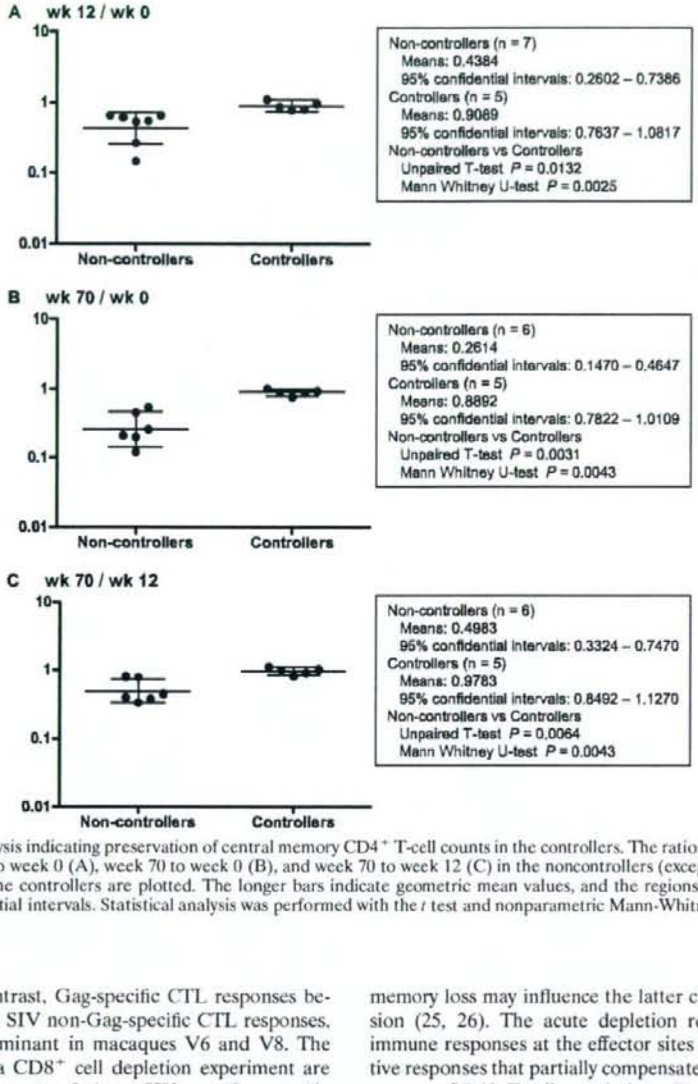

FIG. 6. Statistical analysis indicating preservation of central memory CD4$^+$ T-cell counts in the controllers. The ratios of central memory CD4$^+$ T-cell counts at week 12 to week 0 (A), week 70 to week 0 (B), and week 70 to week 12 (C) in the noncontrollers (except for rapid progressor V2 in panels B and C) and the controllers are plotted. The longer bars indicate geometric mean values, and the regions between the shorter bars indicate the 95% confidential intervals. Statistical analysis was performed with the *t* test and nonparametric Mann-Whitney U-test using the Prizm software.

controllers (10). In contrast, Gag-specific CTL responses became undetectable and SIV non-Gag-specific CTL responses, instead, became predominant in macaques V6 and V8. The results obtained from a CD8$^+$ cell depletion experiment are consistent with involvement of these SIV non-Gag-specific CTL responses in the long-term viral control in both sustained controllers, although there might be involvement of other components, such as NK and CD4$^+$ memory T cells. Thus, it can be speculated that vaccine-based control of primary SIV replication can preserve the ability of the immune system to elicit functional CTL responses, leading to reinforcement or adaptation of protective immunity by postchallenge induction or expansion of effective CTL responses. This may contribute to stable viral containment in the chronic phase.

In the natural courses of HIV and SIV infections, the infected hosts exhibit acute, massive depletion of CCR5$^+$ CD4$^+$ effector memory T cells from mucosal effector sites, and the chronic immune activation with gradual immune disruption that follows leads to AIDS (7, 15, 20, 25). The former acute memory loss may influence the latter chronic disease progression (25, 26). The acute depletion results in compromised immune responses at the effector sites and systemic proliferative responses that partially compensate for the loss of mucosal memory CD4$^+$ T-cell populations. Recent reports indicating amelioration of acute mucosal memory CD4$^+$ T-cell depletion and associated central memory CD4$^+$ T-cell loss in the early phase by CTL-based vaccines have suggested that vaccine-based amelioration of acute memory CD4$^+$ T-cell depletion in mucosal effector sites can delay AIDS progression (13, 19, 35). However, this acute memory CD4$^+$ T-cell depletion is not the only cause of chronic disease progression and persistent viral replication-associated immune activation may be responsible for chronic immune disruption leading to AIDS (7). Indeed, in both of the transient controllers, V3 and V5, central memory CD4$^+$ T cells were preserved during the initial, transient period of viremia control but decreased after the reappearance of plasma viremia. This suggests that there may be an association between persistent viral containment and central memory CD4⁺ T-cell preservation, even in the chronic phase.

Theoretically, protection by CTL-based AIDS vaccines is likely to be nonsterile, and it will be difficult to contain viral replication completely. Additionally, CTL-based viremia control would require CTL activation. Indeed, our $CD8^+$ cell depletion experiment indicated that persistent viral replication was inefficient but not completely contained in the absence of plasma viremia in sustained controllers V6 and V8. Transition of recognition of CTL epitopes from Gag to other non-Gag proteins in the chronic phase suggests that these "new" CTLs were either elicited or expanded by viral replication in the acute phase or by this inefficient persistent viral replication. Nevertheless, these macaques showed long-term viral control with central memory $CD4^+$ T-cell preservation, indicating that nonsterile protection by CTL-based vaccines can result in prevention of chronic central memory $CD4^+$ T-cell loss.

In summary, the present study shows that primary viral control by a CTL-based AIDS vaccine can result in long-term control of SIV replication by adapted CTL responses and preservation of central memory $CD4^+$ T cells without AIDS progression. Our results suggest that CTL-based vaccines can result in long-term viral containment and disease control.

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Original article

Abrogation of AIDS vaccine-induced cytotoxic T-lymphocyte efficacy in vivo due to a change in viral epitope flanking sequences

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Abstract

A current promising AIDS vaccine strategy is to elicit $CD8^+$ cytotoxic T lymphocyte (CTL) responses that broadly recognize highly-diversified HIVs. In our previous vaccine trial eliciting simian immunodeficiency virus (SIV) mac239 Gag-specific CTL responses, a group of Burmese rhesus macaques possessing a major histocompatibility complex haplotype *90-120-Ia* have shown vaccine-based viral control against a homologous SIVmac239 challenge. Vaccine-induced $Gag_{206-216}$ epitope-specific CTL responses exerted strong selective pressure on the virus in this control. Here, we have evaluated in vivo efficacy of vaccine-induced $Gag_{206-216}$-specific CTL responses in two *90-120-Ia*-positive macaques against challenge with a heterologous SIVsmE543-3 that has the same $Gag_{206-216}$ epitope sequence with SIVmac239. Despite efficient $Gag_{206-216}$-specific CTL induction by vaccination, both vaccinees failed to control SIVsmE543-3 replication and neither of them showed mutations within the $Gag_{206-216}$ epitope. Further analysis indicated that $Gag_{206-216}$-specific CTLs failed to show responses against SIVsmE543-3 infection due to a change from aspartate to glutamate at Gag residue 205 immediately preceding the amino terminus of $Gag_{206-216}$ epitope. Our results suggest that even vaccine-induced CTL efficacy can be abrogated by a single amino acid change in viral epitope flanking region, underlining the influence of viral epitope flanking sequences on CTL-based AIDS vaccine efficacy.

Keywords: AIDS vaccine; Simian immunodeficiency virus; Cytotoxic T lymphocyte; Escape

1. Introduction

Development of an effective AIDS vaccine is considered essential for controlling current AIDS pandemic. A current promising AIDS vaccine strategy is to elicit virus-specific $CD8^+$ cytotoxic T lymphocytes (CTLs) that broadly recognize highly-diversified HIVs [1–4]. However, it has remained unclear as to how broadly vaccine-induced CTLs can recognize heterologous viruses in vivo.

Vaccine efficacies have been evaluated in macaque AIDS models. Several vaccine trials eliciting virus-specific CTL responses have successfully shown viral control and prevention of acute AIDS progression after CXCR4-tropic simian-human immunodeficiency virus (SHIV) 89.6P challenge in rhesus macaques [5–9]. In the models of CCR5-tropic simian

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immunodeficiency virus (SIV) infections that induce acute depletion of $CCR5^+CD4^+$ effector memory T cells from mucosal effector sites and following chronic disease progression like HIV-1 infections in humans [10,11], DNA-prime/adenovirus vector-boost vaccine trials have recently shown transient, partial reduction in viral loads in Indian rhesus macaques, although most CTL-based vaccines have failed to show consistent viremia control after SIV challenge [12–15]. However, most of these trials have used SIVmac239 antigens for vaccination and homologous SIVmac239/251 for challenge [16]. There have been a few reports on heterologous challenge [17], but in vivo efficacy of vaccine-induced CTL responses has not yet been compared intensively between in the homologous and in the heterologous CCR5-tropic SIV challenge experiments.

We have developed an Env-independent DNA-prime/SIVmac239 Gag-expressing Sendai virus (SeV-Gag) vector-boost vaccine and shown its protective efficacy in macaque AIDS models [7,18]. A trial of a homologous SIVmac239 challenge has shown vaccine-based control of viral replication in a group of Burmese rhesus macaques possessing a major histocompatibility complex class I (MHC-I) haplotype *90-120-Ia* and suggested involvement of vaccine-induced $Gag_{206-216}$ (IINEEAADWDL) epitope-specific CTL responses in this control [18]. All the SIVmac239-infected macaques possessing MHC-I haplotype *90-120-Ia* selected a viral mutation that results in escape from this $Gag_{206-216}$-specific CTL recognition and loss of viral fitness, indicating strong suppressive pressure on SIVmac239 replication in vivo by this CTL.

In the present study, we have challenged MHC-I haplotype *90-120-Ia*-positive vaccinees with a heterologous SIVsmE543-3 that has the same $Gag_{206-216}$ epitope amino acid sequence with SIVmac239, and have evaluated in vivo efficacy of vaccine-induced $Gag_{206-216}$-specific CTL responses against this heterologous virus in those vaccinees. Remarkably, vaccine-induced $Gag_{206-216}$-specific CTLs failed to show responses against SIVsmE543-3 infection.

2. Materials and methods

2.1. Animal experiments

Burmese rhesus macaques (*Macaca mulatta*) possessing MHC-I haplotype *90-120-Ia* which were used in this study were maintained in accordance with the Guideline for Laboratory Animals of National Institute of Infectious Diseases and National Institute of Biomedical Innovation. Blood collection, vaccination, and virus challenge were performed under ketamine anesthesia.

2.2. Vaccination and challenge

Two MHC-I haplotype *90-120-Ia*-positive rhesus macaques received a DNA vaccine followed by a single boost with SeV-Gag before an intravenous SIVsmE543-3 challenge. The DNA, CMV-SHIVdEN, used for the priming was constructed from an *env*- and *nef*-deleted $SHIV_{MD14YE}$ [19] molecular clone DNA, SIVGP1 [7,18], and has the genes encoding SIVmac239 (GenBank accession no. M33262) Gag, Pol, Vif, and Vpx, SIVmac239-HIV-1_{DH12} chimeric Vpr, and HIV-1_{DH12} Tat and Rev as described previously [18]. At the DNA vaccination, animals received 5 mg of CMV-SHIVdEN DNA intramuscularly. Six weeks after the DNA priming, animals intranasally received a single boost with 1×10^8 cell infectious units (CIU) of replication-competent V-knocked-out SeV-Gag [20,21]. Approximately 3 months after the boost, animals were challenged intravenously with 100 $TCID_{50}$ (50% tissue culture infective dose) of SIVsmE543-3 [22]. An SIVsmE543-3 (GenBank accession number U72748) molecular clone DNA was provided by V. Hirsch, and the virus obtained from COS-1 cells transfected with the molecular clone DNA was propagated on rhesus macaque peripheral blood mononuclear cells (PBMCs) to prepare the SIVsmE543-3 challenge stock.

2.3. Vectors

The plasmid vectors, pEGFP-N1-$Gag_{202-216}$ and pEGFP-N1-$Gag_{202-216}$.205E, were constructed from pEGFP-N1 (Becton Dickinson, Tokyo, Japan) by adding epitope-coding regions into the 5′ end of EGFP cDNA to express $Gag_{202-216}$-EGFP and $Gag_{202-216}$.205E-EGFP fused proteins, respectively. The amino acid sequences added into the N-terminal portion of EGFP are MASRAAAIIRDIINEEAADWDLAAD PPVAT in $Gag_{202-216}$-EGFP and MASRAAAIIREIINEEA ADWDLAADPPVAT in $Gag_{202-216}$.205E-EGFP.

2.4. Quantitation of plasma viral loads

Plasma RNA was extracted using High Pure Viral RNA kit (Roche Diagnostics, Tokyo, Japan). For quantitation of SIVsmE543-3 RNA copies, serial five-fold dilutions of RNA samples were amplified in quadruplicate by reverse transcription (RT) and nested PCR using SIV *gag*-specific primers or SIVsmE543-3 *gag*-specific primers to determine the end point. The SIV *gag*-specific primers were TTGAAGCATGTAG TATGGGCAG and TGGGTAATTTCCTCCTCTGCC for the 1st RT–PCR and GATTAGCAGAAAGCCTGTTGG and TGTTCCTGTTTCCACCACTAG for the 2nd PCR (Sigma–Aldrich Japan, Ishikari, Japan). The SIVsmE543-3 *gag*-specific primers were AGAAACTCCGTCTTGTCAGG and CTAATAATTTGCATGGCTGC for the 1st RT–PCR and GATTAGCAGAAAGCCTGTTGG and TGCAGCCTTCTGA TAGCGC for the 2nd PCR. Plasma SIV RNA levels were calculated according to the Reed-Muench method as described previously [18]. The lower limit of detection is approximately 1×10^3 copies/ml. The plasma viral loads at several time points were confirmed by LightCycler real-time PCR system (Roche Diagnostics) using SIV *gag*-specific primers (GTAG TATGGGCAGCAAATGA and TGTTCCTGTTTCCACCA CTA) and probes (GCATTCACGCAGAAGAGAAAGTGAA ACA and ACTGAGGAAGCAAAACAAATAGTGCAGAGA) (Nihon Gene Research Laboratories Inc., Sendai, Japan).

2.5. Sequencing

A fragment corresponding to nucleotides 973–2690 (containing the entire *gag* region) in SIVsmE543-3 genome was amplified from plasma RNA by nested RT–PCR. For its amplification from plasma with low viral loads, plasma samples were concentrated five-fold by centrifugation at 25,000 × *g* for 2 h before RNA extraction. The PCR products were sequenced using dye terminator chemistry and an automated DNA sequencer (Applied Biosystems, Tokyo, Japan).

2.6. Measurement of virus-specific $CD8^+$ T-cell responses

We measured virus-specific $CD8^+$ T-cell levels by flow-cytometric analysis of interferon-γ (IFN-γ) induction after specific stimulation as described previously [18]. PBMCs were cocultured with autologous herpesvirus papio-immortalized B lymphoblastoid cell lines (B-LCLs) infected with vesicular stomatitis virus G (VSV-G)-pseudotyped SIVGP1 or VSV-G-pseudotyped SIVsmE543-3 for vaccine antigen-specific or SIVsmE543-3-specific stimulation. The pseudotyped viruses were obtained by cotransfection of COS-1 cells with a VSV-G-expression plasmid and SIVGP1 DNA or an SIVsmE543-3 molecular clone DNA. For peptide-specific stimulation, PBMCs were cocultured with B-LCLs pulsed with 1 μM or indicated concentrations of peptides (Sigma–Aldrich Japan). For stimulation with DNA-transfected cells, 10^6 B-LCLs were transfected with 10 μg of DNA by electroporation and 2 days later, one-fifth or half of them were cocultured with 10^6 PBMCs. Parts of the remaining DNA-transfected B-LCLs were subjected to flow-cytometric analysis for examining EGFP expression to confirm the transfection efficiency. Intracellular IFN-γ staining was performed using CytofixCytoperm kit (Becton Dickinson). Fluorescein isothiocyanate-conjugated anti-human CD4, 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 $CD8^+$ T-cell levels were calculated by subtracting non-specific IFN-γ^+ T-cell frequencies from those after antigen-specific stimulation. All the background IFN-γ^+CD8^+ T-cell frequencies in the present study (Figs. 2–4) were less than 100 cells/million PBMCs. Specific T-cell levels less than 100 cells/million PBMCs are considered negative.

3. Results

3.1. Failure in control of heterologous SIVsmE543-3 replication in 90-120-Ia-positive vaccinees

Two Burmese rhesus macaques (R00-018 and R01-006) possessing MHC-I haplotype *90-120-Ia* received a prophylactic DNA-prime/SeV-Gag-boost vaccination consisting of a single intramuscular priming with a DNA encoding SIVmac239 Gag, Pol, Vif, and Vpx followed by a single intranasal booster with an SeV expressing SIVmac239 Gag, and were challenged intravenously with SIVsmE543-3. After challenge, these two vaccinees failed to control SIVsmE543-3 replication with persistent high levels of plasma viremia (Fig. 1). Both of them finally exhibited AIDS-like symptoms and were euthanized around week 115.

We examined antigen-specific $CD8^+$ T-cell frequencies in PBMCs by flow-cytometric detection of IFN-γ induction after stimulation with VSV-G-pseudotyped virus-infected cells (Fig. 2). We used VSV-G-pseudotyped SIVGP1 and VSV-G-pseudotyped SIVsmE543-3 for measurement of frequencies of $CD8^+$ T cells responding to SIVGP1-transduced cells and those responding to SIVsmE543-3-transduced cells, respectively. We call the former vaccine antigen-specific $CD8^+$ T cells and the latter SIVsmE543-3-specific $CD8^+$ T cells, while some of the former cells are expected to respond to SIVsmE543-3-transduced cells and vice versa because of amino acid sequence homology between SIVmac239 and SIVsmE543-3 (e.g., approximately 90% in Gag). Vaccine antigen-specific $CD8^+$ T-cell responses were elicited efficiently after the vaccination but their expansion after SIVsmE543-3 challenge was not observed (in R00-018) or inefficient (in R01-006), suggesting that these vaccine-induced $CD8^+$ T cells did not efficiently respond to SIVsmE543-3 challenge. In

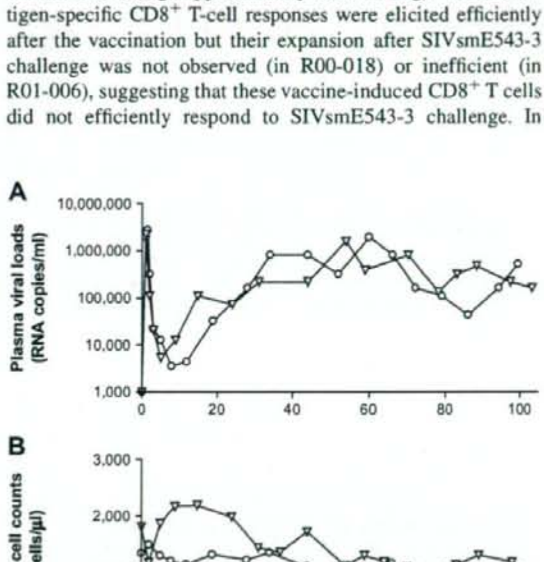

Fig. 1. Follow-up of the *90-120-Ia*-positive vaccinees (R00-018 indicated by circles and R01-006 indicated by triangles) after SIVsmE543-3 challenge. (A) Plasma viral loads (SIV RNA copies/ml plasma). (B) Peripheral $CD4^+$ T-cell counts (cells/μl). (C) Percentage of $CD4^+$ T cells in PBMCs.

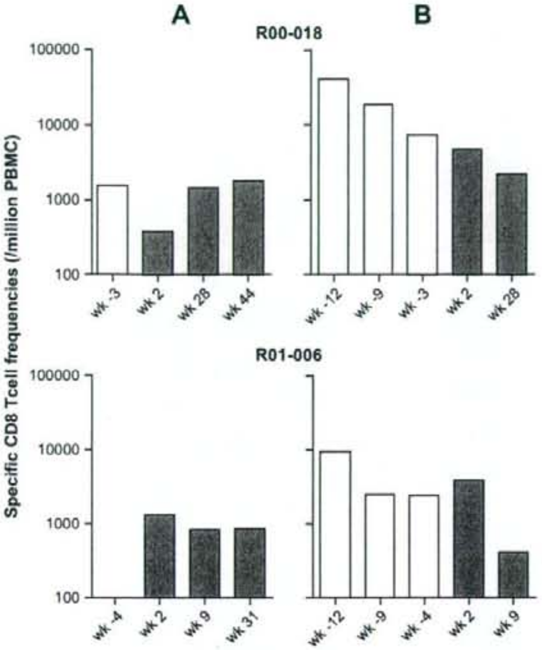

Fig. 2. Virus-specific CD8$^+$ T-cell frequencies in macaques R00-018 (upper panels) and R01-006 (lower panels). (A) SIVsmE543-3-specific CD8$^+$ T-cell frequencies in PBMCs. (B) Vaccine antigen-specific CD8$^+$ T-cell frequencies in PBMCs. The pre-challenge (indicated by minus week) and post-challenge frequencies are indicated by white and black bars, respectively.

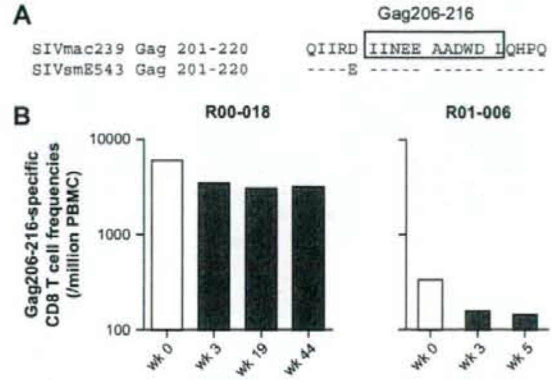

Fig. 3. $Gag_{206-216}$-specific CD8$^+$ T-cell frequencies. (A) Comparison of amino acid sequences around the $Gag_{206-216}$ epitope between SIVmac239 and SIVsmE543-3. (B) $Gag_{206-216}$ peptide-specific CD8$^+$ T-cell frequencies just before challenge (wk 0, white bars) and post-challenge (black bars) in macaques R00-018 (left panel) and R01-006 (right panel). PBMCs were stimulated by coculture with B-LCLs pulsed with $Gag_{206-216}$ peptide (IINEEAADWDL), and specific IFN-γ induction was measured.

R00-018, CD8$^+$ T cells responding to SIVsmE543-3-infected cells were detectable after vaccination but their levels did not increase at week 2 after challenge. In R01-006, these responses were undetectable just before challenge but induced after challenge.

3.2. No suppressive effect of vaccine-induced $Gag_{206-216}$-specific CTL responses on SIVsmE543-3 replication in vivo

Both macaques failed to control SIVsmE543-3 replication despite efficient elicitation of vaccine antigen-specific CD8$^+$ T-cell responses by the DNA-prime/SeV-Gag-boost vaccination. We then examined, in these two macaques, the levels of $Gag_{206-216}$-specific CD8$^+$ T-cell responses that have been indicated to exert suppressive pressure on SIVmac239 replication. Similarly to the previously-reported vaccinees possessing MHC-I haplotype *90-120-Ia* [18], both the vaccinees, R00-018 and R01-006, showed efficient induction of $Gag_{206-216}$-specific CD8$^+$ T-cell responses after vaccination (Fig. 3). Thus, these two macaques failed to control SIVsmE543-3 replication despite efficient induction of $Gag_{206-216}$-specific CTL responses by the prophylactic vaccination.

After SIVsmE543-3 challenge, the $Gag_{206-216}$-specific CD8$^+$ T-cell frequencies did not increase (Fig. 3), indicating no efficient expansion of these responses in these macaques. Sequencing of plasma viral genomes at several time points (Table 1) revealed no detectable mutations within the $Gag_{206-216}$ epitope-coding region even 1 year post-challenge, although all the previously-reported *90-120-Ia*-positive macaques infected with SIVmac239 have shown selection of a mutation within the $Gag_{206-216}$ epitope-coding region resulting in viral escape from recognition by $Gag_{206-216}$-specific CTLs [18]. These results indicate that the vaccine-induced $Gag_{206-216}$-specific CTLs did not respond to the heterologous challenge efficiently, exerting no suppressive pressure on SIVsmE543-3 replication, although the SIVsmE543-3 has the same $Gag_{206-216}$ epitope sequence, IINEEAADWDL, with SIVmac239.

Table 1
SIVsmE543-3 Gag amino acid changes in macaques[a]

R00-018					
Week 5	(no mutation)				
Week 12			V244A*		
Week 19			V244A		D465E
Week 28		T243S	V244A		
Week 60		T243S	V244A	V424I	
Week 100	P221L	T243S	V244A		
R01-006					
Week 9	(no mutation)				
Week 15			V244A		
Week 31		T243S			
Week 54		T243S			

[a] A *gag* fragment was amplified from plasma RNA by nested RT–PCR and subjected to sequencing. Dominant mutations resulting in amino acid changes are shown. Mutations within the $Gag_{206-216}$ epitope-coding region were undetectable. The V244A* at week 12 in R00-018 indicates that the wild-type and the mutant sequences were found equivalently.

3.3. Failure in Gag$_{206-216}$ epitope presentation due to a single amino acid change, Gag D205E, in the epitope flanking region

Comparison of amino acid sequences around the Gag$_{206-216}$ epitope between SIVmac239 and SIVsmE543-3 revealed a single amino acid change at the 205th amino acid in Gag, from aspartate (D) in SIVmac239 to glutamate (E) in SIVsmE543-3 (Fig. 3A). We then examined the effect of this single amino acid difference in the epitope flanking region on recognition of the epitope by Gag$_{206-216}$-specific CTL.

We first prepared amino (N) terminal-extended 15-mer peptides, SIVmac239 Gag$_{202-216}$ (IIRDIINEEAADWDL) and Gag$_{202-216}$.205E (SIVsmE543-3 Gag$_{202-216}$, IIREIINEEAADWDL), and examined frequencies of CD8$^+$ T cells that recognize these peptide-pulsed cells in PBMCs derived from vaccinated *90-120-Ia*-positive macaques (Fig. 4). No significant difference was observed between Gag$_{202-216}$ peptide-specific CD8$^+$ T-cell and Gag$_{202-216}$.205E peptide-specific CD8$^+$ T-cell frequencies, indicating that Gag$_{206-216}$-specific CTLs were able to recognize Gag$_{202-216}$ peptide-pulsed cells and Gag$_{202-216}$.205E peptide-pulsed cells equivalently.

Next, we constructed plasmid vectors, pEGFP-N1-Gag$_{202-216}$ and pEGFP-N1-Gag$_{202-216}$.205E expressing Gag$_{202-216}$-EGFP and Gag$_{202-216}$.205E-EGFP fused proteins, respectively (Fig. 5A). Efficient IFN-γ induction was observed after stimulation with pEGFP-N1-Gag$_{202-216}$-transfected cells but not with pEGFP-N1-Gag$_{202-216}$.205E-transfected cells (Fig. 5B). Thus, Gag$_{206-216}$-specific CTLs were able to recognize the cells expressing Gag$_{202-216}$-EGFP fusion proteins but not those expressing Gag$_{202-216}$.205E-EGFP fused proteins efficiently. These results suggest failure in Gag$_{206-216}$ epitope presentation due to the single amino acid change, Gag205D to Gag205E, in the epitope flanking region of SIVsmE543-3.

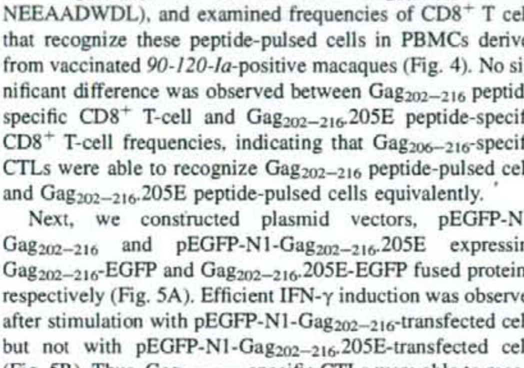

Fig. 4. Recognition of Gag$_{202-216}$ peptide-pulsed cells by Gag$_{206-216}$-specific CD8$^+$ T cells. Results with PBMCs obtained at one week after SeV-Gag boost from a DNA-prime/SeV-Gag-vaccinated macaque (#1) used in other experiment are shown in the left panel (Expt. 1), and those with PBMCs at 4 weeks after boost from another (#2) are in the right (Expt. 2). PBMCs were stimulated by coculture with B-LCLs pulsed with indicated concentrations of the SIVmac239 Gag$_{202-216}$ peptide or the Gag$_{202-216}$.205E peptide (purity: approximately 80%), and specific IFN-γ induction was measured.

4. Discussion

The previous study has shown control of SIVmac239 replication in the group of MHC-I haplotype *90-120-Ia*-positive macaques vaccinated with DNA-prime/SeV-Gag-boost [18]. These controllers showed high levels of Gag$_{206-216}$-specific CTL responses and rapid selection of a mutant escaping from this CTL recognition. The mutation leading to a substitution from leucine to serine at the 216th amino acid in Gag resulted in loss of viral fitness, indicating strong selective pressure of Gag$_{206-216}$-specific CTL responses on homologous SIVmac239 [18,23]. In the present study, we immunized macaques possessing MHC-I haplotype *90-120-Ia* with the vaccine expressing SIVmac239-derived antigens and challenged them with SIVsmE543-3. We then examined the efficacy of vaccine-induced Gag$_{206-216}$-specific CTLs against this heterologous virus that has the same Gag$_{206-216}$ epitope sequence. The vaccinees possibly able to control homologous SIVmac239 replication failed to contain the heterologous SIVsmE543-3 challenge despite efficient Gag$_{206-216}$-specific CTL induction by vaccination.

The vaccinees did not show detectable secondary Gag$_{206-216}$-specific CTL responses after the heterologous SIVsmE543-3 challenge nor exhibited mutations within the Gag$_{206-216}$ epitope-coding region even in the chronic phase. These results indicate that Gag$_{206-216}$-specific CTLs did not efficiently respond to SIVsmE543-3 infection or exert suppressive pressure on SIVsmE543-3 replication in vivo. Involvement of Gag$_{241-249}$ (SSVDEQIQW)-specific CTL responses in vaccine-based SIVmac239 control has also been suggested in the group of MHC-I haplotype *90-120-Ia*-positive macaques [24], but those Gag$_{241-249}$-specific CTLs did not show detectable responses against SIVsmE543-3 that has a different amino acid sequence (STVEEQIQW) within this epitope region (data not shown). Thus, neither vaccine-induced Gag$_{206-216}$-specific CTL nor Gag$_{241-249}$-specific CTL responses were effective against SIVsmE543-3 replication. However, SIVsmE543-3 challenge into unvaccinated *90-120-Ia*-positive control animals showed inefficient viral replication even in the absence of these CTL responses (data not shown). Its mechanism remains unclear, but vaccine-induced dominant CTL responses ineffective against the heterologous SIV may possibly exert worse effect on viral control.

Both SIVmac239-derived Gag$_{202-216}$ peptide-pulsed cells and SIVsmE543-3-derived Gag$_{202-216}$.205E peptide-pulsed cells were recognized equivalently (Fig. 4) whereas Gag$_{202-216}$-EGFP-expressing cells but not Gag$_{202-216}$.205E-EGFP-expressing cells were recognized by Gag$_{206-216}$-specific CTLs (Fig. 5), suggesting failure in recognition of SIVsmE543-3-infected cells by Gag$_{206-216}$-specific CTLs due to a single amino acid change from D in SIVmac239 to E in SIVsmE543-3 at Gag residue 205 immediately preceding the N terminus of Gag$_{206-216}$ epitope. Our results suggest failure in the epitope presentation on SIVsmE543-3-infected cells due to this amino acid change. It may be speculated that the Gag$_{206-216}$ epitope is processed at its carboxy terminus by proteasomes and at its N-terminus by aminopeptidases for

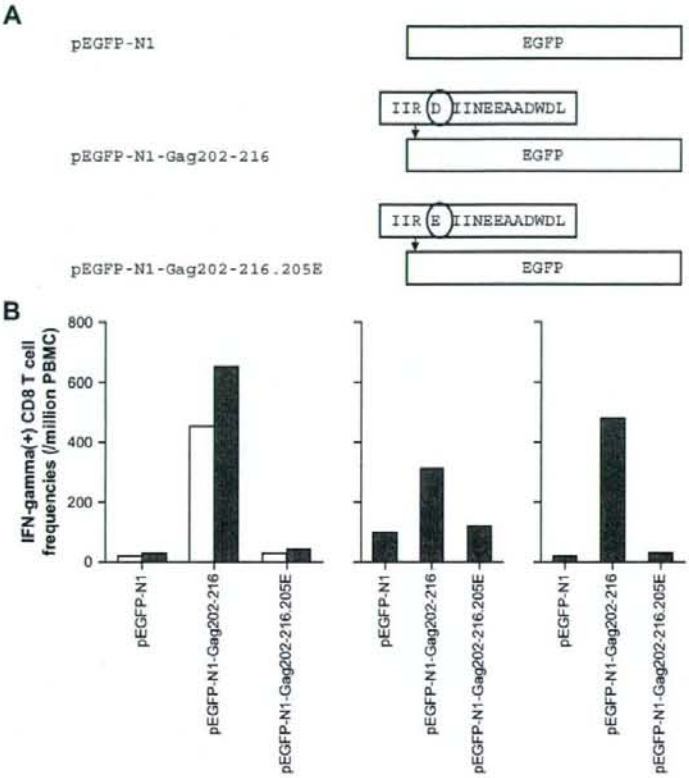

Fig. 5. Recognition of $Gag_{202-216}$-expressing cells by $Gag_{206-216}$-specific $CD8^+$ T cells. (A) Schema of DNA constructs. (B) IFN-γ induction in $CD8^+$ T cells by stimulation with $Gag_{202-216}$-expressing cells but not by $Gag_{202-216}$.205E-expressing cells. Three sets of experiments using PBMCs obtained at 2 weeks after SeV-Gag boost from DNA-prime/SeV-Gag-vaccinated macaques (#1 in Expt. 1, #2 in Expt. 2, and #3 in Expt. 3) are shown. Million PBMCs were stimulated by coculture with one-fifth (white bars in Expt. 1) or half (black bars in Expt. 1, Expt. 2, and Expt. 3) of million B-LCLs transfected with pEGFP-N1, pEGFP-N1-$Gag_{202-216}$, or pEGFP-N1-$Gag_{202-216}$.205E by electroporation and IFN-γ-positive $CD8^+$ T-cell frequencies were measured. The transfection efficiencies determined by EGFP expression in Expt. 1 were 6.1%, 6.2%, and 7.1%, respectively; 3.3%, 3.6%, and 4.1% in Expt. 2; not determined in Expt. 3.

its presentation [25,26] and that the D-to-E change in its N-terminal flanking region results in impairment of its N-terminal processing. However, amino acid changes in epitope flanking region do not always result in impairment of epitope processing [27]. Indeed, there has been no report showing aminopeptidases which do not recognize E but D for processing and the exact mechanism of impairment of the $Gag_{206-216}$ epitope presentation by the D-to-E change in its N-terminal flanking region remains unclear.

This study presents the first case, in macaques, of SIV escape from CTL recognition by changes in viral epitope flanking sequences. HIV-1 escape from CTL recognition by changes in epitope flanking sequences has been shown in HIV-1-infected individuals [28]. The escape observed in patients naturally infected with HIV-1 was from CTLs induced after infection and these post-infection-induced CTLs may not be fully functional because of possible immune impairment after HIV-1 infection [29,30]. Because it is difficult to know whether viral escape from CTL recognition in vivo is complete or not, it is important to clarify how much extent viral escape mutations can abrogate efficacy of functional CTLs in vivo. Indeed, it has remained unclear if changes in epitope flanking sequences can abrogate vaccine-induced CTL efficacy. In this study, the vaccine-induced $Gag_{206-216}$-specific CTLs effective against SIVmac239 did not efficiently respond to or show efficacy against SIVsmE543-3 infection in macaques, indicating a possibility of abrogation of vaccine-induced CTL efficacy in vivo by a single amino acid change in viral epitope flanking region. These results underline the influence of viral epitope flanking sequences on CTL-based AIDS vaccine efficacy, suggesting an important implication for development of an effective vaccine against highly-diversified HIVs.

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Gag-Specific Cytotoxic T-Lymphocyte-Based Control of Primary Simian Immunodeficiency Virus Replication in a Vaccine Trial▽

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Gag-specific cytotoxic T lymphocytes (CTLs) exert strong suppressive pressure on human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) replication. However, it has remained unclear whether they can actually contain primary viral replication. Recent trials of prophylactic vaccines inducing virus-specific T-cell responses have indicated their potential to confer resistance against primary SIV replication in rhesus macaques, while the immunological determinant for this vaccine-based viral control has not been elucidated thus far. Here we present evidence implicating Gag-specific CTLs as responsible for the vaccine-based primary SIV control. Prophylactic vaccination using a Gag-expressing Sendai virus vector resulted in containment of SIVmac239 challenge in all rhesus macaques possessing the major histocompatibility complex (MHC) haplotype *90-120-Ia*. In contrast, *90-120-Ia*-positive vaccinees failed to contain SIVs carrying multiple *gag* CTL escape mutations that had been selected, at the cost of viral fitness, in SIVmac239-infected *90-120-Ia*-positive macaques. These results show that Gag-specific CTL responses do play a crucial role in the control of wild-type SIVmac239 replication in vaccinees. This study implies the possibility of Gag-specific CTL-based primary HIV containment by prophylactic vaccination, although it also suggests that CTL-based AIDS vaccine efficacy may be abrogated in viral transmission between MHC-matched individuals.

Despite tremendous efforts to develop AIDS vaccines eliciting virus-specific T-cell responses, whether this approach actually does result in controlling human immunodeficiency virus (HIV) replication remains unknown. Recent trials have shown reductions in postchallenge viral loads by prophylactic vaccination eliciting virus-specific T-cell responses in macaque AIDS models (19, 22, 34), but the first advanced human trial of a T-cell-based vaccine was halted because of a lack of efficacy (5). Hence, it is quite important to determine which T-cell responses are responsible for primary HIV control.

Cytotoxic T-lymphocyte (CTL) responses have been indicated to play an important role in the control of HIV and simian immunodeficiency virus (SIV) infections (2, 9, 10, 17, 23, 29). Above all, the potential of Gag-specific CTL responses to contribute to viral control has been suggested by a cohort study indicating an association of HIV control with the breadth of Gag-specific CTL responses (15). In support of this, a recent in vitro study revealed their ability to rapidly respond to SIV infection (28). However, it has remained unclear whether Gag-specific CTL-based viral containment can be achieved by prophylactic vaccination.

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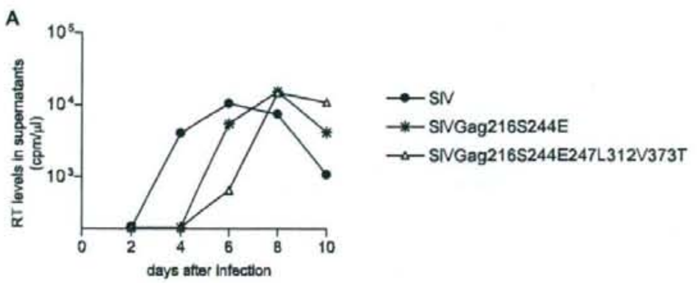
We previously developed a prophylactic AIDS vaccine regimen consisting of a DNA prime followed by a boost with a Sendai virus (SeV) vector expressing SIVmac239 Gag (SeV-Gag) (22, 32). Our trial showed potential for efficiently inducing Gag-specific T-cell responses and containment of SIVmac239 challenge in a group of Burmese rhesus macaques sharing the major histocompatibility complex class I (MHC-I) haplotype *90-120-Ia* (22). A follow-up study revealed the reappearance of plasma viremia at >1 year postchallenge in some of these *90-120-Ia*-positive SIV controllers. In these transient controllers, multiple CTL escape mutations were accumulated in the viral *gag* gene, resulting in viremia reappearance and thus suggesting the involvement of $Gag_{206-216}$ (IINEEAADWDL) epitope-specific, $Gag_{241-249}$ (SSVDEQIQW) epitope-specific, and $Gag_{373-380}$ (APVPIPFA) epitope-specific CTLs in sustained viral control (12). Nonetheless, it has remained undetermined whether such Gag-specific CTL responses were responsible for the vaccine-based primary SIV control in *90-120-Ia*-positive vaccinees. In the present study, we challenged the *90-120-Ia*-positive vaccinees with SIVs carrying the *gag* CTL escape mutations to determine the role of Gag-specific CTLs in primary SIVmac239 control.

MATERIALS AND METHODS

Viral competition assay. SIV molecular clone DNAs with *gag* mutations were constructed by site-directed mutagenesis from the wild-type SIVmac239 (14) molecular clone DNA. Virus stocks were obtained by transfection of COS-1 cells with wild-type or mutant SIV molecular clone DNAs, and their titers were

A



B

Competition between SIVmac239 vs SIVmac239Gag216S244E

inoculated dose ratio 1/1

	day 6	day 18
Exp 1	Wt	Wt
Exp 2	Wt	Wt
Exp 3	Wt (mt)	Wt

Competition between SIVmac239Gag216S244E vs SIVmac239Gag216S244E247L312V373T

	day 6	day 18
Exp 1	Wt	Wt
Exp 2	Wt (mt)	Wt
Exp 3	Wt (mt)	Wt

C

inoculated dose ratio 1/4

	day 6	day 18
SIVmac239	6/13	15/16
SIVmac239Gag216S244E	7/13	1/16

	day 6	day 18
SIVmac239Gag216S244E	7/18	12/16
SIVmac239Gag216S244E247L312V373T	11/18	3/16
*SIVmac239Gag216S244E312V373T		1/16

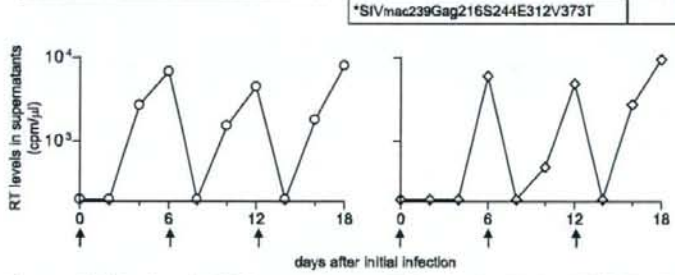


FIG. 1. Replication of mutant SIVs in vitro. (A) Wild-type and mutant SIV replication kinetics in HSC-F cells. HSC-F cells were infected with SIVmac239 (closed circles), SIVmac239Gag216S244E (asterisks), or SIVmac239Gag216S244E247L312V373T (open triangles). Virus production was monitored by measuring RT activity in the culture supernatants. Representative results from three sets of experiments are shown. (B) Viral competition assay. HSC-F cells were coinfected with SIVmac239 and SIVmac239Gag216S244E (left) or with SIVmac239Gag216S244E and SIV mac239Gag216S244E247L312V373T (right) at a ratio of 1:1. Viral *gag* fragments were amplified by RT-PCR from viral RNAs from the culture supernatants at days 6 and 18 postinfection and then sequenced. Dominant amino acid sequences at the 216th and 244th aa (left) or the 247th, 312th, and 373rd aa (right) in Gag in three sets of experiments are shown. Wt, only the wild-type sequence was detected; Wt (mt), the wild type was dominant, but the mutant was detectable (the mutant/wild-type ratio was <1/2). (C) Viral competition assay. HSC-F cells were coinfected with SIVmac239 and SIVmac239Gag216S244E (left) or with SIVmac239Gag216S244E and SIVmac239Gag216S244E247L312V373T (right) at a ratio of 1:4. The amplified *gag* fragments were subcloned into plasmids and sequenced. Frequencies of the indicated SIV clones (number of indicated clone per total number of clones) are shown. Changes in RT levels in the culture supernatants are shown in the bottom panels. The arrows indicate the time points of coinfection (at day 0) and viral passage for the second (at day 6) and the third (at day 12) cultures.

measured by reverse transcription (RT) assay as described previously (25, 33). For analysis of viral replication, HSC-F cells (herpesvirus saimiri-immortalized macaque T-cell line) (1) were infected with wild-type or mutant SIVs (normalized by RT activity), and virus production was monitored by measuring RT activity in the culture supernatants. For competition, HSC-F cells were coinfected with two SIVs at a ratio of 1:1 or 1:4, and the culture supernatants were harvested every other day and used for RT assays. On day 6, the supernatant was added to fresh HSC-F cells to start the second culture. Similarly, on day 12 after the initial coinfection, the second culture supernatant was added to fresh HSC-F cells to start the third culture. RNAs were extracted from the initial culture supernatant on day 6 and from the third culture supernatant on day 18 postcoinfection. The fragment (nucleotides 1231 to 2958 in SIVmac239 [GenBank accession number M33262]) containing the entire *gag* region was amplified from the RNA by RT-PCR and sequenced. Alternatively, it was subcloned into plasmids to determine dominant sequences.

Animal experiments. Burmese rhesus macaques (*Macaca mulatta*) were maintained in accordance with the guidelines for animal experiments performed at the National Institute of Infectious Diseases (26). Three animals, R01-007, R02-003, and R02-012, that received a prophylactic DNA prime/SeV-Gag boost vaccine and contained SIVmac239 challenge have been reported previously (22). In the present study, macaques R06-015, R06-035, R06-041, R05-004, R05-027, and R07-005 also received the DNA prime/SeV-Gag boost vaccine. The DNA used for the vaccination, CMV-SHIVdEN, was constructed from *env*- and *nef*-deleted simian-human immunodeficiency virus $SHIV_{MD14YE}$ molecular clone

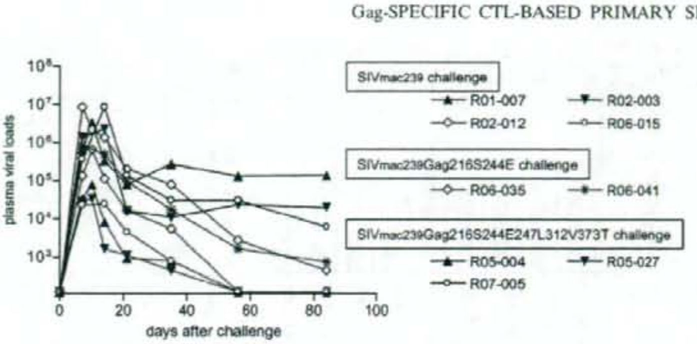


FIG. 2. Plasma viral loads after wild-type or mutant SIV challenge. The *90-120-Ia*-positive vaccinees were challenged with SIVmac239 (red lines), SIVmac239Gag216S244E (blue lines), or SIVmac239Gag216S244E247L312V373T (black lines). Plasma viral loads (SIV *gag* RNA copies/ml plasma) were determined as described before (22). The lower limit of detection is approximately 4×10^2 copies/ml.

DNA (SIVGP1) (31, 32) and has the genes encoding SIVmac239 Gag, Pol, Vif, and Vpx, SIVmac239-HIV chimeric Vpr, and HIV Tat and Rev. At the DNA vaccination, animals received 5 mg of CMV-SHIVdEN DNA intramuscularly. Six weeks after the DNA prime step, animals received a single boost intranasally with 6×10^9 cell infectious units of F-deleted replication-defective SeV-Gag (21, 32). Approximately 3 months after the boost, animals were challenged intravenously with 1,000 50% tissue culture infective doses of SIVmac239, SIVmac239 Gag216S244E, or SIVmac239Gag216S244E247L312V373T. The challenge virus stocks were prepared by virus propagation on rhesus macaque peripheral blood mononuclear cells (PBMCs). Sequence analysis confirmed the absence of *gag* mutations except for the two or five mutations in the challenge viruses.

Immunostaining of CD4⁺ T-cell memory subsets. PBMCs were subjected to immunofluorescence staining by using fluorescein isothiocyanate-conjugated anti-human CD28, phycoerythrin-conjugated anti-human CD95, peridinin chlorophyll protein-conjugated anti-human CD4, and allophycocyanin-conjugated anti-human CD3 monoclonal antibodies (Becton Dickinson, Tokyo, Japan). The central memory subset of $CD4^+$ T cells was defined by possession of a $CD28^+$ $CD95^+$ phenotype, as described previously (13, 27).

Measurement of virus-specific $CD8^+$ T-cell responses. We measured virus-specific $CD8^+$ T-cell levels by flow cytometric analysis of gamma interferon (IFN-γ) induction after specific stimulation, as described previously (13, 22). In brief, PBMCs were cocultured with autologous herpesvirus papio-immortalized B-lymphoblastoid cell lines infected with a vaccinia virus vector expressing SIVmac239 Gag for Gag-specific stimulation or a vesicular stomatitis virus G protein-pseudotyped SIVGP1 for SIV-specific stimulation. The pseudotyped virus was obtained by cotransfection of COS-1 cells with a vesicular stomatitis virus G protein expression plasmid and the SIVGP1 DNA. Alternatively, B-lymphoblastoid cell lines were pulsed with 1 to 10 μM peptides for peptide-specific stimulation (11, 12). The 15-mer $Gag_{367-381}$ peptide was used to detect $Gag_{367-381}$-specific CTLs, including $Gag_{373-380}$-specific CTLs. Intracellular IFN-γ staining was performed using a Cytofix Cytoperm kit (Becton Dickinson). 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 nonspecific IFN-γ^+ T-cell frequencies from those after Gag-specific, SIV-specific, or peptide-specific stimulation. Specific T-cell levels of <100 cells per million PBMCs were considered negative.

Statistical analysis. Statistical analysis was performed with Prism software, version 4.03, with significance set at P values of <0.05 (GraphPad Software, Inc., San Diego, CA). Central memory $CD4^+$ T-cell counts before challenge were not significantly different between the wild-type SIV-challenged ($n = 4$) and the mutant SIV-challenged ($n = 5$) macaques ($P = 0.70$ by unpaired two-tailed t test with Welch's correction and $P = 0.73$ by nonparametric Mann-Whitney U test). Ratios of the central memory $CD4^+$ T-cell counts from a few months postchallenge to those prechallenge were log transformed and compared between the two groups by an unpaired two-tailed t test and the Mann-Whitney U test. Gag-specific $CD8^+$ T-cell frequencies postvaccination (prechallenge) or postchallenge were also log transformed and compared between the two groups in the same statistical manner.

RESULTS

Comparison of viral fitness in wild-type and mutant SIVs. We used two mutant SIVs for challenge of the *90-120-Ia*-positive vaccinees. The first, designated SIVmac239Gag216S244E, carries two *gag* mutations, GagL216S and GagD244E, leading to a leucine (L)-to-serine (S) substitution at the 216th amino acid (aa) and an aspartic acid (D)-to-glutamic acid (E) substitution at the 244th aa in Gag. The second, designated SIVmac239Gag216S244E247L312V373T, carries five *gag* mutations, GagL216S, GagD244E, 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). In our previous study (12), the former became dominant in the early phase (at approximately 4 months postchallenge) during the period of viral control, and the latter was dominant at viremia reappearance in a transient controller. GagL216S, GagD244E and GagI247L, and GagA373T mutations result in viral escape from recognition by $Gag_{206-216}$-specific, $Gag_{241-249}$-specific, and $Gag_{373-380}$-specific CTLs, respectively, while it remains unclear whether GagA312V was selected for by CTLs.

We first compared viral fitness in wild-type and mutant SIVs. In HSC-F cells (a macaque T-cell line), not only the wild type but also the mutant SIVs were able to replicate, but SIVmac239Gag216S244E replication was less efficient than that of wild-type SIVmac239, and SIVmac239Gag216S244E247L312V373T replication was even less efficient (Fig. 1A). In competitions between two SIVs, HSC-F cells were coinfected with both viruses, and viral genome sequences in the culture supernatants were assessed to establish which SIV became predominant. In culture supernatants of HSC-F cells after coinfection with SIVmac239 and SIVmac239Gag216S244E inoculated at a ratio of 1:1, the wild type rapidly became dominant (at day 6) (Fig. 1B). Coinfection at a ratio of 1:4 resulted in equivalence at day 6, but the wild type again dominated by day 18 (Fig. 1C). These results indicate a lower replicative ability of SIVmac239 Gag216S244E than of wild-type SIVmac239. In addition, competition between SIVmac239Gag216S244E and SIVmac239 Gag216S244E247L312V373T showed the lower replicative ability of the latter (Fig. 1B and C).

Challenge of *90-120-Ia*-positive vaccinees with wild-type or mutant SIVs. Next, we challenged *90-120-Ia*-positive macaques

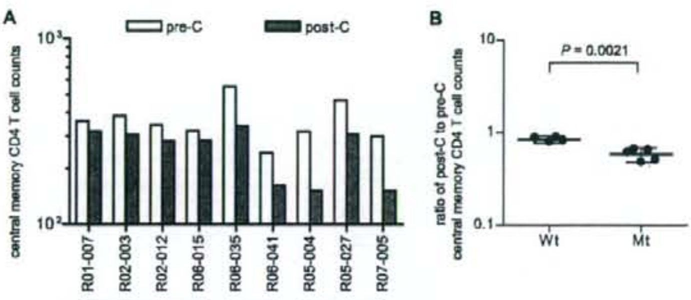


FIG. 3. Changes in central memory CD4$^+$ T-cell counts after wild-type or mutant SIV challenge. (A) Peripheral central memory CD4$^+$ (CD4$^+$ CD95$^+$ CD28$^+$) T-cell counts (/μl) prechallenge (pre-C) and a few months postchallenge (post-C). (B) Statistical comparison of central memory CD4$^+$ T-cell loss between the wild-type SIV-challenged (Wt) and the mutant SIV-challenged (Mt) macaques. The ratios of central memory CD4$^+$ T-cell counts postchallenge to those prechallenge are plotted. The longer bars indicate geometric mean values, and the regions between the shorter bars indicate the 95% confidence intervals. The ratios in the mutant group (n = 5) were significantly lower than those in the wild-type group (n = 4) (P = 0.0021 by unpaired t test and P = 0.0159 by Mann-Whitney U test).

with the mutant SIVs after DNA prime/SeV-Gag vaccination. Remarkably, all three vaccinees (R05-004, R05-027, and R07-005) challenged with SIVmac239Gag216S244E247L312V373T failed to control viral replication and showed high set point plasma viral loads, while all four vaccinees (R01-007, R02-003, R02-012, and R06-015) challenged with wild-type SIVmac239 contained viral replication, with undetectable set point plasma viral loads (Fig. 2). Even the two vaccinees (R06-035 and R06-041) challenged with SIVmac239Gag216S244E failed to contain viral replication, although with lower plasma viral loads, at approximately 10^3 RNA copies/ml at 3 months postchallenge. Central memory CD4$^+$ T-cell counts before challenge were not significantly different between the wild-type SIV-challenged (n = 4) and mutant SIV-challenged (n = 5) macaques, but ratios of the counts at a few months postchallenge to prechallenge for the latter group were significantly lower than those for the former (P = 0.0021 by unpaired t test and P = 0.0159 by Mann-Whitney U test) (Fig. 3). Thus, *90-120-Ia*-positive vaccinees can contain wild-type SIVmac239 but not SIVmac239Gag216S244E or SIVmac239Gag216S244E247L312V373T challenge.

Viral *gag* sequence analysis confirmed the rapid selection for the GagL216S mutation in all wild-type SIVmac239-challenged macaques, as described previously (22). All of the *gag* mutations in the challenge mutant viruses were maintained during the observation period (Table 1). SIVmac239Gag216S244E247L312V373T-challenged macaques showed no additional dominant *gag* mutations, whereas animals challenged with SIVmac239Gag216S244E rapidly selected viruses with a GagV145A (V to A at the 145th aa) mutation. Recovery of viral fitness by this mutation was not observed, and whether it was selected for by CTLs was unclear in our previous study (12).

Gag-specific CTL responses were induced after SeV-Gag boost in all vaccinees, and there was no significant difference in the levels between the wild-type and mutant challenges (P = 0.1198 by unpaired t test and P = 0.1111 by Mann-Whitney U test). However, secondary Gag-specific CTL responses were

TABLE 1. Dominant sequences in SIV Gag in macaques after challenge

Macaque	Time (wk) of plasma sample	Amino acid change in Gag at position[a]:								
		140	145	206	216	244	247	312	341	373
R01-007	5				L216S					
R02-003	5				L216S					
R02-012	5				L216S					
R06-015	5			(I206M)	L216S					
R06-035	5				L216S*	D244E*				
	12		V145A		L216S*	D244E*			(N341Y)	
R06-041	5		(V145A)		L216S*	D244E*				
	12		V145A		L216S*	D244E*				
R05-004	5				L216S*	D244E*	I247L*	A312V*		A373T*
	12	(I140V)			L216S*	D244E*	I247L*	A312V*		A373T*
R05-027	5				L216S*	D244E*	I247L*	A312V*		A373T*
	12				L216S*	D244E*	I247L*	A312V*		A373T*
R07-005	5				L216S*	D244E*	I247L*	A312V*		A373T*
	12				L216S*	D244E*	I247L*	A312V*		A373T*

[a] A fragment containing the entire *gag* region was amplified from plasma RNA by nested RT-PCR and then sequenced. We were unable to amplify the fragment from plasmas obtained at week 12 from the wild-type SIVmac239-challenged macaques with undetectable viremia. Dominant *gag* mutations resulting in amino acid changes are shown. Asterisks indicate the mutations included in the challenge inoculums. Parentheses indicate that both the wild-type and mutant sequences were detected equivalently at that position.

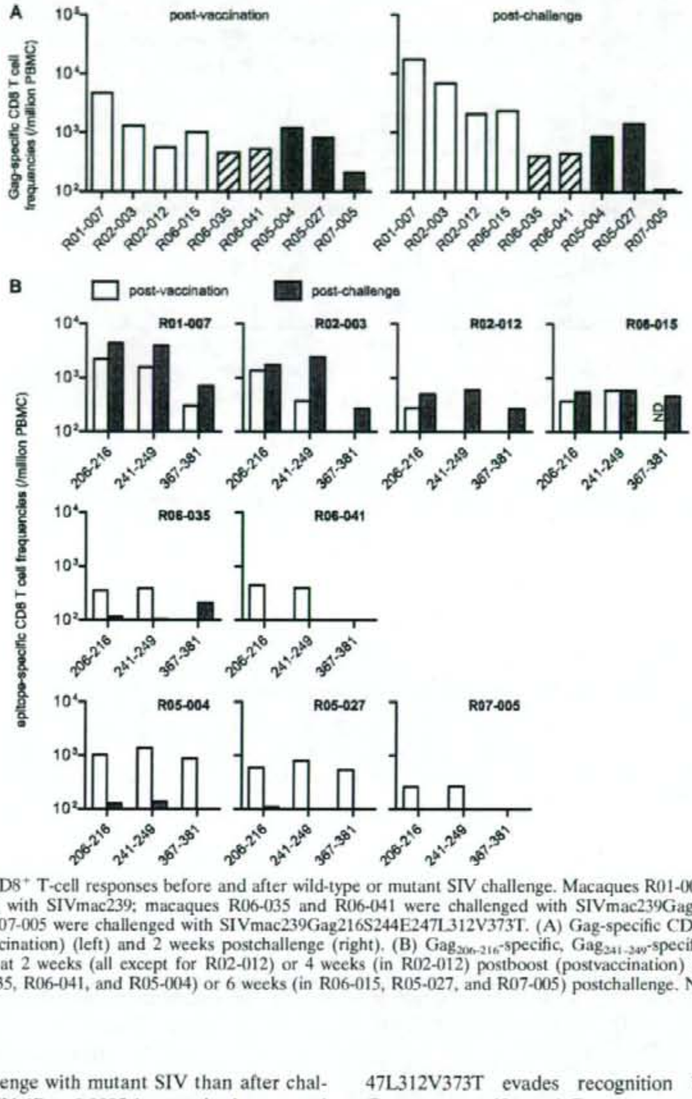


FIG. 4. Gag-specific CD8⁺ T-cell responses before and after wild-type or mutant SIV challenge. Macaques R01-007, R02-003, R02-012, and R06-015 were challenged with SIVmac239; macaques R06-035 and R06-041 were challenged with SIVmac239Gag216S244E; and macaques R05-004, R05-027, and R07-005 were challenged with SIVmac239Gag216S244E247L312V373T. (A) Gag-specific CD8⁺ T-cell frequencies at 2 weeks postboost (postvaccination) (left) and 2 weeks postchallenge (right). (B) $Gag_{206-216}$-specific, $Gag_{241-249}$-specific, and $Gag_{367-381}$-specific CD8⁺ T-cell frequencies at 2 weeks (all except for R02-012) or 4 weeks (in R02-012) postboost (postvaccination) and 5 weeks (in R01-007, R02-003, R02-012, R06-035, R06-041, and R05-004) or 6 weeks (in R06-015, R05-027, and R07-005) postchallenge. ND, not determined.

less efficient after challenge with mutant SIV than after challenge with wild-type SIV ($P = 0.0095$ by unpaired t test and $P = 0.0159$ by Mann-Whitney U test) (Fig. 4A).

SeV-Gag boost induced efficient $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses in all vaccinees and $Gag_{367-381}$-specific CTL responses in some of them (Fig. 4B). Challenge with wild-type SIVmac239 resulted in efficient secondary responses of these three epitope-specific CTLs, whereas SIVmac 239Gag216S244E247L312V373T challenge evoked none of them (Fig. 4B). SIVmac239Gag216S244E challenge did not result in secondary responses of $Gag_{206-216}$-specific or $Gag_{241-249}$-specific CTLs but did induce $Gag_{367-381}$-specific CTL responses in one case (Fig. 4B). These results indicate that SIVmac239 Gag216S244E evades recognition by $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTLs and that SIVmac239Gag216S244E247L312V373T evades recognition by $Gag_{206-216}$-specific, $Gag_{241-249}$-specific, and $Gag_{367-381}$-specific CTLs.

We next examined Gag-specific and SIV-specific CTL responses after mutant SIV challenge (Fig. 5A). We used an *env*- and *nef*-deleted SHIV molecular clone DNA, SIVGP1, that has the genes encoding SIVmac239 Gag, Pol, Vif, Vpx, and a part of Vpr and measured the frequencies of CTLs responding to SIVGP1-transduced cells (referred to as SIV-specific CTLs) as described previously (13, 32). SIV-specific CTL frequencies at week 12 were much higher than those at week 2 for all five macaques challenged with mutant SIVs. In contrast, Gag-specific CTL frequencies at week 12 were lower than those at week 2 for four of five animals; the remaining macaque, R06-035, mounted $Gag_{367-381}$-specific CTL responses. Importantly, in all animals challenged with mutant SIVs, SIV-specific CTL

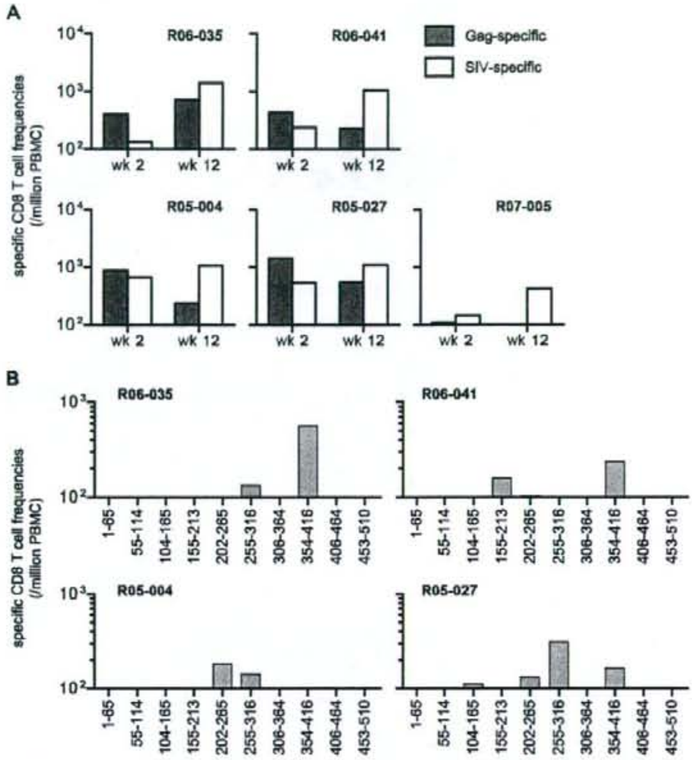


FIG. 5. SIV-specific CD8$^+$ T-cell responses after mutant SIV challenge. (A) Gag-specific (closed boxes) and SIV-specific (open boxes) CD8$^+$ T-cell frequencies at 2 weeks or 12 weeks postchallenge. (B) Frequencies of CD8$^+$ T cells specific for pools of SIV Gag peptides. A panel of 117 overlapping peptides (15 to 17 aa in length and overlapping by 10 to 12 aa) spanning the entire SIV Gag amino acid sequence were divided into the following 10 pools (each consisting of 11 or 12 peptides): pool 1, 1st to 65th aa in SIV Gag; pool 2, 55th to 114th aa; pool 3, 104th to 165th aa; pool 4, 155th to 213th aa; pool 5, 202nd to 265th aa; pool 6, 255th to 316th aa; pool 7, 306th to 364th aa; pool 8, 354th to 416th aa; pool 9, 406th to 464th aa; and pool 10, 453rd to 510th aa. The pools were used for stimulation to detect peptide pool-specific CD8$^+$ T cells.

frequencies were at marginal levels or lower than Gag-specific CTL frequencies at week 2, but the former became higher than the latter at week 12. These results indicate an induction of CTL responses specific for SIV antigens other than Gag in all five macaques after mutant SIV challenge.

At week 12 after mutant SIV challenge, Gag-specific CTL responses were undetectable in macaque R07-005 but were still detected in the other four macaques. We then analyzed Gag-specific CTL responses in these four macaques by using a panel of overlapping peptides spanning the entire SIV Gag amino acid sequence (Fig. 5B). In both SIVmac239Gag216S244E-challenged animals, R06-035 and R06-041, exhibiting detectable $Gag_{367-381}$-specific CTL responses (data not shown), CTL responses specific for the peptide mixture corresponding to the 354th to 416th aa in SIV Gag were detected at week 12. In addition, we found $Gag_{255-316}$-specific CTL responses in macaque R06-035 and $Gag_{155-213}$-specific CTL responses in macaque R06-041. SIVmac239Gag216S244E247L312V373T-challenged macaques R05-004 and R05-027 showed responses specific for several Gag peptide mixtures, including $Gag_{202-265}$-specific and $Gag_{255-316}$-specific CTL responses. These results indicate an induction of CTL responses specific for Gag epitopes other than the $Gag_{206-216}$, $Gag_{241-249}$, and $Gag_{373-380}$ epitopes after mutant SIV challenge.

DISCUSSION

In the present study, SIVs carrying multiple *gag* CTL escape mutations showed lower replicative abilities than that of the wild type; nonetheless, the *90-120-Ia*-positive vaccinees were able to contain only the latter. This demonstrates that Gag-specific CTL responses did play a central role in the vaccine-based primary containment of wild-type SIVmac239 replication in *90-120-Ia*-positive macaques.

Elicitation of virus-specific T-cell responses by prophylactic vaccination is believed to be a promising strategy for HIV control (3, 24); whether this approach can actually result in HIV control remains unknown. Recent studies have indicated the possibility of reductions in set point viral loads after SIV challenge by prophylactic vaccination inducing T-cell responses in rhesus macaques (19, 22, 34), yet the immune component crucial for the vaccine-based viral control has not been determined. No clear evidence for a contribution of vaccine-induced CTLs to this viral control has been forthcoming to date, although virus-specific CTL responses have been implicated in exerting strong suppressive pressure on HIV/SIV infection (9, 22). Indeed, viral replication persists even in the presence of CTL responses in the natural course of infection; it has thus remained unclear whether HIV/SIV replication can be controlled by vaccine-induced CTLs. The evidence from the present study now strongly implicates Gag-specific CTL responses as responsible for vaccine-based primary SIV control. This offers the possibility of Gag-specific CTL-based HIV containment by prophylactic vaccination and provides insight into the development of CTL-based AIDS vaccines.

The containment of SIVmac239 but failure to contain SIV mac239Gag216S244E in the vaccinees documents a crucial role for $Gag_{206-216}$-specific and/or $Gag_{241-249}$-specific CTL responses in vaccine-based SIVmac239 containment. Furthermore, challenge with SIVmac239Gag216S244E247L312V373T, possessing diminished viral fitness compared to SIVmac239Gag216S244E, tended to result in higher viral loads, indicating the involvement of $Gag_{373-380}$-specific CTL responses in viral control, while more complete viral evasion of $Gag_{241-249}$-specific CTL recognition by addition of the GagI247L mutation may also contribute to the difference between SIVmac239 Gag216S244E and SIVmac239Gag216S244E247L312V373T challenge. Taken together, we conclude that these two or three epitope-specific CTL responses are crucial for primary SIVmac239 control in *90-120-Ia*-positive vaccinees. Conversely, this study implies that viral evasion of recognition by two dominant epitope-specific CTLs can result in failure of primary viral containment but may not be sufficient for abrogation of vaccine efficacy. Thus, analysis of CTL-based vaccine efficacy against SIVs carrying single or multiple CTL escape mutations could contribute to an evaluation of its potential for controlling the replication of highly diversified HIVs.

Our results suggest that SIV- but non-Gag-specific CTLs became predominant after mutant SIV challenge. Additionally, CTLs recognizing Gag regions other than the $Gag_{206-216}$, $Gag_{241-249}$, and $Gag_{373-380}$ epitopes were detected in most cases. These CTL responses may exert suppressive pressure on viral replication but are considered insufficient for controlling replication of the mutant SIVs with lower viral fitness.

Finally, this study also provides evidence indicating a possible abrogation of CTL-based AIDS vaccine efficacy in viral transmission between MHC-I-matched individuals. Indeed, even the mutant SIVs carrying multiple CTL escape mutations were able to replicate persistently in vivo, despite their diminished replicative ability. Transmission of these viruses can result in persistent viral infection and AIDS progression (30). CTL escape mutations resulting in a loss of viral fitness may revert to the wild-type sequence after transmission into MHC-I-mismatched hosts (4, 8, 9, 16, 18, 20), but such reversion does not occur rapidly; alternatively, some may be retained with additional compensatory mutations (6, 7, 30). Thus, there may be a risk of transmission and accumulation of HIV CTL escape variants even among MHC-I-mismatched individuals, resulting in abrogation of CTL-based AIDS vaccine efficacy in a population.

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Enhanced Replication of Human T-Cell Leukemia Virus Type 1 in T Cells from Transgenic Rats Expressing Human CRM1 That Is Regulated in a Natural Manner▿

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Human T-cell leukemia virus type 1 (HTLV-1) is the etiologic agent of adult T-cell leukemia (ATL). To develop a better animal model for the investigation of HTLV-1 infection, we established a transgenic (Tg) rat carrying the human CRM1 (hCRM1) gene, which encodes a viral RNA transporter that is a species-specific restriction factor. At first we found that CRM1 expression is elaborately regulated through a pathway involving protein kinase C during lymphocyte activation, initially by posttranscriptional and subsequently by transcriptional mechanisms. This fact led us to use an hCRM1-containing bacterial artificial chromosome clone, which would harbor the entire regulatory and coding regions of the CRM1 gene. The Tg rats expressed hCRM1 protein in a manner similar to expression of intrinsic rat CRM1 in various organs. HTLV-1-infected T-cell lines derived from these Tg rats produced 100- to 10,000-fold more HTLV-1 than did T cells from wild-type rats, and the absolute levels of HTLV-1 were similar to those produced by human T cells. We also observed enhancement of the dissemination of HTLV-1 to the thymus in the Tg rats after intraperitoneal inoculation, although the proviral loads were low in both wild-type and Tg rats. These results support the essential role of hCRM1 in proper HTLV-1 replication and suggest the importance of this Tg rat as an animal model for HTLV-1.

Human T-cell leukemia virus type I (HTLV-1) is etiologically associated with human adult T-cell leukemia (ATL), a chronic progressive neurological disorder termed HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (17, 27, 54, 55), and several other human diseases (23, 40, 42, 48). Examination of the viral nucleotide sequences associated with different disease groups has not revealed any specific determinants that distinguish a particular HTLV-1-associated disease (11, 35, 67). Thus, a primary determinant of HTLV-1-associated disease may be host related.

In order to investigate HTLV-1 infection and related disease development in detail, suitable animal models are required. HTLV-1 can immortalize simian, feline, rat, and rabbit lymphocytes in vitro (2, 29, 46). HTLV-1 can also infect experimental animals, such as rabbits, monkeys, and rats (2, 45, 53, 62). Using these susceptible animals, several models have been developed to study HTLV-1-associated diseases. The HAM/TSP-like disease model in strain WKA rats is well established and has been used to dissect the pathogenic mechanisms of the disease (31, 39). In contrast, only a few ATL model systems have been established using rabbits and rats, and their utility is limited. For instance, the rabbit ATL model shows reproducible development of an ATL-like disease in adult animals (58), but few immunological studies can be performed with this animal, primarily because of the difficulty of obtaining inbred strains of rabbits. In the rat models, the development of ATL-like disease was observed only in newborn animals, with a very short period of disease onset (64), making it difficult to perform oncological and immunological studies at the same time. Ohashi et al. have established a rat model of ATL-like disease in which they were able to examine the growth and spread of HTLV-1-infected cells, as well as to assess the effects of T cells on the development of the disease in T-cell-deficient nude rats (51). This model system has been used to assess DNA- or peptide-based vaccine development (25, 52) and to study the effects of Tax-directed small interfering RNA on HTLV-1-induced tumors (50). However, since the growth of HTLV-1 tumors could be monitored only in immune-deficient nude rats in this model system, better animal models are still necessary.

HTLV-1 replicates poorly in rats, which may be one of the reasons why previously established models could not completely reproduce the features of HTLV-1-related diseases. We have previously examined the differences in the pattern of viral gene expression between human and rat T cells infected with HTLV-1 (69). In rat cells, the levels of viral mRNAs

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