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Broadening of CD8⁺ cell responses in vaccine-based simian immunodeficiency virus controllers

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Objective: In our prior study on a prophylactic T-cell-based vaccine, some vaccinated macaques controlled a simian immunodeficiency virus (SIV) challenge. These animals allowed viremia in the acute phase but showed persistent viral control after the setpoint. Here, we examined the breadth of postchallenge virus-specific cellular immune responses in these SIV controllers.

Design: We previously reported that in a group of Burmese rhesus macaques possessing the MHC haplotype *90-120-1a*, immunization with a Gag-expressing vaccine results in nonsterile control of a challenge with SIVmac239 but not a mutant SIV carrying multiple cytotoxic T lymphocyte (CTL) escape *gag* mutations. In the present study, we investigated whether broader cellular immune responses effective against the mutant SIV replication are induced after challenge in those vaccinees that maintained wild-type SIVmac239 control.

Methods: We analyzed cellular immune responses in these SIV controllers ($n=8$).

Results: These controllers elicited CTL responses directed against SIV non-Gag antigens as well as Gag in the chronic phase. Postvaccinated, prechallenge CD8⁺ cells obtained from these animals suppressed wild-type SIV replication *in vitro*, but mostly had no suppressive effect on the mutant SIV replication, whereas CD8⁺ cells in the chronic phase after challenge showed efficient antimutant SIV efficacy. The levels of *in vitro* antimutant SIV efficacy of CD8⁺ cells correlated with Vif-specific CD8⁺ T-cell frequencies. Plasma viremia was kept undetectable even after the mutant SIV superchallenge in the chronic phase.

Conclusion: These results suggest that vaccine-based wild-type SIV controllers can acquire CD8⁺ cells with the potential to suppress replication of SIV variants carrying CTL escape mutations.

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AIDS 2010, **24**:2777–2787

Keywords: AIDS vaccine, cytotoxic T lymphocyte, major histocompatibility complex, mutation, simian immunodeficiency virus

Introduction

Virus-specific CD8⁺ cytotoxic T lymphocyte (CTL) responses are crucial for the control of HIV and simian immunodeficiency virus (SIV) replication [1–6]. Cumulative studies on HIV-infected individuals have shown association of HLA genotypes with rapid or delayed AIDS progression [7,8]. For instance, most of the HIV-infected

individuals possessing *HLA-B*57* have been indicated to show a better prognosis with lower viral loads, implicating *HLA-B*57*-restricted epitope-specific CTL responses in this viral control [9–11]. Indian rhesus macaques possessing particular major histocompatibility complex class I (MHC-I) alleles such as *Mamu-B*17* tend to show SIV control [12–14]. These imply possible HIV control by induction of particular effective CTL responses.

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Received: 19 June 2010; revised: 23 August 2010; accepted: 1 September 2010.

DOI:10.1097/QAD.0b013e3283402206

Recent trials of prophylactic T-cell-based vaccines in macaque AIDS models have indicated a possibility of reduction in postchallenge viral loads [15–20]. We previously developed a prophylactic AIDS vaccine consisting of a DNA prime followed by a boost with a Sendai virus (SeV) vector expressing SIVmac239 Gag (SeV-Gag) [21,22]. Our trial showed vaccine-based control of a SIVmac239 challenge in a group of Burmese rhesus macaques sharing the MHC-I haplotype *90-120-Ia*; these *90-120-Ia*-positive vaccinees dominantly elicited Gag₂₀₆₋₂₁₆ (IINEEAADWDL) epitope-specific and Gag₂₄₁₋₂₄₉ (SSVDEQIQW) epitope-specific CTL responses and contained SIVmac239 replication after challenge [15,23]. In contrast, *90-120-Ia*-positive vaccinees failed to control a challenge with a mutant virus, SIVmac239Gag216S244E247L312V373T (referred to as SIV-G64723mt), which carries five *gag* mutations resulting in escape from recognition by Gag-specific CTLs including Gag₂₀₆₋₂₁₆-specific and Gag₂₄₁₋₂₄₉-specific CTLs. This indicates that these CTL responses play a crucial role in the vaccine-based primary control of wild-type SIVmac239 replication [24]. Furthermore, in a SIVmac239 challenge experiment of *90-120-Ia*-positive rhesus macaques that received a prophylactic vaccine expressing the Gag₂₄₁₋₂₄₉ epitope fused with enhanced green fluorescent protein (EGFP), this single epitope vaccination resulted in control of SIVmac239 replication with dominant induction of Gag₂₄₁₋₂₄₉-specific CTL responses in the acute phase after challenge [25]. We refer to these vaccinated animals that controlled viral replication after wild-type SIVmac239 challenge as SIV controllers in the present study.

Administration of SIV controllers with a monoclonal anti-CD8 antibody (i.e., CD8 depletion after the establishment of primary viral control) has suggested that CD8⁺ cell responses play an important role in maintaining the viral control in the chronic phase [26,27]. Then, it is of great concern whether these wild-type SIV controllers can acquire CD8⁺ cells effective against replication of SIV variants escaping from dominant CTL responses. In the present study, we have analyzed *90-120-Ia*-positive vaccinees controlling a SIVmac239 challenge in order to examine whether *90-120-Ia*-positive animals can elicit cellular immune responses effective against the mutant SIV, SIV-G64723mt, carrying multiple CTL escape *gag* mutations. Our analyses in these vaccine-based SIV controllers revealed dynamics of virus-specific cellular immune responses during persistent viral control and suggested postchallenge induction of CD8⁺ cells able to suppress replication of SIV variants carrying CTL escape mutations.

Materials and methods

SIV-G64723mt

The SIV-G64723mt (SIVmac239Gag216S244E247L312V373T) carries five *gag* mutations, GagL216S (leading

to a leucine [L]-to-serine [S] substitution at the 216th amino acid in Gag, GagD244E (aspartic acid [D]-to-glutamic acid [E] at the 244th amino acid), GagI247L (isoleucine [I] to L at the 247th amino acid), GagA312V (alanine [A] to valine [V] at the 312th amino acid), and GagA373T (A to threonine [T] at the 373rd amino acid), which were selected, at the cost of viral fitness, in a SIVmac239-infected macaque possessing the MHC-I haplotype *90-120-Ia*, as described previously [23,28]. GagL216S, GagD244E, GagI247L, and GagA373T mutations, which became dominant mostly in SIVmac239-infected *90-120-Ia*-positive rhesus macaques, result in viral escape from recognition by Gag₂₀₆₋₂₁₆-specific, Gag₂₄₁₋₂₄₉-specific, and Gag₃₇₃₋₃₈₀-specific CTLs, respectively, whereas it remains unclear whether GagA312V was selected for by CTLs.

Animal experiments

Eight Burmese rhesus macaques (*Macaca mulatta*) possessing the MHC-I haplotype *90-120-Ia*, which showed vaccine-based control of a SIVmac239 challenge, were used in this study and divided into two groups (Fig. 1a). Five macaques, R06-015, R03-014, R03-012, R02-002, and R02-003, in group I received a prophylactic DNA prime/SeV-Gag boost vaccine (referred to as DNA/SeV-Gag vaccine) and contained SIVmac239 challenge as reported previously [15,24,29]. The DNA used for the vaccination, CMV-SHIVdEN [15], was constructed from *env*-deleted and *nef*-deleted simian-human immunodeficiency virus SHIV_{MD14YE} [30] molecular clone DNA (SIVGP1) 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, animals received a single boost intranasally with 6×10^9 cell infectious units (CIUs) of F-deleted replication-defective SeV-Gag [31,32]. At week 1 after SIV challenge, macaque R03-014 was inoculated with nonspecific immunoglobulin G (IgG), and macaques R03-012 and R02-002 with IgG purified from neutralizing antibody-positive plasma of chronically SIV-infected macaques in our previous study [29]. Two macaques R04-016 and R06-007 in group II received a prophylactic prime-boost vaccine eliciting single Gag₂₄₁₋₂₄₉ epitope-specific CTL responses (referred to as DNA/SeV-Gag₂₃₆₋₂₅₀-EGFP vaccine) and contained SIVmac239 challenge as reported previously [25]. In this vaccine protocol, animals were primed with 5 mg of pGag₂₃₆₋₂₅₀-EGFP-N1 DNA expressing a Gag₂₃₆₋₂₅₀-EGFP fusion protein, followed by a boost with 6×10^9 CIU of F-deleted SeV expressing the Gag₂₃₆₋₂₅₀-EGFP fusion protein (SeV-Gag₂₃₆₋₂₅₀-EGFP). Macaque R04-015 in group II received a prophylactic prime-boost vaccine eliciting Gag₂₀₆₋₂₁₆ epitope-specific and Gag₂₄₁₋₂₄₉ epitope-specific CTL responses (referred to as DNA/SeV-Gag₂₀₂₋₂₁₆-EGFP and DNA/SeV-Gag₂₃₆₋₂₅₀-EGFP vaccine); this animal was primed with pGag₂₀₂₋₂₁₆-EGFP-N1 and pGag₂₃₆₋

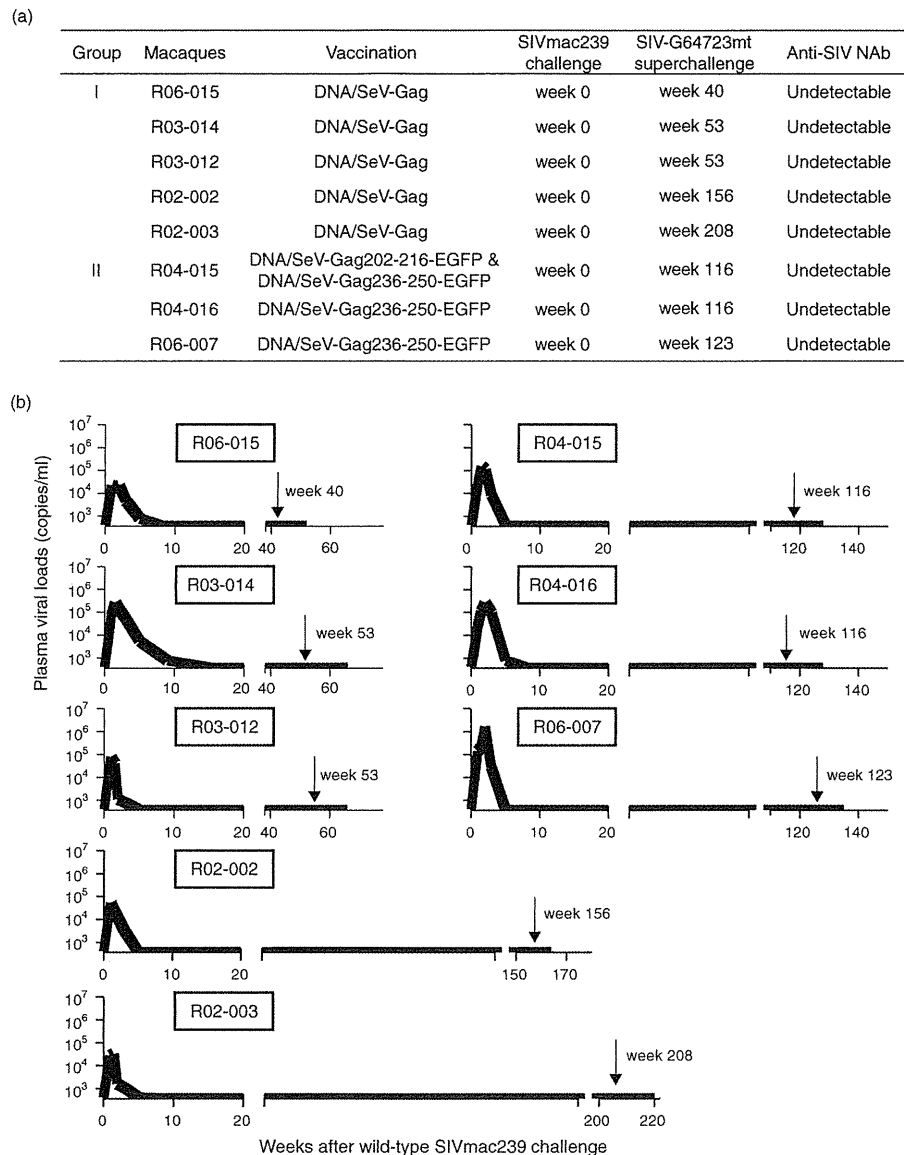


Fig. 1. Plasma viral loads in simian immunodeficiency virus controllers. (a) The list of rhesus macaques used in this study. All are *90-120-Ia*-positive. SIVmac239-specific neutralizing antibody (anti-SIV NAb) responses just before the mutant SIV superchallenge were undetectable. (b) Plasma viral loads (SIV *gag* RNA copies/ml plasma) determined as described previously [15]. The lower limit of detection is approximately 4×10^2 copies/ml. The arrows indicate the time points of SIV-G64723mt superchallenge. SIV, simian immunodeficiency virus.

₂₅₀-EGFP-N1 DNAs, followed by a boost with SeV-Gag₂₀₂₋₂₁₆-EGFP and SeV-Gag₂₃₆₋₂₅₀-EGFP. Both pGag₂₀₂₋₂₁₆-EGFP-N1 and SeV-Gag₂₀₂₋₂₁₆-EGFP express a Gag₂₀₂₋₂₁₆-EGFP fusion protein [33]. These vaccinated animals were challenged intravenously with 1000 50% tissue culture infective doses (TCID₅₀) of SIVmac239 [34] approximately 3 months after the boost and were superchallenged intravenously with 1000 TCID₅₀ of SIV-G64723mt in the chronic phase. The challenge virus stocks were prepared by virus propagation on rhesus macaque peripheral blood mononuclear cells

(PBMCs). All animals were maintained in accordance with the guidelines for animal experiments at the National Institute of Infectious Diseases.

In-vitro viral suppression assay

To evaluate in-vitro anti-SIVmac239 or anti-SIV-G64723mt efficacy of CD8⁺ cells, we examined SIVmac239 or SIV-G64723mt replication on CD8-depleted PBMCs in the presence of CD8⁺ cells positively selected from macaque PBMCs as described previously [27,35]. In brief, PBMCs were separated into CD8⁺ and

CD8⁻ cells by using Macs CD8 MicroBeads (Miltenyi Biotec, Tokyo, Japan). For preparing target cells, the CD8⁻ cells selected from PBMCs obtained before SIVmac239 challenge were cultured in the presence of 2 µg/ml phytohemagglutinin L and 20 IU/ml recombinant human interleukin-2 (Roche Diagnostics, Tokyo, Japan) and infected with SIVmac239 at a multiplicity of infection (MOI) of 1:10³ TCID₅₀/cell or with SIV-G64723mt at MOI of 1:10² TCID₅₀/cell, using the virus stocks prepared by virus propagation on HSC-F cells (herpesvirus saimiri-immortalized macaque T-cell line) [36]. SIV-G64723mt with lower replicative ability was added at higher MOI to show similar replication kinetics with SIVmac239 replication in the control culture without CD8⁺ cells. Target cells were cultured for 2 days and then effector CD8⁺ cells selected from PBMCs obtained 1 week after boost or at several time points after the challenge were added to the target cells at an effector : target (*E* : *T*) ratio of 1 : 4. Reverse transcriptase activities in these culture supernatants were measured [37] to determine the peak of viral production in the control culture of target cells without CD8⁺ cells. RNA was extracted from culture supernatants at the peak using the high pure viral RNA Kit (Roche Diagnostics) and viral RNA levels were measured by LightCycler system (Roche Diagnostics) using SIV *gag*-specific primers (GTAGTATGGGCAGCAAATGA and TGTTCTGT TTTCCACCACTA) and probes (GCATTCACGCA GAAGAGAAAGTGAAACA-Flu and LCRed-ACTG AGGAAGCAAAACAGATAGTGCAGAGA) (Nihon Gene Research Laboratories Inc., Sendai, Japan). Reduction in viral production by addition of each group of CD8⁺ cells was shown as reduction (fold) in viral RNA level compared with that in the supernatant from virus-infected CD8⁻ cell culture without CD8⁺ cells.

Analysis 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 [15]. In brief, PBMCs were cocultured for 6 h with autologous herpesvirus papio-immortalized B-lymphoblastoid cell lines pulsed with 1 µmol/l SIVmac239 Gag₂₀₆₋₂₁₆, Gag₂₄₁₋₂₄₉, or Gag₃₆₇₋₃₈₁ peptides for Gag₂₀₆₋₂₁₆-specific, Gag₂₄₁₋₂₄₉-specific, or Gag₃₆₇₋₃₈₁-specific stimulation. Alternatively, PBMCs were cocultured with B-lymphoblastoid cell lines pulsed with peptide pools using panels of overlapping peptides spanning the entire SIVmac239 Gag, Pol, Vif, Vpx, Vpr, Tat, Rev, Nef, and Env amino acid sequences. Intracellular IFN-γ staining was performed using a CytofixCytoperm kit (BD, Tokyo, Japan) and fluorescein isothiocyanate-conjugated antihuman CD4, peridinin chlorophyll protein-conjugated antihuman CD8, allophycocyanin-conjugated antihuman CD3, and phycoerythrin-conjugated antihuman IFN-γ monoclonal antibodies (BD). Specific CD8⁺ T-cell levels were calculated by subtracting nonspecific IFN-γ⁺ CD8⁺ T-cell fre-

quencies from those after peptide-specific stimulation. Specific CD8⁺ T-cell levels lower than 100 per million PBMCs were considered negative.

Analysis of virus-specific neutralizing antibody responses

SIVmac239-specific neutralizing antibody responses were examined by determining the end point plasma titers for inhibiting 10 TCID₅₀ virus replication as described previously [26]. Serial two-fold dilutions of heat-inactivated plasma were prepared in quadruplicate and mixed with 10 TCID₅₀ of SIVmac239. In each culture, 5 µl of virus was incubated with 5 µl of plasma for 45 min and was added to 5 × 10⁴ MT4 cells. Reverse transcriptase activities in the culture supernatants on day 12 were measured to determine the 100% neutralizing endpoint. The lower limit of detection is a titer of 1 : 2.

Statistical analysis

Statistical analysis was performed using Prism software version 4.03 (GraphPad Software Inc., San Diego, California, USA) with significance levels set at a *P* value of less than 0.05. Specific CD8⁺ T-cell frequencies and in-vitro anti-SIV efficacy levels (fold of reduction in viral production) were log transformed and correlation was analyzed by the Pearson test.

Results

Anti-SIVmac239 and anti-SIV-G64723mt efficacy *in vitro* of CD8⁺ cells in simian immunodeficiency virus controllers

We analyzed eight 90-120-*Ia*-positive rhesus macaques that showed vaccine-based control of a SIVmac239 challenge (Fig. 1a). These SIV controllers were divided into group I consisting of five animals (R06-015, R03-014, R03-012, R02-002, and R02-003) vaccinated with DNA/SeV-Gag [15] and group II consisting of one animal (R04-015) vaccinated with DNA/SeV-Gag₂₀₂₋₂₁₆-EGFP and DNA/SeV-Gag₂₃₆₋₂₅₀-EGFP and two (R04-016 and R06-007) vaccinated with DNA/SeV-Gag₂₃₆₋₂₅₀-EGFP [25]. After an intravenous challenge with SIVmac239, all of these macaques showed viremia in the acute phase, but then controlled viral replication; plasma viremia was undetectable after the setpoint (Fig. 1b).

First, we investigated the potential of macaque CD8⁺ cells obtained at several time points, after boost but before SIVmac239 challenge (referred to as postboost) and after challenge, to suppress SIVmac239 (Fig. 2) or SIV-G64723mt (Fig. 3) replication by in-vitro viral suppression assay [27,38-40]. In this assay, PBMC-derived CD8⁻ target cells infected with SIVmac239 or SIV-G64723mt were cocultured with effector CD8⁺ cells from PBMCs obtained at several time points at an *E*/*T*

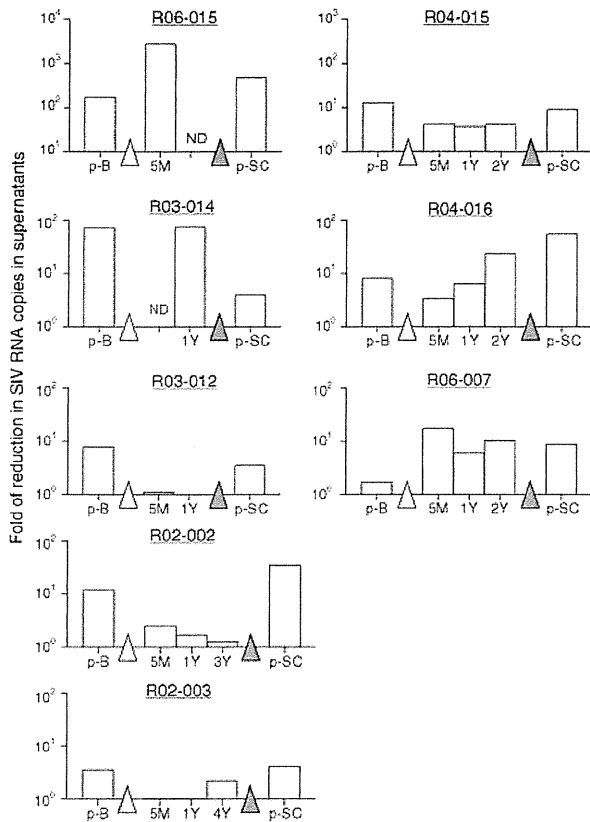


Fig. 2. Anti-SIVmac239 efficacy *in vitro* of CD8⁺ cells in simian immunodeficiency virus controllers. PBMC-derived CD8⁻ (target) cells infected with SIVmac239 were cultured alone or cocultured with autologous PBMC-derived CD8⁺ (effector) cells at several time points at an *E:T* ratio of 1:4. The ratios of viral RNA levels in the supernatants from the coculture to those without CD8⁺ cells are shown. ND: not determined. p-B: 1 week after boost; 5M, 1Y, 2Y, 3Y, and 4Y: 5 months, 1, 2, 3, and 4 years after challenge, respectively; p-SC: 1 or 2 months after superchallenge. Open triangles indicate the time points of SIVmac239 challenge and closed triangles SIV-G64723mt superchallenge. PBMC, peripheral blood mononuclear cell; SIV, simian immunodeficiency virus.

ratio of 1:4, and viral production in culture supernatants was examined to assess suppressive effect of CD8⁺ cells on viral replication *in vitro*.

CD8⁺ cells 1 week after boost mostly suppressed wild-type SIVmac239 replication efficiently. In contrast, these postboost CD8⁺ cells failed to show efficient suppressive effect on SIV-G64723mt replication. These results suggest that Gag₂₀₆₋₂₁₆-specific, Gag₂₄₁₋₂₄₉-specific, and Gag₃₆₇₋₃₈₁-specific CTL responses play a central role in the suppression of SIVmac239 replication by postboost CD8⁺ cells.

After SIVmac239 challenge, all these animals showed efficient *in vitro* anti-SIV-G64723mt efficacy (more than

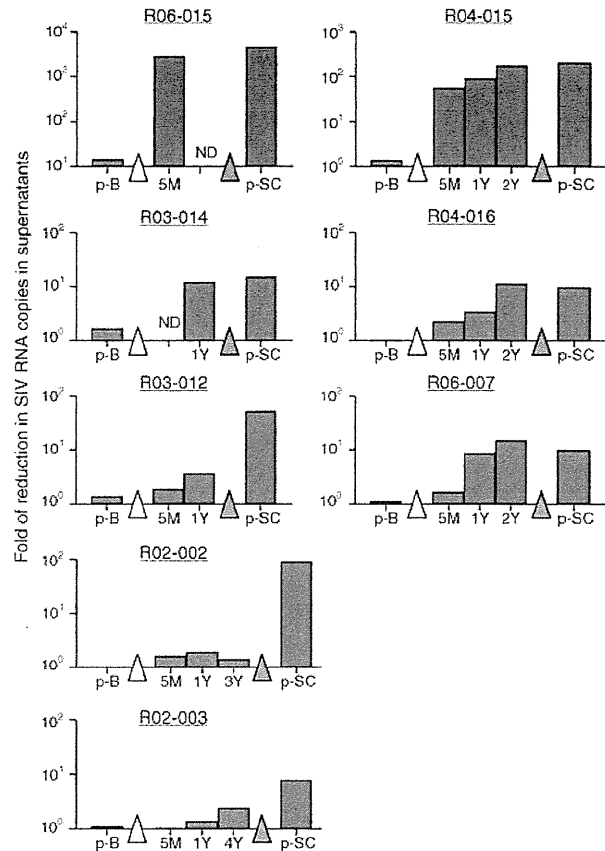


Fig. 3. Anti-SIV-G64723mt efficacy *in vitro* of CD8⁺ cells in simian immunodeficiency virus controllers. PBMC-derived CD8⁻ cells infected with SIV-G64723mt were cultured alone or cocultured with autologous PBMC-derived CD8⁺ cells at several time points at an *E:T* ratio of 1:4. The ratios of viral RNA levels in the supernatants from the coculture to those without CD8⁺ cells are shown. ND: not determined. p-B: 1 week after boost; 5M, 1Y, 2Y, 3Y, and 4Y: 5 months, 1, 2, 3, and 4 years after challenge, respectively; p-SC: 1 or 2 months after superchallenge. Open triangles indicate the time points of SIVmac239 challenge and closed triangles SIV-G64723mt superchallenge. PBMC, peripheral blood mononuclear cell; SIV, simian immunodeficiency virus.

two-fold reduction in viral production) of CD8⁺ cells, sooner or later, in the chronic phase. The levels of *in vitro* anti-SIV-G64723mt efficacy of CD8⁺ cells tended to become higher in the chronic phase. Anti-SIVmac239 efficacy of CD8⁺ cells was not associated with anti-SIV-G64723mt efficacy. For instance, some CD8⁺ cells efficiently suppressed SIV-G64723mt but not SIVmac239 replication. After all, all SIV controllers acquired CD8⁺ cells able to suppress the mutant SIV-G64723mt replication *in vitro* in the chronic phase.

Control of a mutant simian immunodeficiency virus superchallenge

These animals were superchallenged with a mutant SIV, SIV-G64723mt, that has five *gag* mutations resulting in

escape from recognition by Gag₂₀₆₋₂₁₆-specific, Gag₂₄₁₋₂₄₉-specific, and Gag₃₆₇₋₃₈₁-specific CTLs around 1 year (R06-015, R03-014, and R03-012), 2 years (R04-015, R04-016, and R06-007), 3 years (R02-002), or 4 years (R02-003) after SIVmac239 challenge. The replicative ability of SIV-G64723mt is significantly lower than that of wild-type SIVmac239, but SIV-G64723mt challenge of naive 90-120-Ia-negative rhesus macaques can result in persistent viral replication and AIDS progression [23,28]. It has previously been shown that 90-120-Ia-positive macaques vaccinated with DNA-prime/SeV-Gag-boost are unable to contain a SIV-G64723mt challenge, whereas they can control replication of wild-type SIVmac239 [24]. Indeed, we confirmed that CD8⁺ cells obtained from these 90-120-Ia-positive vaccinees before challenge efficiently suppressed wild-type SIVmac239 but not SIV-G64723mt replication *in vitro*. In the present study, however, all eight wild-type SIV controllers contained the SIV-G64723mt superchallenge without detectable viremia (Fig. 1b). SIVmac239-specific neutralizing antibody responses were undetectable around the superchallenge in any of these controllers (Fig. 1a). These results indicate that, after SIVmac239 challenge, the SIV controllers acquired the potential to control SIV-G64723mt replication in the absence of neutralizing antibody responses, although to what extent CD8⁺ cell responses may contribute to this containment of SIV-G64723mt superchallenge remains unclear. Postsuperchallenge CD8⁺ cells suppressed both SIVmac239 and SIV-G64723mt replication *in vitro* efficiently (Figs. 2 and 3).

Simian immunodeficiency virus Gag-specific cytotoxic T lymphocyte responses in simian immunodeficiency virus controllers

Then, in these SIV controllers, we examined Gag₂₀₆₋₂₁₆-specific, Gag₂₄₁₋₂₄₉-specific, and Gag₃₆₇₋₃₈₁-specific CTL responses, which have previously been indicated responsible for control of SIVmac239 replication in 90-120-Ia-positive vaccinees [24] (Fig. 4a). In DNA/SeV-Gag vaccinated animals (R06-015, R03-014, R03-012, and R02-002), SIV-specific CTL responses were undetectable before SeV-Gag boost (data not shown), but Gag₂₀₆₋₂₁₆-specific, Gag₂₄₁₋₂₄₉-specific, and Gag₃₆₇₋₃₈₁-specific responses were efficiently induced 1 week after the boost. After SIVmac239 challenge, these animals showed efficient responses of these CTLs in the acute phase. These CTL levels were reduced in the chronic phase, but Gag₂₄₁₋₂₄₉-specific CTL responses were detectable even 1 year after challenge. In macaque R04-015 vaccinated with DNA/SeV-Gag₂₀₂₋₂₁₆-EGFP and DNA/SeV-Gag₂₃₆₋₂₅₀-EGFP, Gag₂₀₆₋₂₁₆-specific CTL responses were induced dominantly 1 week after boost and 2 weeks after SIVmac239 challenge, whereas Gag₂₄₁₋₂₄₉-specific CTL responses were detected predominantly in the chronic phase. In macaques R04-016 and R06-007 vaccinated with DNA/SeV-Gag₂₃₆₋₂₅₀-EGFP, Gag₂₄₁₋₂₄₉-specific CTL responses were induced dominantly 1 week after boost and 2 weeks after SIVmac239 challenge and

were maintained in the chronic phase. No significant enhancement of these CTL responses was observed after SIV-G64723mt superchallenge.

We also examined Gag-specific CTL responses in SIV controllers at several time points by using a panel of overlapping peptides (Gag peptide pools 1–10) spanning the entire SIVmac239 Gag (Fig. 4b). Group I macaques vaccinated with DNA/SeV-Gag elicited CTL responses directed against not only Gag peptide pool 5 (including Gag₂₀₆₋₂₁₆ and Gag₂₄₁₋₂₄₉) and 7 (including Gag₃₆₇₋₃₈₁) but also other Gag peptide pools after boost and after challenge; some peptide pool-specific CTLs were diminished, whereas others appeared in the chronic phase. In contrast, group II macaques eliciting CTL responses directed against single Gag₂₀₆₋₂₁₆ (R04-015) or Gag₂₄₁₋₂₄₉ (R04-016 and R06-007) epitope after boost showed predominant Gag peptide pool 5-specific CTL responses after challenge and accumulated multiple Gag epitope-specific CTL responses in the chronic phase. These results indicate dynamics of postchallenge Gag-specific CTL responses in vaccine-based SIV controllers. After SIV-G64723mt superchallenge, changes in the pattern of Gag-specific CTL responses were observed in some animals.

Simian immunodeficiency virus non-Gag antigen-specific cytotoxic T lymphocyte responses in simian immunodeficiency virus controllers

Next, in SIV controllers, we examined CTL responses directed against SIV non-Gag antigens by using panels of overlapping peptides spanning the entire SIVmac239 antigens other than Gag (Fig. 5a). These SIV controllers showed SIV non-Gag-specific CTL responses from the early phase after challenge. After SIV-G64723mt superchallenge, broadening or changes in the pattern of these CTL responses were observed in some animals; Vif-specific or Nef-specific CTL responses were detected predominantly, although we did not find common CTL epitopes in Vif or Nef.

Correlation of antigen-specific cytotoxic T lymphocyte levels with in-vitro antiviral efficacy levels

Finally, we analyzed correlation of antigen-specific CTL levels with in-vitro anti-SIVmac239 or anti-SIV-G64723mt efficacy levels of CD8⁺ cells (Fig. 5b). We found a correlation of anti-SIVmac239 efficacy levels with Gag₂₀₆₋₂₁₆-specific and Gag₂₄₁₋₂₄₉-specific CTL levels but not with total Gag-specific CTL levels. The anti-SIVmac239 efficacy levels did not correlate with either Gag₂₀₆₋₂₁₆-specific or Gag₂₄₁₋₂₄₉-specific CTL levels alone (data not shown), although our previous study [25] indicated inverse correlation between peak plasma viral loads and the levels of Gag₂₄₁₋₂₄₉-specific CTLs dominantly induced in DNA/SeV-Gag₂₃₆₋₂₅₀-EGFP-vaccinated animals in the acute phase after

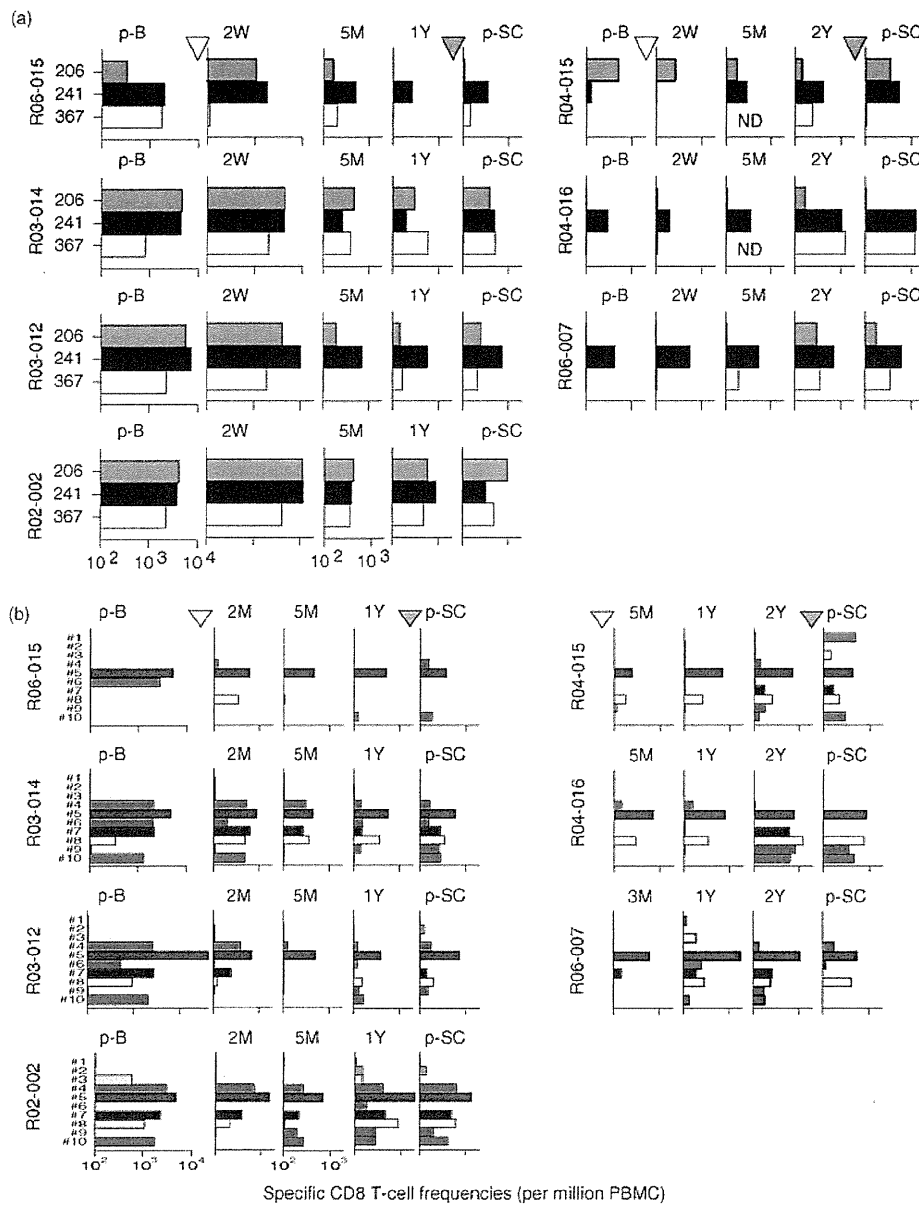


Fig. 4. Gag-specific CD8⁺ T-cell responses in simian immunodeficiency virus controllers. (a) Gag₂₀₆₋₂₁₆-specific (206), Gag₂₄₁₋₂₄₉-specific (241), and Gag₃₆₇₋₃₈₁-specific (367) CD8⁺ T-cell frequencies at several time points are shown. Regarding macaque R02-003, we confirmed efficient responses of these CTLs after boost and in the acute phase as reported previously [24] but did not have enough PBMC samples for the analyses in the chronic phase. (b) A panel of 117 overlapping peptides (15–17 amino acid in length and overlapping by 10–12 amino acid) spanning the entire SIV Gag amino acid sequence was divided into the following 10 pools (each consisting of 11 or 12 peptides): pool 1, first to 65th amino acid in SIV Gag; pool 2, 55th to 114th amino acid; pool 3, 104th to 165th amino acid; pool 4, 155th to 213th amino acid; pool 5, 202nd to 265th amino acid; pool 6, 255th to 316th amino acid; pool 7, 306th to 364th amino acid; pool 8, 354th to 416th amino acid; pool 9, 406th to 464th amino acid; and pool 10, 453rd to 510th amino acid. These Gag peptide pool-specific CD8⁺ T-cell frequencies at several time points are shown. ND: not determined. p-B: 1 week after boost; 2W, 5M, 1Y, and 2Y: 2 weeks, 5 months, 1, and 2 years after challenge, respectively; p-SC: 1 or 2 months after superchallenge. Open triangles indicate the time points of SIVmac239 challenge and closed triangles SIV-G64723mt superchallenge. CTL, cytotoxic T lymphocyte; PBMC, peripheral blood mononuclear cell; SIV, simian immunodeficiency virus.

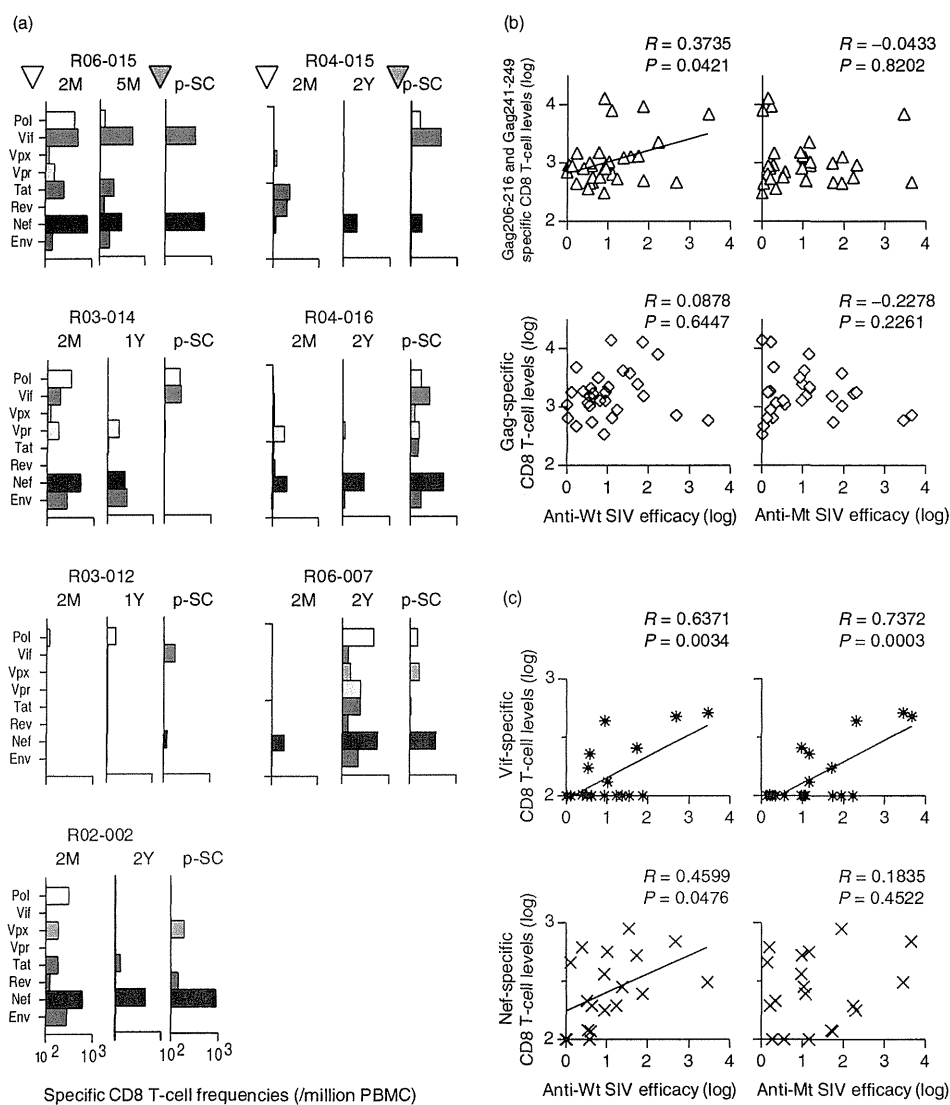


Fig. 5. Analysis of correlation between anti-SIVmac239 or anti-SIV-G64723mt efficacy *in vitro* and simian immunodeficiency virus antigen-specific CD8⁺ T-cell levels in simian immunodeficiency virus controllers. (a) SIV non-Gag antigen-specific CD8⁺ T-cell responses. Pol-specific, Vif-specific, Vpx-specific, Vpr-specific, Tat-specific, Rev-specific, Nef-specific, and Env-specific CD8⁺ T-cell frequencies at several time points were measured by using panels of overlapping peptides spanning the entire SIVmac239 Pol, Vif, Vpx, Vpr, Tat, Rev, Nef, and Env amino acid sequences, respectively. R02-003 PBMC samples were unavailable. 2M, 5M, 1Y, and 2Y: 2, 5 months, 1, and 2 years after challenge, respectively; p-SC: 1 or 2 months after superchallenge. Open triangles indicate the time points of SIVmac239 challenge and closed triangles SIV-G64723mt superchallenge. (b) Analysis of correlation between anti-SIVmac239 (Wt SIV) efficacy (left panels) or anti-SIV-G64723mt (Mt SIV) efficacy (right panels) levels and Gag₂₀₆₋₂₁₆-specific plus Gag₂₄₁₋₂₄₉-specific CTL (upper panels) or Gag-specific CTL (lower panels) levels ($n = 30$ in each panel). A correlation between anti-SIVmac239 efficacy levels and Gag₂₀₆₋₂₁₆-specific plus Gag₂₄₁₋₂₄₉-specific CTL levels is indicated ($P = 0.0421$, $R = 0.3735$). (c) Analysis of correlation between after challenge anti-SIVmac239 efficacy (left panels) or anti-SIV-G64723mt efficacy (right panels) levels and Vif-specific CTL (upper panels) or Nef-specific CTL (lower panels) levels ($n = 19$ in each panel). Correlations of anti-SIVmac239 efficacy levels with Vif-specific CTL ($P = 0.0034$, $R = 0.6731$) and with Nef-specific CTL levels ($P = 0.0476$, $R = 0.4599$) and a strong correlation between anti-SIV-G64723mt efficacy levels and Vif-specific CTL levels ($P = 0.0003$, $R = 0.7372$) are indicated. CTL, cytotoxic T lymphocyte; SIV, simian immunodeficiency virus.

challenge. Correlations of anti-SIVmac239 efficacy levels after challenge with Vif-specific CTL levels and with Nef-specific CTL levels were indicated. On the contrary,

anti-SIV-G64723mt efficacy levels after challenge strongly correlated with Vif-specific CTL levels, although any correlation of these levels with other SIV antigen-

specific CTL levels was not indicated. These results suggest that Vif-specific CTL induction may contribute in part to acquisition of the potential to suppress SIV-G64723mt replication efficiently.

Discussion

We have previously shown that 90-120-*Ia*-positive macaques eliciting Gag-specific CTL responses by vaccination can control SIVmac239 replication but are unable to contain a challenge with a mutant SIV, SIV-G64723mt, carrying multiple *gag* mutations that result in escape from recognition by Gag₂₀₆₋₂₁₆-specific and Gag₂₄₁₋₂₄₉-specific CTLs [24]. The present study revealed, by in-vitro viral suppression assay, that those 90-120-*Ia*-positive vaccinees can acquire, after wild-type SIVmac239 challenge, CD8⁺ cells able to suppress the mutant SIV replication. Induction of these CD8⁺ cell responses may have some supportive effect on the maintenance of viral control after the initial viral containment [4,26,27]. Such dynamics of anti-SIV responses have not been shown clearly even in live attenuated SIV infection [41–44]. Recently, HIVs have been suggested to accumulate mutations escaping from dominant CTL responses [45–51], but our results imply a possibility of induction of cellular immune responses effective against even those HIV variants escaping from dominant CTL responses.

The group I animals induced multiple Gag epitope-specific CTL responses after boost (before challenge) and after challenge, whereas the group II animals elicited only Gag₂₀₆₋₂₁₆-specific or Gag₂₄₁₋₂₄₉-specific CTL responses before challenge and showed induction of additional CTL responses directed against Gag epitopes other than Gag₂₀₆₋₂₁₆ and Gag₂₄₁₋₂₄₉ after challenge. Furthermore, both groups elicited SIV non-Gag-specific CTL responses after challenge. These results indicate post-challenge accumulation of broader CTL responses. The in-vitro anti-SIVmac239 efficacy levels correlated with Vif-specific and Nef-specific CTL as well as Gag₂₀₆₋₂₁₆-specific and Gag₂₄₁₋₂₄₉-specific CTL levels but not with total Gag-specific or total SIV-specific CTL levels, suggesting that not all but some particular epitope-specific CTL responses were involved in suppression of SIVmac239 replication. Nef-specific CTL responses were detected more frequently than Vif-specific ones, whereas the latter showed stronger correlation with antiviral efficacy levels (Fig. 5). We did not find common CTL epitopes in Vif or Nef. These may imply higher frequencies of effective CTLs in Vif-specific ones; conversely, Nef-specific CTLs may include effective ones but with higher frequencies of ineffective ones.

Postboost CD8⁺ cells able to suppress SIVmac239 replication failed to show suppressive effect on SIV-

G64723mt replication. We confirmed it also in two 90-120-*Ia*-positive vaccinated animals that had failed to control the mutant SIV challenge in our previous studies [24] (data not shown). However, CD8⁺ cells in the chronic phase suppressed SIV-G64723mt replication efficiently. This indicates postchallenge induction of CD8⁺ cells with the potential to suppress SIV-G64723mt replication in vaccine-based SIVmac239 controllers, although it remains unclear whether these CD8⁺ cells with antimutant SIV efficacy are responsible for the control of mutant SIV superchallenge *in vivo*. The in-vitro anti-SIV-G64723mt efficacy levels correlated with Vif-specific CTL levels and CD8⁺ cells with detectable Vif-specific CTL responses showed suppressive effect on SIV-G64723mt replication. These results implicate Vif-specific CTL responses in the suppression of SIV-G64723mt replication *in vitro* by CD8⁺ cells in the chronic phase, although other factors may also be involved in this suppression. Preservation of memory CD4⁺ T cells by vaccine-based SIV control [26] may contribute to induction of these effective CTL responses.

We found dynamics of cellular immune responses during viral control in vaccine-based SIV controllers, but the exact mechanism for broadening or changes in dominance patterns of CTL responses remains unclear. All the group I animals and macaque R04-015 showed rapid selection of a CTL escape *gag* mutation, L216S, at week 5 after challenge, whereas no *gag* mutations were selected at week 5 in macaques R04-016 or R06-007 (data not shown). We failed to recover viral genome cDNAs for sequencing from plasma after week 5 due to undetectable viral loads, but selection of viral CTL escape mutations and reversions [23,28,52–57] under undetectable levels of viral replication may contribute to induction of broader CTL responses in SIV controllers.

It is difficult to directly compare anti-SIVmac239 and anti-SIV-G64723mt efficacy of CD8⁺ cells because of difference in their replicative ability, but the ratios of the latter level to the former 1 year after challenge were higher than those after boost in all animals. Indeed, CD8⁺ cells 1 year after challenge in macaques R03-012 and R02-003 showed suppressive effect on SIV-G64723mt but not on wild-type SIVmac239 replication, although R03-012 CD8⁺ cells at 5 months and 1 year after challenge efficiently suppressed SIVmac239 replication at higher *E/T* ratio of 1:1 (R02-003 CD8⁺ cells in the chronic phase for this analysis were unavailable). Because no SIV controllers elicited CTL responses specific for peptides with mutated amino acid sequences (data not shown), all CTLs specific for SIV-G64723mt antigens in SIV controllers are expected to recognize SIVmac239 antigens also. Thus, our observation that some post-challenge CD8⁺ cells showed efficient suppressive effect on SIV-G64723mt but not on SIVmac239 replication *in vitro* may be explained by higher replicative ability of SIVmac239 compared with SIV-G64723mt; it could

be more difficult for CD8⁺ cells to suppress replication of the wild-type SIVmac239 than the mutant SIV-G64723mt, implying a possible requirement of more potent CTL responses for SIVmac239 control than for SIV-G64723mt control.

In summary, this study showed dynamics of postchallenge cellular immune responses in vaccine-based SIV controllers. Our results suggest that, during persistent viral control, vaccine-based SIV controllers can acquire CD8⁺ cells with the potential to suppress replication of SIV variants carrying CTL escape mutations. Elucidation of the mechanism for induction of broader responses in these controllers may contribute to development of a vaccine effective against highly diversified HIV infection.

Acknowledgements

This work was supported by grants-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology, a grant-in-aid from the Japan Society for the Promotion of Science, grants-in-aid from the Ministry of Health, Labor, and Welfare, and a grant from Takeda Science Foundation in Japan. N.I. is a Research Fellow of the Japan Society for the Promotion of Science. The animal experiments were conducted through the Cooperative Research Program in the Tsukuba Primate Research Center (TPRC), National Institute of Biomedical Innovation, with the help of the Corporation for Production and Research of Laboratory Primates (CPRLP). We thank Dr H. Akari, A. Saito, Y. Yasutomi, A. Hiyaoka, K. Komatsuzaki, K. Oto, and F. Ono for their assistance in animal experiments, T. Naruse and A. Kimura for MHC-I haplotyping, and Dनावेक Corporation for providing SeV vectors.

Author contributions: N.I., T.T., M.K., and T.M. designed the study. T.M. ordered animal maintenance and experimental support to TPRC and CPRLP. N.I., T.T., and M.K. contributed to vaccination and challenge experiments. A.T. contributed to blood processing and measurement of plasma viral loads. N.I., T.T., and H.T. contributed to analyses of anti-SIV efficacy of CD8⁺ cells. N.I., T.T., M.K., and H.Y. analyzed SIV-specific immune responses. N.I. and T.M. analyzed the data and wrote the article.

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RESEARCH

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A structural constraint for functional interaction between N-terminal and C-terminal domains in simian immunodeficiency virus capsid proteins

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Abstract

Background: The Gag capsid (CA) is one of the most conserved proteins in highly-diversified human and simian immunodeficiency viruses (HIV and SIV). Understanding the limitations imposed on amino acid sequences in CA could provide valuable information for vaccine immunogen design or anti-HIV drug development. Here, by comparing two pathogenic SIV strains, SIVmac239 and SIVsmE543-3, we found critical amino acid residues for functional interaction between the N-terminal and the C-terminal domains in CA.

Results: We first examined the impact of Gag residue 205, aspartate (Gag205D) in SIVmac239 and glutamate (Gag205E) in SIVsmE543-3, on viral replication; due to this difference, Gag₂₀₆₋₂₁₆ (IINEEAADWDL) epitope-specific cytotoxic T lymphocytes (CTLs) were previously shown to respond to SIVmac239 but not SIVsmE543-3 infection. A mutant SIVmac239, SIVmac239Gag205E, whose Gag205D is replaced with Gag205E showed lower replicative ability. Interestingly, however, SIVmac239Gag205E passaged in macaque T cell culture often resulted in selection of an additional mutation at Gag residue 340, a change from SIVmac239 valine (Gag340V) to SIVsmE543-3 methionine (Gag340M), with recovery of viral fitness. Structural modeling analysis suggested possible intermolecular interaction between the Gag205 residue in the N-terminal domain and Gag340 in the C-terminal in CA hexamers. The Gag205D-to-Gag205E substitution in SIVmac239 resulted in loss of in vitro core stability, which was recovered by additional Gag340V-to-Gag340M substitution. Finally, selection of Gag205E plus Gag340M mutations, but not Gag205E alone was observed in a chronically SIVmac239-infected rhesus macaque eliciting Gag₂₀₆₋₂₁₆-specific CTL responses.

Conclusions: These results present in vitro and in vivo evidence implicating the interaction between Gag residues 205 in CA NTD and 340 in CA CTD in SIV replication. Thus, this study indicates a structural constraint for functional interaction between SIV CA NTD and CTD, providing insight into immunogen design to limit viral escape options.

Background

One of the characteristics of human immunodeficiency virus (HIV) is to induce persistent viral replication resulting in AIDS progression. HIV has enormous capacity to mutate and escape from host immune recognition, driving genetic diversification of the circulating viruses [1-3]. The Gag capsid (CA), comprising the N-terminal (NTD) and the C-terminal domains (CTD)

[4-6], is one of the most conserved proteins in highly-diversified HIVs [7]. Understanding structural constraints in such viral proteins could provide valuable information for immunogen design in AIDS vaccine development.

Virus-specific cytotoxic T-lymphocyte (CTL) responses play a central role in the control of immunodeficiency virus infection [7-12]. CTLs exerting strong suppressive pressure on HIV replication select for viral mutations resulting in escape from CTL recognition [13-16]. Escape mutations in viral proteins with structural constraints are often selected with viral fitness costs, possibly facilitating subsequent immune control

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