$CD4^+$ T lymphocyte subsets enriched with CXCR4 expression [11,16], vaccine-based viral control has in fact been achieved consistently [17–22]. In contrast, in the latter model of CCR5-tropic SIV infection, vaccine-based successful viremia control has not consistently been attained [23–28] but observed under the conditions with specific host genetic backgrounds affecting CTL efficacies [29].

ANTI-HIV CTL POTENCY

Virus-specific CTLs play an indispensable role in control of immunodeficiency virus infection [30–35]. Enrolment of $CD8^+$ CTLs in primary control of HIV/SIV replication is directly visible as the initial decline of viral loads from the peak to the set point. Yet infection persists, and eventually the host progresses to AIDS (Figure 1). Hence the question remains as to what determines the persistence of viremia even under vigorous CTL pressure. To this important question, several explanations have been posed to date; some have suggested possible contribution of the aberrance of virus-specific $CD4^+$ T cell responses to failure in viral control [36,37]. Other reports have indicated that ongoing immune activation itself may fuel infection persistence, in part in a hen-and-egg fashion of CTL dysfunction or exhaustion [38–40]. Analyses on the various aspects of difficulty in HIV/SIV control by CTLs have newly highlighted several important issues related to its functionality, including competitive equilibrium between CTL potency and viral escape, influence of viral and host major histocompatibility complex (MHC) polymorphisms, and kinetics of infected cell killing. These may provide important clues for development of effective CTL-based AIDS vaccines.

HIV infections mostly exhibit chronic disease progression, but occasionally show rapid AIDS progression or, in reverse, delayed disease courses with viral control. Genetic analyses attributed some of the latter cases of inefficient viral replication to viral polymorphisms such as *nef* deletion [41] or host polymorphisms such as CCR5Δ32 variants [42,43]. These were followed by a finding of worse prognoses in HIV-infected human leukocyte antigen (HLA) homozygotes compared to heterozygotes [44] and a wave of cumulative analyses indicating association of HLA/MHC genotypes with rapid or delayed AIDS progression [45–49]. For instance, most of the HIV-1-infected individuals possessing *HLA-B*57* have been indicated to show a better prognosis with lower viral loads, and involvement of HLA-B*57-restricted epitope-specific CTL responses in this viral control has been suggested [50]. This notion is back to back with the existence of HLA alleles associated with rapid disease progression such as *HLA-B*35* [44]. Similarly, association between MHC class I (MHC-I) genotypes and viral loads has been intensively investigated in macaque models of SIV infection. In Indian rhesus macaques, association of *Mamu-A*01*, *Mamu-A*02*, *Mamu-B*08* and

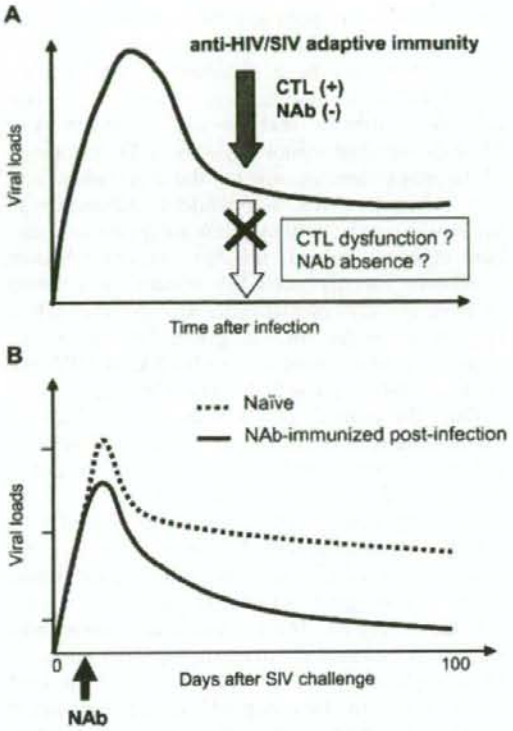

Figure 1. Schema for indicating changes in viral loads in HIV/SIV infections. (A) Persistent HIV/SIV natural infection. Virus-specific CTL responses play a central role in the resolution from viremia peak to the set point (indicated by a black arrow), whereas NAb induction is inefficient in this early phase. Yet viral replication persists and its mechanism has been unclear; speculations include CTL dysfunction, lack of memory $CD4^+$ T-cell help and absence of NAb responses. (B) Effect of post-infection passive immunisation with polyclonal NAbs on primary SIVmac239 replication in rhesus macaques [94]. NAb-immunised macaques showed lower or undetectable viral loads compared to naive controls

*Mamu-B*17* with enhanced SIV control has been indicated [35,51–54], while *Mamu-B*01* and *Mamu-A*08* have been observed as alleles associated with rapid disease progression [35]. It is reasonable to surmise that the overall spectrum of MHC-I-restricted epitope-specific CTL responses is the major determinant for the association, and this inherent, MHC-I-dependent difference in host resistance to viral infection might modify vaccine efficacy. Indeed, we have recently shown consistent vaccine-based SIV control in a group of Burmese rhesus macaques sharing an MHC-I haplotype, suggesting a possibility of MHC-dependent attainment of vaccine-based HIV/SIV control with effective CTL induction [29].

Thus the potency of CTLs largely depends on the host genetic backgrounds, while viral genome polymorphisms can also affect CTL efficacy. Selection of viral mutations resulting in escape from CTL recognition is frequently observed in HIV infections, and potent CTLs often select for escape mutations even at the cost of viral fitness [29,55–59], suggesting that CTLs specific for viral epitopes derived from structurally conserved regions may exert stronger suppressive effect on viral replication. Viral replication *in vivo* may likely be affected by a single CTL escape mutation [50,60–62], while a clear association of viremia recrudescence with accumulation of multiple CTL escape mutations has been observed in HIV/SIV infections, indicating possible involvement of multiple epitope-specific CTLs in viral control [59].

Broad, multiple epitope-specific CTL induction seems preferable for viral control, but it may not always result in advantageous induction of effective CTL responses due to the inherent hierarchy of CTLs [63]. For instance, there may be a possibility of disturbance in subdominant effective CTL responses by dominant ineffective CTL induction. Furthermore, whether oligoclonal immunodominant CTL responses or polyclonal subdominant responses are superior in viral control remains unclear. Analysis of SIVmac239-infected Indian rhesus macaques exhibiting low viral loads (elite controllers) has shown dominant Gag-specific CTL responses during primary viral control followed by induction of subdominant CTL responses responsible for the control in the chronic phase [64]. Accordingly, our long-term follow up of Burmese rhesus macaques exhibiting vaccine-based control of SIVmac239 replication has shown disappearance of Gag-specific CTL responses dominant during primary viral control and subsequent appearance of non-Gag-specific CTL responses responsible for sustained control in the chronic phase [65].

Turning to the potency of individual CTLs *in vivo*, numbers of attempts have been made for its evaluation. In addition to the analyses of CTL frequency, its pattern of cytokine secretion, its direct cytolytic activity, and avidity of its T cell receptor (TCR) with the epitope-MHC complex, the importance of examining its suppressive effect on viral replication *in vitro* has been suggested, although the levels of *in vitro* anti-viral efficacy resulting in viral control *in vivo* have not been determined [66,67]. Our recently established assay for measurement of *in vitro* anti-viral efficacy of bulk $CD8^+$ cells derived from peripheral blood may be helpful for evaluation of *in vivo* anti-viral efficacy of overall CTL responses [68]. Interestingly, a report has recently indicated that Gag-specific CTLs can respond to SIV infection more rapidly *in vitro* compared with Tat- and Env-specific CTLs, presumably by recognising virion-derived Gag epitopes presented in the early phase post-viral entry, suggesting a possibility of modification of CTL anti-viral efficacy by its recognition/killing kinetics during the viral replication cycle [69]. In support of this result, an inverse correlation between the breadth of Gag-specific CTL responses and viral loads has been indicated in a large-scale African cohort and SIV-infected rhesus macaques [69,70].

ANTI-HIV NAB POTENCY

Another major issue to be addressed in natural HIV/SIV infections is the characteristic lack of potent neutralising antibody (NAb) responses in the acute phase (Figure 1) [71]. To date, disoriented and delayed virus-specific antibody responses have been observed in the subacute phase, which permit consequent viral escape from neutralisation [72–74]. Monoclonal anti-CD20 antibody-mediated B-cell depletion experiments in macaques during the acute phase of SIV infection have suggested a possibility of modest contribution of such inefficient humoral immune responses to partial resolution from primary infection [75–77], but the effect of the absence of efficient post-infection NAb responses on

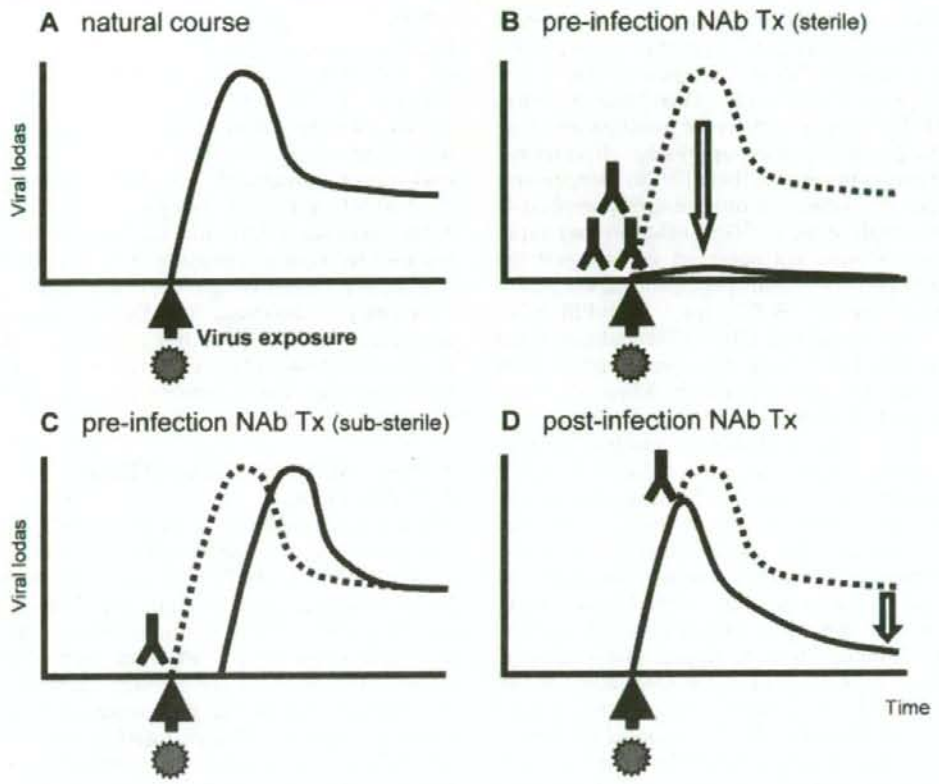

Figure 2. A schematic comparison of viral loads in NAb passive immunisation experiments. (A) In HIV/SIV natural infections, absence of NAb induction accompanies failure in primary viral control. (B) In pre-infection passive NAb immunisation, NAb titers overwhelming the challenged virus inoculum in theory provide an ultimate reduction in the peak viral load. (C) Pre-infection passive immunisation with insufficient doses of NAbs for sterile protection may cause transient delay or reduction in viral replication but permit persistent viral replication. (D) Post-infection passive NAb immunisation results in reduction of set-point viral loads beyond the period of detection of the administered NAb responses

primary HIV/SIV replication has not been formally scrutinised.

In vivo anti-viral activity of several monoclonal anti-HIV-1 NAbs such as b12, 2F5, 4E10 and 2G12 has been intensively assessed by pre-challenge passive NAb immunisation experiments against CXCR4-tropic SHIV infection in macaques [78–81]. These studies indicated a possibility of dose-dependent sterile protection (Figure 2), but the neutralising titers required for the protection were relatively high, suggesting difficulty in attainment and maintenance of those titers by prophylactic vaccination [82]. Sterile protection against CCR5-tropic SIV has not consistently been achieved by pre-challenge administration of antiserum or polyclonal antibodies [83,84] while protection against CCR5-tropic SHIV by pre-challenge monoclonal NAb administration has been indicated [85–87].

Compared with the appreciable sterile protective activity of pre-existing NAbs against viral challenge, post-infection NAb efficacy against established immunodeficiency virus infection has been a field of ambiguity. In one study, NAb administration at 6 h after CXCR4-tropic $SHIV_{DH12}$ challenge provided sterile protection while passive immunisation at 24 h post-challenge did not, suggesting a moment between 6 and 24 h

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post-infection as the threshold of post-exposure prophylaxis efficacy [88]. In another, NAb passive immunisation of rhesus macaques at day 1 and day 14 after CCR5-tropic SIVsmE660 challenge exhibited divergent patterns of infection and suggested a possibility of its suppressive effect on viral replication in some of them [89,90]. Suppressive activities of passively infused anti-SIV antibodies on viral replication in SIVmac251-infected rapid progressors were not observed *in vivo* past the set point [91]. In human peripheral blood leukocyte-reconstituted SCID mice (hu-PBL-SCID mice), monoclonal anti-HIV-1 NAbs administered 3 days after HIV-1 challenge were overwhelmed by established viral replication whereas several hours post-challenge as well as pre-challenge NAb infusion provided sterile protection [92,93].

Our recent results have, however, clearly shown the potency of post-infection NAbs against immunodeficiency virus replication *in vivo*. We performed passive NAb immunisation post-infection in SIVmac239-challenged rhesus macaques by using polyclonal antibodies purified from chronically infected macaques with NAb induction. The passive immunisation at day 7 post-challenge, preceding peak replication, resulted in significant suppression of viral replication past the limited duration of detectable NAb responses (Figures 1 and 2D) [94]. *De novo* SIVmac239-NAb responses were not involved in this viral control. Analysis revealed immediate accumulation of viral RNA to peripheral lymph node dendritic cells (DCs) after NAb passive immunisation, and pulsing of DCs with antibody-neutralised SIV activated virus-specific $CD4^+$ T lymphocytes *in vitro* in an antibody Fc-dependent manner, suggesting antibody-mediated virion uptake to DCs and a possibility of enhanced T cell priming. This study provided evidence indicating that potent NAb induction post-infection can in fact result in primary immunodeficiency virus control, and points out a possibility of non-sterile HIV control by prophylactic vaccine-induced, sub-sterile titers of NAbs post-infection, lending support for current efforts on vaccine induction of NAbs. Reversely, it suggested direct and indirect contribution of primary NAb absence to viral persistence.

The current mainstream of vaccine strategy for NAb induction is a rational design of immunogens mimicking broadly reactive neutralisation epitopes based on structural approaches on the HIV/SIV envelope protein and NAbs [71,95–99]. Broadly reactive monoclonal NAbs such as b12, 447-52D, 2G12, 2F5 and 4E10 have been characterised [100–103], and some have recently been confirmed to be potent to suppress rebound viremia when administered to HIV-infected humans undergoing structured treatment interruption (STI) [104]. Yet viral escape from autologous NAb responses in HIV-infected humans has been reported to occur in a manner of rapid and repetitive chase accompanying conformational masking, which may continuously limit the total neutralisation spectrum [72–74,105]. It is of our great interest if or how vaccine-induced primary and secondary NAb responses can overcome this propensity of escape and provide viral control.

A POSSIBILITY OF NON-STERILE HIV CONTROL

While given the difficulty in sterile HIV control, our passive immunisation study has indicated a possibility of non-sterile viral control by transient NAb responses post-infection [94], which may reflect another essence of immune dysfunction at the initial stage of HIV infection. Inefficient primary NAb induction can be seen as the lack of one effector out of the two wings of adaptive immune responses, humoral and cellular, but in addition may also affect the remaining cellular immune responses and contribute to their dysfunction due to abrogated antigen presentation.

Activities of specific antibody-mediated pathogen internalisation conventionally categorised in the genre of opsonisation are well known to occur in responses against bacterial and parasitic infections [106], but their involvement in antigen presentation have been clarified only recently [107]. Possible involvement of antibody-mediated antigen presentation in modification of cellular immunity [108,109] has not been discussed directly in the context of antiviral adaptive immunity [110,111], and this synergistic mechanism has not drawn primary attention in self-remitting acute viral infections, where the appearance of anti-viral NAbs mostly results in viral control. NAbs may facilitate induction of virus-specific $CD4^+$ T lymphocytes via efficient virion uptake into DCs, in turn functionally supporting virus-specific cellular immunity behind the scenes and providing consistent, fail-safe control of viral replication in combination with augmented CTLs. Reversely,

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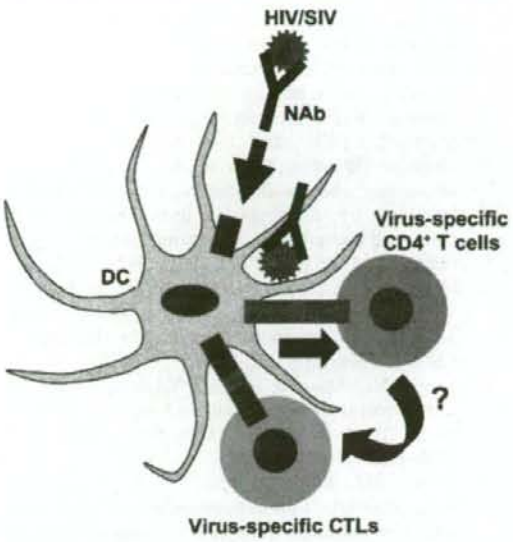

Figure 3. A putative model of NAbs synergising with virus-specific cellular immunity for HIV/SIV control. In the acute phase of viral infection, non-neutralising virus-specific antibodies may enhance antibody-mediated virion uptake into DCs leading to efficient antigen presentation to virus-specific $CD4^+$ T lymphocytes but fail to protect these cells from viral infection. In contrast, virus-specific NAbs, if induced, can not only induce but also protect virus-specific $CD4^+$ T cells, resulting in elicitation of virus-specific $CD4^+$ T-cell responses. Whether the protection of virus-specific $CD4^+$ T cells results in modification of virus-specific CTL function is an open question

abrogation of this process in infections of neutralisation-resistant viruses such as HIV and SIV may result in establishment of persistent viral infection in the absence of potent cellular immune responses [112].

Elicitation of virus-specific $CD4^+$ T lymphocytes could result in expansion of the targets of HIV infection [113]. In the antibody-mediated enhancement of antigen presentation leading to cellular immune induction described above, virus neutralisation activity would be required at least during the acute phase for protection of the induced virus-specific $CD4^+$ T lymphocytes from HIV/SIV infection via transmission from virus-exposed DCs [114,115]. Thus, our passive NAb immunisation study highlighted the active and protective role of NAbs in induction of functional virus-specific $CD4^+$ T-cell responses (Figure 3).

PERSPECTIVES

Elucidation of the determinants for HIV persistence would contribute to clarification of the requisites for viral control. Full commitment of potent CTLs under MHC restriction in concert with MHC-independent NAb responses would result in inhibition of persistent HIV infection. Further analyses determining the requisites for viral control may reveal novel endpoints for well-concerted elicitation of anti-HIV immunity by prophylactic vaccination.

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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.

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

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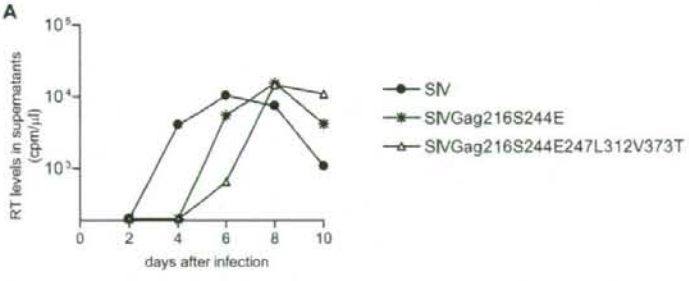

B

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

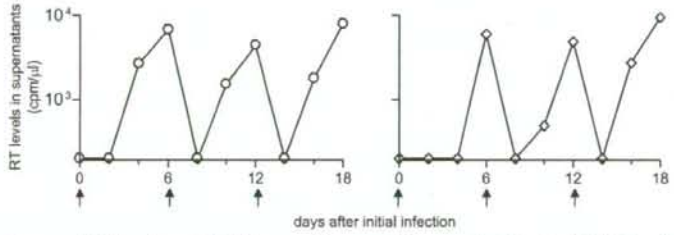

FIG. 1. Replication of mutant SIVs in vitro. (A) Wild-type and mutant SIV replication kinetics in HSC-F cells. HSC-F cells were infected with SIVmac239 (closed circles), SIVmac239Gag216S244E (asterisks), or SIVmac239Gag216S244E247L312V373T (open triangles). Virus production was monitored by measuring 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 $SHIV_{MDH4YF}$ molecular clone

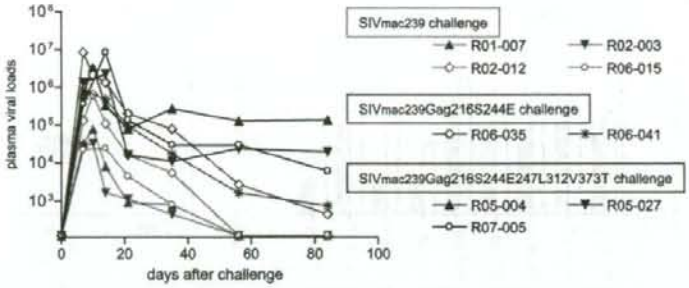

FIG. 2. Plasma viral loads after wild-type or mutant SIV challenge. The *90-120-Ia*-positive vaccinees were challenged with SIVmac239 (red lines), SIVmac239Gag216S244E (blue lines), or SIVmac239Gag216S244E247L312V373T (black lines). Plasma viral loads (SIV *gag* RNA copies/ml plasma) were determined 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 $Gag_{367\text{-}381}$ peptide was used to detect $Gag_{367\text{-}381}$-specific CTLs, including $Gag_{373\text{-}380}$-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 $Gag_{206\text{-}216}$-specific, $Gag_{241\text{-}249}$-specific, and $Gag_{373\text{-}380}$-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 SIVmac 239Gag216S244E replication was less efficient than that of wild-type SIVmac239, and SIVmac239Gag216S244E247L312 V373T 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

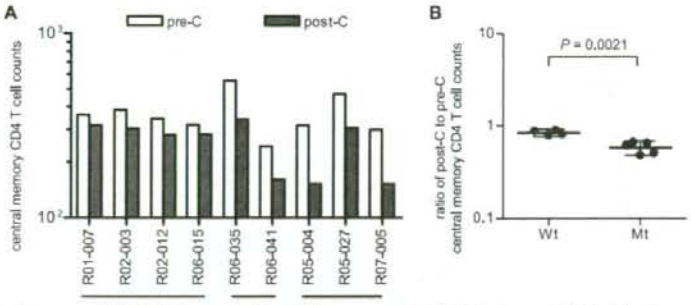

FIG. 3. Changes in central memory CD4$^+$ T-cell counts after wild-type or mutant SIV challenge. (A) Peripheral central memory CD4$^+$ (CD4$^+$ CD95$^+$ CD28$^+$) T-cell counts (/μl) prechallenge (pre-C) and a few months postchallenge (post-C). (B) Statistical comparison of central memory CD4$^+$ T-cell 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.

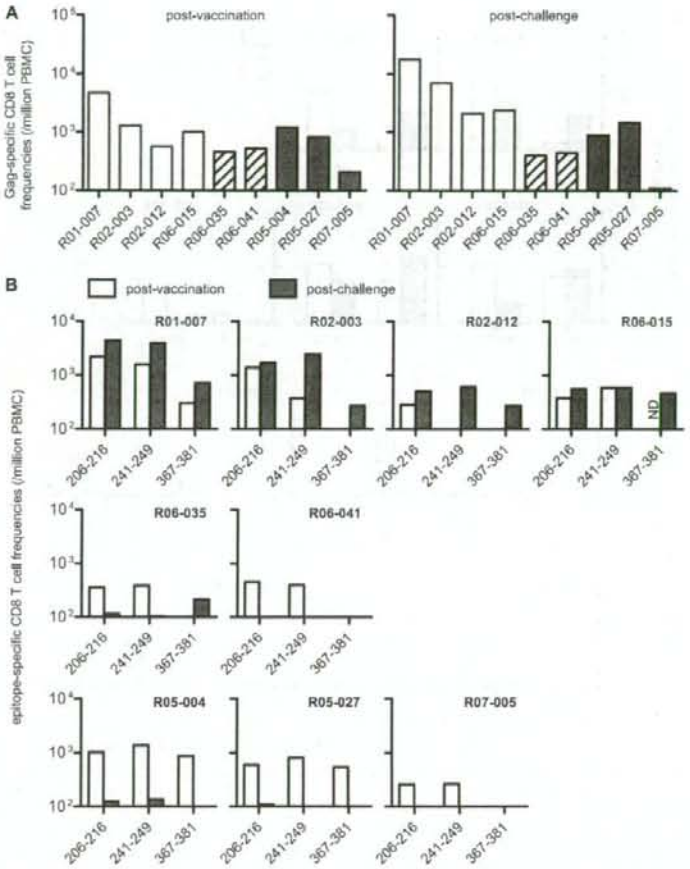

FIG. 4. Gag-specific CD8$^+$ T-cell responses before and after wild-type or mutant SIV challenge. Macaques R01-007, R02-003, R02-012, and R06-015 were challenged with SIVmac239; macaques R06-035 and R06-041 were challenged with SIVmac239Gag216S244E; and macaques R05-004, R05-027, and R07-005 were challenged 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 SIVmac239Gag216S244E247L312V373T 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 SIVmac239Gag216S244E 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

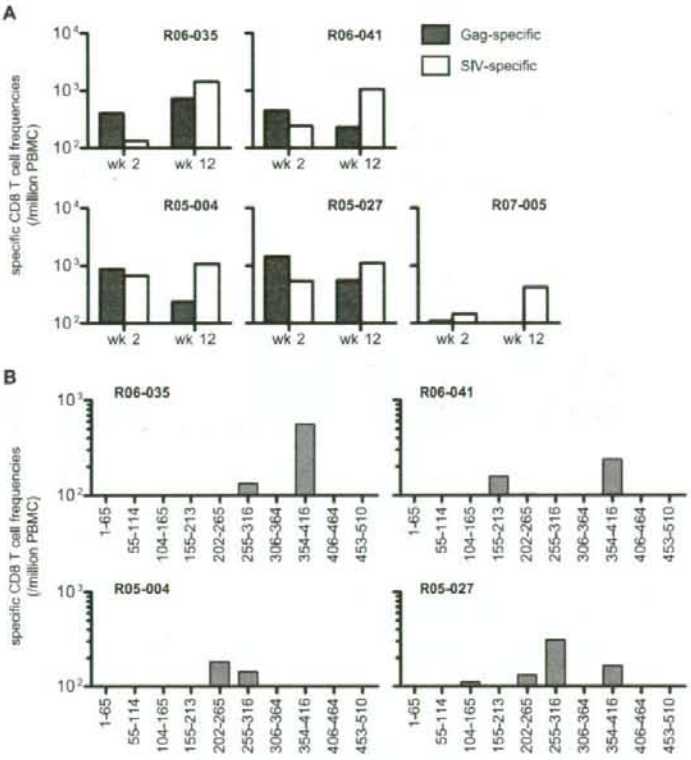

FIG. 5. SIV-specific $CD8^-$ T-cell responses after mutant SIV challenge. (A) Gag-specific (closed boxes) and SIV-specific (open boxes) $CD8^+$ T-cell frequencies at 2 weeks or 12 weeks postchallenge. (B) Frequencies of $CD8^+$ T cells specific for pools of SIV Gag peptides. A panel of 117 overlapping 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 SIVmac239Gag216S244E 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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Evaluation of the immunogenicity of replication-competent V-knocked-out and replication-defective F-deleted Sendai virus vector-based vaccines in macaques

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ABSTRACT

Viral vectors are promising vaccine tools for eliciting antigen-specific T-cell responses. We previously showed the potential of recombinant Sendai virus (SeV) vectors to induce virus-specific T-cell responses in macaque AIDS models. Here, we have evaluated the immunogenicity of replication-competent V-knocked-out and replication-defective F-deleted SeV vectors in macaques. Intranasal replication-competent and replication-defective SeV immunizations both elicited robust systemic antigen-specific T-cell responses, whereas the responses induced by the former were more durable than those by the latter. However, even the latter-induced T-cell responses remained detectable in a local, retropharyngeal lymph node two months after the immunization. These findings are useful for establishment of a vaccine protocol using SeV vectors.

1. Introduction

Virus-specific T-cell responses play an important role in the control of human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus (SIV) infection [1–7]. Especially, virus-specific $CD8^+$ cytotoxic T lymphocyte (CTL) responses exert suppressive pressure on HIV-1 and SIV replication. Hence, efficient T-cell induction is a key strategy for AIDS vaccine development, and recombinant viral vectors are promising tools for its elicitation [8].

We previously developed an AIDS vaccine using a recombinant Sendai virus (SeV) vector and showed the potential of this vector for efficient CTL induction in macaques [9–11]. SeV, murine parainfluenza virus type 1 (PIV-1), is an enveloped virus with a negative-sense RNA genome. Its natural host is mice and it is considered nonpathogenic for primates including humans [12]. Indeed, our analysis showed no disease progression by SeV infection of macaques or no detectable SeV transmission from SeV-infected to uninfected macaques [10]. A clinical phase I trial of SeV as a vaccine against human PIV-1 indicated its safety in humans [13].

We have two types of SeV vectors expressing SIV Gag, a replication-competent V-knocked-out V(-)SeV-Gag and a replication-defective F-deleted F(-)SeV-Gag [9,10,14–18]. The former V(-)SeV vector carrying a genome whose V gene, an SeV accessory gene, is knocked out is transmissible; i.e., infectious V(-)SeV virions can be produced from V(-)SeV-infected cells and transmitted to another cells. This V(-)SeV has attenuated virulence in mice but keeps the ability to induce efficient gene transfer [15,19]. In contrast, the latter F(-)SeV vector is non-transmissible; i.e., F(-)SeV-infected cells are unable to produce infectious, transmissible F(-)SeV virions [17]. This F(-)SeV vector also maintains efficient transduction ability with a high level of transgene expression. Therefore, the non-transmissible F(-)SeV vector, which may be advantageous in safety issues, is a promising delivery tool for gene therapy and vaccination in humans.

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In our previous studies [9,11,18], V(-)SeV-Gag and F(-)SeV-Gag immunizations both elicited Gag-specific T-cell responses in macaques, but we have not precisely compared their abilities to induce T-cell responses. In the present study, to evaluate their immunogenicity, we have examined systemic and local antigen-specific T-cell responses after immunization with replication-competent V(-)SeV-Gag or replication-defective F(-)SeV-Gag in macaques.

2. Materials and methods

2.1. Animals

Cynomolgus macaques (*Macaca fascicularis*) and rhesus macaques (*Macaca mulatta*) were maintained in accordance with the guidelines for laboratory animals of the National Institute of Infectious Diseases and the National Institute of Biomedical Innovation. Blood collection, lymph node (LN) biopsy, and vaccination were performed under ketamine anesthesia. Animals received a DNA vaccine followed by a single boost with V(-)SeV-Gag or F(-)SeV-Gag as described previously [11]. The DNA, CMV-SHIVdEN, used for the vaccination was constructed from an *env*- and *nef*-deleted $SHIV_{MD14YE}$ molecular clone DNA [20] and has the genes encoding SIVmac239 Gag, Pol, Vif, and Vpx, SIVmac239-HIV-1 chimeric Vpr, and HIV-1 Tat and Rev [9,11]. 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 (CIU) of replication-competent V(-)SeV-Gag or 6×10^9 CIU of replication-defective F-deleted F(-)SeV-Gag [9,10,18].

2.2. Measurement of virus-specific T-cell responses

We measured virus-specific T-cell levels by flow-cytometric analysis of interferon-γ (IFN-γ) induction after specific stimulation as described previously [11,21]. In brief, peripheral blood mononuclear cells (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 SeV for SeV-specific stimulation. Intracellular IFN-γ staining was performed using CytofixCytoperm kit (Becton Dickinson, Tokyo, Japan). 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 (Becton Dickinson, Tokyo, Japan) were used. Specific T-cell levels were calculated by subtracting nonspecific IFN-γ$^+$ T-cell frequencies from those after Gag-specific or SeV-specific stimulation. Specific T-cell frequencies less than 100 cells per million PBMCs were considered negative, those between 100 and 200 borderline, and those greater than 200 positive.

2.3. Statistical analysis

Statistical analysis was performed by Prism software version 4.03 with significance levels set at $p < 0.05$ (GraphPad Software, Inc., San Diego, CA). Gag-specific CD8$^+$ T-cell frequencies were log-transformed and compared between the two groups, V(-)SeV-Gag-vaccinated and F(-)SeV-Gag-vaccinated, by unpaired two-tailed *t* test.

3. Results

3.1. Both replication-competent V(-)SeV-Gag and replication-defective F(-)SeV-Gag elicited robust Gag-specific T-cell responses

Four cynomolgus macaques received a DNA vaccine followed by a boost with replication-competent V(-)SeV-Gag, while seven macaques were boosted with replication-defective F(-)SeV-Gag after the DNA prime. We then examined Gag-specific T-cell responses in these macaques by flow-cytometric analysis of IFN-γ induction after specific stimulation. All four V(-)SeV-Gag-boosted and all seven F(-)SeV-Gag-boosted macaques showed efficient induction of Gag-specific T-cell responses after the boost (Fig. 1A). While Gag-specific CD4$^+$ T-cell responses were predominant in some animals such as macaque C97-018, Gag-specific CD8$^+$ T-cell responses were detectable in all macaques one week after the boost (Fig. 1A), although these responses were undetectable before the boost (data not shown). There was no significant difference in Gag-specific CD4$^+$ T-cell or CD8$^+$ T-cell levels one week after the boost between the V(-)SeV-Gag-boosted and the F(-)SeV-Gag-boosted macaques ($p = 0.3779$ [CD4] and $p = 0.7963$ [CD8] by unpaired two-tailed *t* test). We also examined SeV-specific T-cell responses and found efficient induction of SeV-specific T-cell responses in

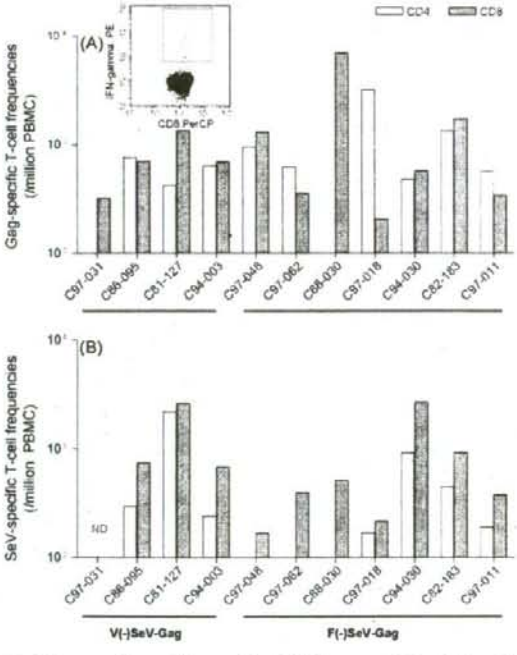

Fig. 1. Antigen-specific T-cell frequencies in PBMCs one week after SeV boost. (A) Gag-specific CD4$^+$ T-cell (open boxes) and CD8$^+$ T-cell (closed boxes) frequencies one week after an intranasal boost with V(-)SeV-Gag (macaques C97-031, C86-095, C81-127, and C94-003) or F(-)SeV-Gag (macaques C97-048, C97-062, C88-030, C97-018, C94-030, C82-183, and C97-011). A representative dot plot gated on CD3$^+$ CD8$^+$ lymphocytes (macaque C86-095) after Gag-specific stimulation is shown at the top. Geometric means of Gag-specific CD4$^+$ T-cell and CD8$^+$ T-cell frequencies were 3.8×10^2 cells/million PBMCs and 6.7×10^2 cells/million PBMCs in the V(-)SeV-Gag-boosted group and 6.8×10^2 cells/million PBMCs and 8.1×10^2 cells/million PBMCs in the F(-)SeV-Gag-boosted group. (B) SeV-specific CD4$^+$ T-cell (open boxes) and CD8$^+$ T-cell (closed boxes) frequencies one week after a boost with V(-)SeV-Gag or F(-)SeV-Gag. ND, not determined.