of CTL clones and CTL assay. $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL clones were obtained from macaque V5 PBMCs cocultured with irradiated, V5-derived B-LCL pulsed with the corresponding peptides. Cytotoxicity was measured in a standard ^{51}Cr release assay. In brief, target cells (5×10^5) were incubated with 150 μCi $Na_2{}^{51}CrO_4$ for 1 h, pulsed with the corresponding peptides for 1 h, and cocultured with effector cells for 4 h. The culture supernatants were analyzed with a gamma counter. The spontaneous ^{51}Cr release (cpm spn) was determined by measuring the ^{51}Cr release from the culture containing only target cells. The maximum release (cpm max) was determined by measuring the ^{51}Cr release from target cells in the presence of 2.5% Triton X-100. Percent specific lysis was calculated as follows: percent specific lysis = 100 × (cpm exp − cpm spn)/(cpm max − cpm spn), where cpm exp is the ^{51}Cr release from the culture containing both target and effector cells.

Viral competition assay. SIV molecular clone DNAs with mutations in *gag* were constructed by site-directed mutagenesis from the wild-type SIV molecular clone DNA pBRmac239, provided by T. Kodama and R. C. Desrosiers. COS1 cells were transfected with mutant SIV molecular DNAs to obtain mutant SIV stocks. Two million cells of a herpesvirus saimiri-immortalized macaque T-cell (MTC) line (1) were infected with one of the mutant SIVs at the dose of 2 ng of SIV CA (p27), and 1 day later, half of them were cocultured with those infected with another mutant SIV. Two million MTCs were added into the culture on days 8, 12, 16, and 20 after infection. RNA was extracted from the culture supernatant on day 24. The fragment (nt 1231 to nt 3016 in SIVmac239) containing the entire *gag* region was amplified from the RNA by RT-PCR and was subcloned into plasmids for sequencing to determine dominant sequences.

RESULTS

Reappearance of viremia after 1 year of control in two of the five controllers. Twelve Burmese rhesus macaques used in our previous SIVmac239 challenge experiment (28) were followed up in the present study (Table 1). Of the 12, eight macaques descended from a male breeder, R-90-120, and four of them shared an MHC-I haplotype, *90-120-Ia*. Four macaques were naive whereas eight macaques received a DNA vaccine followed by a single boost with SeV-Gag before an intravenous SIVmac239 challenge. All four naive animals and three of the vaccinees failed to control SIV replication, but five of eight vaccinees controlled SIV replication with undetectable levels of plasma viremia (less than 400 RNA copies/ml) after 5 weeks of infection. We have termed the former seven animals noncontrollers and the latter five controllers in the present study.

During 2 years of follow-up, all the seven noncontrollers maintained high levels of plasma viremia (Fig. 1A). Four of them developed AIDS and had to be euthanized. By contrast, plasma viremia was undetectable and peripheral $CD4^+$ T-cell counts were maintained even after 2 years of infection in three (V4, V6, and V8) of five controllers (Fig. 1A and B). In the other two controllers (V5 and V3), however, plasma viremia reappeared and was detectable (more than 400 RNA copies/ml) at week 58 after challenge (Fig. 1A). Thus, three of five controllers maintained control of SIV replication for more than 2 years, whereas the other two controllers lost control after 1 year of infection. We have termed the former three animals sustained controllers and the latter two transient controllers in the present study.

Of four macaques possessing the MHC-I haplotype *90-120-Ia*, all of the three vaccinees, V5, V3, and V4, successfully controlled SIV replication, although one naive macaque, N2, failed. Remarkably, two of the three controllers possessing *90-120-Ia* lost control around week 60.

We examined viral loads in the controllers by detection of viral genomes in concentrated plasma (Fig. 1C). The cutoff line of this assay is about 80 RNA copies/ml whereas that of our standard assay for quantitation of plasma viral RNA is approximately 400 RNA copies/ml. In both of the transient control-

TABLE 1. SIVmac239 challenge experiments

Macaque	MHC-I haplotype[a]	Naive or vaccinee[b]	Set point VL[c] around wk 12	CTL escape[d] at wk 5	VL around wk 60
R-90-120 descendants					
N2	*90-120-Ia*	Naive	10^4–10^6		10^4–10^6
V5	*90-120-Ia*	Vaccinee	<400	GagL216S	$>10^3$
V3	*90-120-Ia*	Vaccinee	<400	GagL216S	$>10^3$
V4	*90-120-Ia*	Vaccinee	<400	GagL216S	<400
V2	*90-120-Ib*	Vaccinee	10^4–10^6		Dead[e]
N3	*90-122-Ie*	Naive	10^4–10^6		10^4–10^6
V7	*90-122-Ie*	Vaccinee	10^4–10^6		10^4–10^6
V6	*90-122-Ie*	Vaccinee	<400	GagI377T	<400
R-90-088 descendants					
N1	*90-088-Ij*	Naive	10^4–10^6		10^4–10^6
V1	*90-088-Ij*	Vaccinee	10^4–10^6		10^4–10^6
R-90-010 descendants					
N4	*90-010-Id*	Naive	10^4–10^6		10^4–10^6
V8	*90-010-Id*	Vaccinee	<400	GagQ58K	<400

[a] MHC-I haplotype was determined by reference strand-mediated conformation analysis (4) as described previously (28). Macaques N2, V3, and V2 are sons of male breeder R-90-120; V5, V4, N3, V7, and V6 are sons of R-94-027; N1 and V1 are sons of R-90-088; N4 and V8 are sons of R-90-010. Breeder R-94-027 is the son of male R-90-120 and female R-90-122 and possesses *90-120-Ia* and *90-122-Ie* haplotypes. MHC-I haplotypes *90-120-Ia* and *90-120-Ib* are derived from breeder R-90-120, *90-122-Ie* is from R-90-122, *90-088-Ij* is from R-90-088, and *90-010-Id* is from R-90-010.

[b] All the animals were challenged intravenously with SIVmac239. Vaccinees received a prophylactic DNA prime/SeV-Gag boost vaccine before challenge.

[c] Plasma viral load (RNA copies/ml plasma). VL, viral load.

[d] Rapidly selected CTL escape mutations in Gag as described previously (28).

[e] Macaques N3, V1, V2, and V7 developed AIDS and were euthanized at weeks 104, 105, 42, and 77, respectively.

lers, viral RNA was detected in the concentrated plasma during the period of control although it was undetectable by our standard assay. In contrast, viral RNA was undetectable even in the concentrated plasma in all of the sustained controllers. These results indicate that SIV replication was contained to much lower levels in the sustained controllers compared to the rather high levels in the transient controllers.

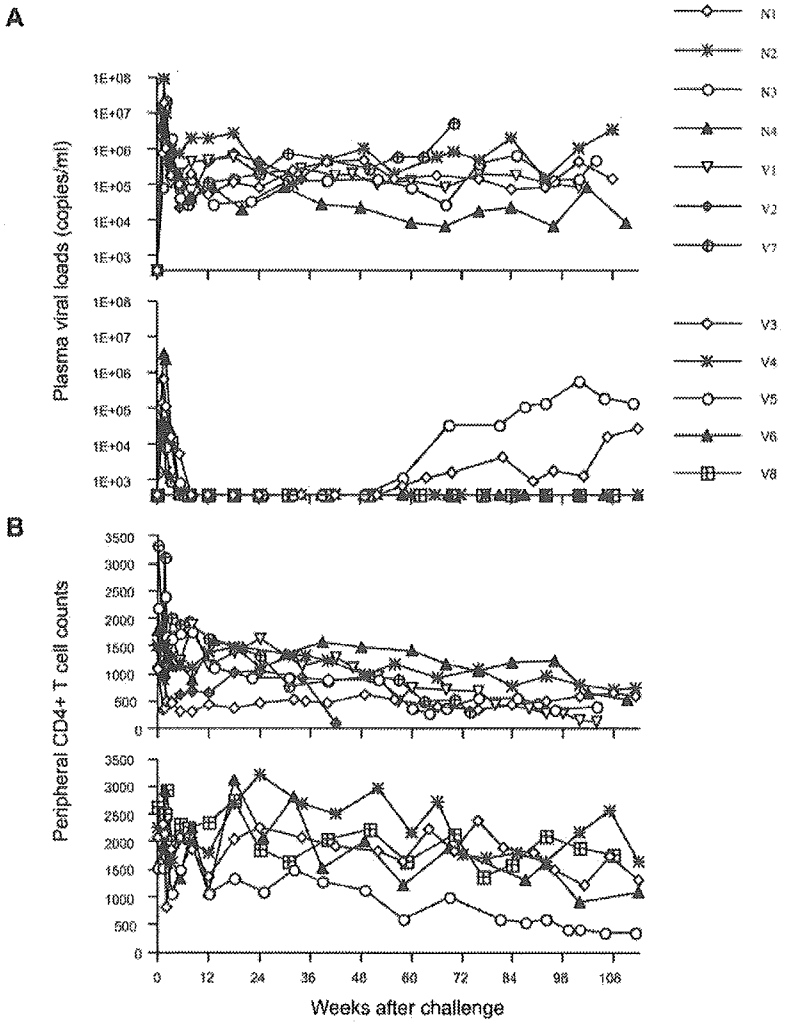
Viral mutations in the transient controllers. The previous study (28) showed rapid selection of CTL escape mutations in *gag* in all of the controllers (Table 1), indicating the importance of the CTL responses in the control of SIV replication. We then examined *gag* sequences to see if additional viral mutations were involved in the loss of control in the transient-controllers (Table 2). In a sustained controller (V4) possessing the MHC-I haplotype *90-120-Ia*, we observed rapid selection of the GagL216S mutation leading to escape from $Gag_{206–216}$-specific CTL responses (referred to as $Gag_{206–216}$-CTL-escape mutation) both in plasma viral RNA and in proviral DNA of PBMCs. This mutation was maintained, but no other mutation became dominant even at week 85. In the other two sustained controllers (V6 and V8), the rapidly selected CTL escape mutations were observed in viral RNA but not in proviral DNA. This may reflect the possibility that accumulated mutant copies were too small for their detection in provirus compared to the wild type in these two macaques.

In both of the transient controllers (V5 and V3) possessing the MHC-I haplotype *90-120-Ia*, the $Gag_{206–216}$-CTL-escape mutation was rapidly selected and still maintained at approximately week 60. In contrast to the sustained controllers, we found multiple additional mutations in the reemerged viruses in both of these macaques. In macaque V5, viral genomes with GagL216S, GagD244E (aspartic acid [D]-to-glutamic acid [E] alteration at the 244th aa in Gag), GagI247L (isoleucine [I] to L at the 247th aa), GagA312V (alanine [A] to valine [V] at the 312th aa), and GagA373T (A to threonine [T] at the 373rd aa) mutations were dominant at week 58. In macaque V3, viral genomes with GagV145A (V to A at the 145th aa), GagL216S, GagD244E, and GagP376S (proline [P] to serine[S] at the 376th aa) mutations were dominant, but those with GagP172S (P to S at the 172nd aa), GagL216S, GagD244E, and GagV375A (V to A at the 375th aa) mutations were also detected at week 64.

We then examined *gag* sequences during control in both of the transient controllers (Tables 3 and 4). This analysis showed that, in addition to the GagL216S mutation, the GagD244E mutation was initially selected, followed by selection of the mutations leading to alterations around the 375th aa in Gag in both of these macaques. In this regard, the two transient controllers showed similar patterns of sequential accumulation of mutations.

Accumulation of CTL escape mutations in the transient controllers. To see if the mutations observed in the transient controllers were CTL escape mutations, we examined IFN-γ induction after stimulation with peptides corresponding to the regions around the mutation sites. In addition to the $Gag_{206–216}$ epitope, we mapped two CTL epitopes, $Gag_{241–249}$ (SSVDEQ IQW) and $Gag_{373–380}$ (APVPIPFA). High levels of these three epitope-specific ($Gag_{206–216}$-specific, $Gag_{241–249}$-specific, and $Gag_{373–380}$-specific) CTL responses were observed in all the three controllers possessing MHC-I haplotype *90-120-Ia* in the early phase of infection (Fig. 2A). The $Gag_{206–216}$-specific and $Gag_{241–249}$-specific CTL responses were especially dominant. These CTL levels were considerably reduced in the chronic phase, probably reflecting diminished SIV replication during the control. Reduction in $Gag_{206–216}$-specific CTL responses was faster, consistent with the fastest selection of the $Gag_{206–216}$-CTL-escape mutation.

Both of the transient controllers (V5 and V3) showed di-



C

SIV *gag* RNA in concentrated plasma

V5	wk 12 positive	wk 25 positive		
V3	wk 12 positive	wk 24 positive		
V4	wk 12 negative	wk 24 negative	wk 60 negative	wk 100 negative
V6	wk 12 negative	wk 25 negative	wk 58 negative	wk 100 negative
V8	wk 12 negative	wk 24 negative	wk 50 negative	wk 100 negative

FIG. 1. Follow-up of 12 macaques after SIVmac239 challenge. (A) Plasma viral loads. Top, noncontrollers; bottom, controllers. (B) Peripheral $CD4^+$ T-cell counts (per μl). Top, noncontrollers; bottom, controllers. (C) Detection of viral genomes in concentrated plasma obtained from the controllers. Positive, detected ($>8 \times 10^1$ copies/ml); negative, undetectable.

minished recognition of the peptide with the GagD244E mutation, $Gag_{241-249}$.244E (SSVEEQIQW), by $Gag_{241-249}$-specific CTL responses (Fig. 2B and 2C). The peptide with the GagI247L mutation in addition to the GagD244E (SSVEEQLQW) showed further-reduced sensitivity to CTL recognition. This indicates that the GagD244E and GagI247L mutations were selected for by $Gag_{241-249}$-specific CTLs (referred to as $Gag_{241-249}$-CTL-escape mutations). Furthermore, the GagA373T, GagV375A, and GagP376S mutations in the $Gag_{373-380}$ peptide (APVPIPFA) resulted in diminished recognition by $Gag_{373-380}$-specific CTL responses (Fig. 2B and 2C), indicating that the GagA373T, GagV375A, and GagP376S mutations were selected for by $Gag_{373-380}$-specific CTLs (referred to as $Gag_{373-380}$-CTL-escape mutations). Thus, viruses in both of the transient controllers accumulated the $Gag_{241-249}$-CTL-escape mutation and the $Gag_{373-380}$-CTL-es-