

Fig. 1. Plasma viral loads in naïve controls and NAB-immunized macaques. Changes in geometric mean plasma viral loads in naïve controls (squares with dotted lines) and NAB-immunized macaques (triangles with bold lines) are shown. Error bars show the 95% confidence interval at each time point. The geometric mean plasma viral loads between weeks 12 and 60 were 5×10^4 copies/ml in naïve controls and $\leq 1 \times 10^3$ copies/ml NAB-immunized macaques.

2. Non-sterile SIV control via NABs

As an answer to this delineated question, we recently provided evidence for the clear potency of NABs to control established immunodeficiency virus replication *in vivo* by performing a post-infection NAB passive immunization in SIVmac239-challenged rhesus macaques [19]. While most SIVmac239-infected naïve macaques usually fail to elicit NAB responses during the early phase of infection, some acquire detectable levels of NABs against the challenge strain in the late phase. IgG purified from plasma of such SIVmac239-infected macaques with NAB induction, showing *in vitro* SIVmac239-specific neutralizing activity, was used for passive immunization as polyclonal anti-SIV NABs. These NABs were administered intravenously at day 7 post-SIVmac239 challenge, just before peak replication. The NAB passive immunization resulted in significant reduction of set-point viral loads (Fig. 1); this suppressive effect on viral replication became apparent (after week 5) past the limited duration (<1 week) of detectable NAB titers (Fig. 2). A notable observation in the NAB-immunized macaques was an accumulation of viral RNA in peripheral lymph node dendritic cells (DCs) within 24 h after the NAB infusion. Pulsing of DCs with NAB-bound SIV activated virus-specific CD4⁺ T cells *in vitro* with Fc-dependence, pointing out to a possibility of antibody-mediated virion uptake to DCs and facilitation of T-cell priming. This study thus unraveled that the existence of sub-sterile NABs near peak infection can indeed render significant suppressive activity against establishment of immunodeficiency virus persistent infection.

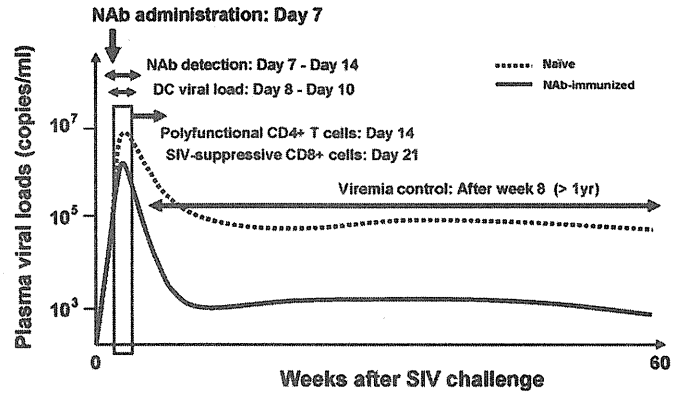


Fig. 2. Time-course events in NAB-mediated SIV control. The limited detection of plasma NAB titers between days 7 and 14 are concurrent with the rise in DC-viral loads (days 8–10), which is followed by appearance of Gag-specific CD4⁺ T cells with higher polyfunctionality at day 14 and CD8⁺ cells possessing higher anti-SIV efficacy at day 21 post-challenge. Plasma viral loads in the two groups start to show significant differences at week 8, and this is withheld up to week 60 post-challenge shown in Fig. 1. The box shows the critical time zone (weeks 1–2 post-challenge) to prime SIV-specific cellular immunity via NAB coexistence. Viral loads up to week 2 are drawn in a wider scale to ease visualization.

In our second follow-up study [20], the functional phenotypes of virus-specific T-cell responses in NAB-immunized macaques and naïve controls were further evaluated. Peripheral blood mononuclear cells (PBMCs) from both groups were pulsed with recombinant SIV Gag proteins *in vitro*, and SIV Gag-specific CD4⁺ T cells were assessed of their polyfunctionality via measurement of antigen-specific interferon- γ (IFN- γ), tumor necrosis factor- α (TNF- α), interleukin-2 (IL-2), macrophage inflammatory protein-1 β (MIP-1 β), and CD107a expression. The frequencies of polyfunctional Gag-specific CD4⁺ T cells, defined here as the upregulation of ≥ 3 of these five markers, were significantly elevated in the NAB-immunized macaques at day 14 post-challenge compared with naïve controls. Frequencies of these polyfunctional Gag-specific CD4⁺ T cells showed an inverse correlation with plasma viral loads at week 5, implying that early induction of these effectors was involved in the subsequent reduction of viremia.

In the chronic phase of infection (around week 30), Gag-specific CD4⁺ T-cell responses were detected in the NAB-immunized animals with higher polyfunctionality. These cells also showed their enhanced proliferative capacity as determined by SIV Gag-specific BrdU uptake. Accompanying these Gag-specific CD4⁺ T-cell responses, viral replication in the chronic phase remained significantly contained in the NAB-immunized macaques (Table 1 and Fig. 1). Three out of five animals exhibited undetectable plasma

Table 1
Summary of the passive NAB immunization experiment.

Animal	MHC-I haplotype	Ab-Tx at week 1 post-challenge ^a	Set-point VL (copies/ml)
Naïve controls			
R01-011	90-010- <i>le</i>	–	2×10^4 to 2×10^5
R01-012	90-010- <i>ld</i>	–	2×10^4 to 2×10^5
R03-005	90-030- <i>lg</i>	–	1×10^3 to 2×10^4
R02-004	90-088- <i>lj</i>	–	5×10^4 to 5×10^5
R02-021	ND ^b	Control Ab	3×10^5 to 6×10^6
R06-038	90-010- <i>le</i>	Control Ab	1×10^4 to 2×10^5
NAB-immunized			
R03-011	90-010- <i>le</i>	Anti-SIV NAB	$<4 \times 10^2$
R06-023	90-010- <i>ld</i>	Anti-SIV NAB	$<4 \times 10^2$
R03-020	ND	Anti-SIV NAB	1×10^3 to 2×10^4
R02-020	ND	Anti-SIV NAB	1×10^3 to 2×10^4
R03-013	90-030- <i>lh</i>	Anti-SIV NAB	$<2 \times 10^3$

^a Macaques received no immunization (–) or passive immunization with control Abs or anti-SIVmac239 NABs intravenously at week 1 post-SIVmac239 challenge.

^b Not determined.

viral loads up to 60 weeks post-challenge, while the other two also maintained them at low levels lacking any palpable sign of control failure. De novo NABs were not detected in the NAb-immunized group, together suggesting that a single administration of NABs in acute SIV infection can result in long-term viremia control with appearance of robust virus-specific CD4+ T-cell responses.

Gag-specific CD8+ T-cell frequencies at day 14 post-challenge were simultaneously assessed in both groups, which revealed no significant differences in polyfunctionality between the two. The caveat here may have been the stimulation protocol, relying on cross-presentation of the pulsed recombinant SIV Gag protein which left room for a possibility of suboptimal virus-specific CTL examination. Therefore we alternatively attempted to assess their direct anti-SIV efficacy by performing an *in vitro* viral suppression assay (VSA). In this assay, CD8+ effector cells positively selected from PBMCs at week 3 post-infection were cocultured with autologous CD8-negative target cells pre-infected with SIV and peak virus production in the culture supernatant was measured [20]. CD8+ cells from three out of four examined NAb-immunized macaques showed nearly complete suppression of progeny virus production, a phenomenon not observed in any of the examined naïve controls. Notably, none of the NAb-immunized macaques possessed an MHC class I (MHC-I) haplotype *90-120-1a* which has been previously shown to mount potent anti-SIV CTL responses. These data suggest that the NAb administration may help de novo induction of CD8+ cells exerting enhanced anti-SIV efficacy *in vivo*.

3. Significance of T-cell induction in NAb-mediated SIV control

Collectively these findings have depicted a previously undocumented pattern of immunodeficiency virus control, in that the early existence of NABs preceding peak SIV replication was followed by de novo appearance of polyfunctional Gag-specific CD4+ T cells (at week 2) and subsequent robust viremia control (after week 5) (Fig. 2). While the direct virus-neutralizing activity of the antibodies, as well as other effector functions such as antibody-dependent cell-mediated virus inhibition (ADCVI) [21,22] and/or complement activation [23] may have additively provided the protection of induced virus-specific CD4+ T cells from events such as DC trans-infection [24], the above sequence also coheres with our proposed possibility of NAb-mediated antigen presentation [19,25]. Antibody binding to soluble antigens is known to facilitate Fc-mediated uptake and resultant MHC class II antigen presentation in DCs [26,27], which renders the appearance of virus-specific polyfunctional CD4+ T cells in NAb-immunized macaques likely being a result of direct induction via NAb-mediated virion uptake into DCs.

An enigmatic role for HIV-1/SIV-specific CD4+ T cells has overall been posed in this regard of their potential vulnerability. While having been found of their presence as an inverse correlate of chronic phase viremia in HIV-1-infected humans [28], memory CD4+ T-cell subsets themselves (which likely include the virus-specific effectors) have been later determined as the primary target of CCR5-tropic primary AIDS-virus infections [15,29–32]. Detectable HIV-1-specific effector CD4+ T-cell responses in humans show an agreeable decline as viremia progresses [33], which may be driven by selective infection to some extent [34]. These are also in agreement with one study which documents prophylactic induction of Env-specific CD4+ T cells exerting a detrimental influence on the otherwise self-remitting course of SIVsmE660 viremia within that system [35]. In CTL-based prophylactic vaccines, acute and long-term preservation of (total and central) memory CD4+ T-cell counts has accordingly been defined as a passive correlate of protection status [36–38]. Hence the entity of truly contributable

HIV-1/SIV-specific CD4+ T-cell responses has overall not been clarified, although implications of their enrollment exist such as in relatively benign HIV-2 infection [39]. The current study newly supports two possibilities. One is that de novo polyfunctional Gag-specific effector CD4+ T cells, if induced, may be potent of actively driving primary viremia control; in other words, their existence can be taken as a cause rather than a result of protection. The other is that the antigen-specific activation potential of central memory CD4+ T cells is reinforced as a detailed protective correlate during chronic infection as in humans [28], in addition to the quantitative preservation of the subset. These two hallmarks in NAb-mediated SIV control sharply contrast Gag-specific CD4+ T-cell responses in naïve controls which, importantly, exhibit neither of the two.

It is important to question how these Gag-specific polyfunctional CD4+ T cells had exerted anti-SIV activity. Possibilities include roles such as conventional helper cells providing cognate assistance in priming CTLs for viremia clearance [40], effectors directly suppressing infected cells with [41,42] or without cytolytic activity, or both. Results of the CD8+ cell VSA in NAb-immunized macaques do not contradict participation of SIV-specific CTLs in acute phase viremia control, partially supporting the first candidate. When we performed VSA with CD8+ cells from the chronic phase (around week 20 post-challenge) in NAb-immunized macaques, no comparable *in vitro* virus suppression had been observed (unpublished results). This may also concur with the contracting levels of SIV-specific CTLs at low viral replication levels *in vivo*. Augmented virus-specific CTLs being a major, though maybe not the only, determinant of viremia suppression in the acute phase may be a reasonable explanation; if so, how their commitment may differ from viremia control in vaccinated and naïve SIV elite controllers (ECs) [43,44], or HIV-1 ECs in humans [45], is still unknown. Another factor to consider may be the Gag-specificity of these CD4+ T cells. As in CTLs, where importance of their Gag-biased induction in HIV-1/SIV viremia control has been emphasized both *in vitro* and *in vivo* [46,47], inducing CD4+ T-cell responses with a preference for Gag epitopes may also be advantageous in HIV-1/SIV control.

Regardless of the precise mechanism, there certainly lies a limitation to directly extrapolating results of this post-infection NAB passive immunization study to the patterns of protection likely afforded by endogenous NAB-inducing regimens. Nevertheless it is still important to recognize that the concordance of primary SIV control with de novo induction of polyfunctional Gag-specific CD4+ T cells, near the normal peak of systemic memory CD4+ T-cell destruction [14], does strengthen the rationale for prophylactic NAB induction. Even if induced NABs do not prevent the initial establishment of HIV-1/SIV infection, they may well exhibit their potential against systemic infection by a non-sterile protective mechanism. This narrow 1-week window around peak plasma viremia (Box in Fig. 2) is indeed some watershed for the HIV-1/SIV-infected host to impact viremia thereafter.

4. Perspectives: humoral and cellular immune concert for HIV-1 control

Whether or how cellular and humoral immune responses may collectively exert their effect against CCR5-tropic HIV-1/SIV acute infections had been unexplored, while systems involving other retroviruses have given some implications for this question. In Friend murine leukemia virus infection, high doses of administered pre-exposure NABs have provided an augmentation to memory T-cell-based control [48]. A similar augmentation does not, however, appear to happen in CXCR4-tropic SHIV89.6P infection [49]. Turning to a self-remitting benign SHIV infection (SHIV DH12R clone 8), the negative effect of CD20-depletion had only become apparent in macaques lacking a highly protective MHC-I allele

(Mamu-A*01), suggesting a seemingly interdependent rather than synergistic cooperation [50].

It is noteworthy that CD20+ B-cell depletion in the SIV/SHIV-macaque model is an interesting but intrinsically sensitive approach. What is problematic is that the outcome is complicated in a virus strain-dependent manner. In a pathogenic virus challenge (e.g., SIVmac) model the set-point viremia is significantly high. This seemingly blunts the effect of transient B-cell depletion from an incompetent baseline [51], while an impact on disease course appears to be partially observed in the subacute phase by constitutive B-cell depletion [52]. On the contrary, in models where relatively benign SIV (or SHIV) strains are challenged, host CTL responses reaching a certain threshold of potency may readily control viremia by themselves. The need of humoral immunity assistance is sufficiently compensated here, which again dampens the impact of B-cell depletion [53]. The propensity in such models seems to be that B-cell depletion effects only become appreciable in non-elite cellular immunity-eliciting controllers [50,54]. Since no detectable NABs are elicited in acute to subacute SIV infection, it likely follows that the cases of accelerated pathology via B-cell depletion may derive from the deprival of non-NAB-mediated effects, such as ADCVI. Taken together, the endogenous existence of antiviral non-neutralizing antibodies may change the outcome from rapid progression to persistent infection, whereas the exogenous administration of NABs may change the outcome from persistent infection to relative viremia control.

The entity of humoral immunity modulation against cellular immunity in HIV-1/SIV infection is hence still unclear; nevertheless one thing is becoming evident which is that, at least, NABs and T cells do not diminish each other in mere competitive coexistence for target elimination. Especially near peak SIV replication, NAB-dependent modification of T-cell immunity does exert a significant impact on viremia control.

In conclusion, there appears to be a preference of a balance, perhaps a temporal one, between induction of HIV-1/SIV-specific CD8+, CD4+ T cells and NABs. Determining the requisites for NAB-triggered T-cell immunity-based SIV replication control shall further reveal rational endpoints for prophylactic HIV-1 vaccines.

Conflict of interest statement

The authors state that they have no conflict of interest.

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Diversity of MHC class I genes in Burmese-origin rhesus macaques

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Abstract Rhesus macaques (*Macaca mulatta*) are widely used in developing a strategy for vaccination against human immunodeficiency virus by using simian immunodeficiency virus infection as a model system. Because the genome

diversity of major histocompatibility complex (MHC) is well known to control the immune responsiveness to foreign antigens, MHC loci in Indian- and Chinese-origin macaques used in the experiments have been characterized, and it was revealed that the diversity of MHC in macaques was larger than the human MHC. To further characterize the diversity of *Mamu-A* and *Mamu-B* loci, we investigated a total of 73 different sequences of *Mamu-A*, 83 sequences of *Mamu-B*, and 15 sequences of *Mamu-I* cDNAs isolated from Burmese-origin macaques. It was found that there were one to five expressing genes in each locus. Among the *Mamu-A*, *Mamu-B*, and *Mamu-I* sequences, 44 (60.2%), 45 (54.2%), and 8 (53.3%), respectively, were novel, and most of the other known alleles were identical to those reported from Chinese- or Indian-origin macaques, demonstrating a genetic mixture between the geographically distinct populations of present day China and India. In addition, it was found that a *Mamu* haplotype contained at least two highly transcribed *Mamu-A* genes, because multiple *Mamu-A1* cDNAs were obtained from one haplotype. These findings further revealed the diversity and complexity of MHC locus in the rhesus macaques.

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Introduction

The rhesus macaque (*Macaca mulatta*) is a member of the old world monkey. It is estimated that the ancestor of macaques was diverged from the human-chimpanzee ancestor approximately 25 million years ago (Stewart and Disotell 1998). The habitat of the rhesus macaque extends from Pakistan and India to the southern part of China

(Timmins et al. 2008), wider than that of the other nonhuman primates. It is known that the genome diversity in rhesus macaques is quite unique, because more than 60% of the rhesus macaque-specific expansions are found in the protein coding sequences (Gibbs et al. 2007). The increase in the gene copy number in the rhesus macaque, relative to that in humans, can also be observed in the major histocompatibility complex (MHC) locus (Gibbs et al. 2007).

The rhesus macaque is widely used as a nonhuman primate species model in biomedical researches for human diseases including acquired immunodeficiency syndrome (AIDS). Particularly, the development of vaccines against the human immunodeficiency virus (HIV) in part depends on the results of experiments using macaques, because the simian immunodeficiency virus (SIV) infection causes AIDS-like syndrome (Barouch et al. 2000; Schmitz et al. 1999; Yasutomi et al. 1993). Previous SIV challenge studies indicated association of MHC class I genotypes with rapid or delayed AIDS progression in rhesus macaques like HIV-1 infection in humans (Mothe et al. 2003; Yant et al. 2006; Loffredo et al. 2008; Reynolds et al. 2008). In addition, effective vaccination was associated with specific MHC class I alleles called as “elite controller” alleles, by which prevention of viral replication could be achieved in macaques challenged by SIVmac239 (Loffredo et al. 2007; Maness et al. 2008). In these experiments, macaques of Indian or Chinese origin have been widely used, and macaques from different regions such as Burma have also been used recently.

To evaluate the efficacy of SIV vaccination, it is necessary to characterize the MHC alleles because the presentation of antigenic peptides by MHC molecules to T cells, more specifically the binding of antigenic peptide to the MHC molecule, depends on the structure of the MHC allele. We have previously developed a reference strand conformation analysis-based typing system for *Mamu* class I genes and reported that the number of expressing genes varies among macaques of Burmese or Laotian origin; we could identify at least 16 different *Mamu* class I locus haplotypes that were composed of different numbers of *Mamu* class I genes (Tanaka-Takahashi et al. 2007). In addition, we reported that a haplotype of *Mamu* class I genes, *90-120-Ia*, exerted a protective vaccination against

SIVmac239 challenge (Matano et al. 2004). Furthermore, it was revealed that one of highly expressed *Mamu-A* allele of the *90-120-Ia* haplotype, *Mamu-A1*065:01* (previously designated as *Mamu-A*90120-5*), encoded a *Mamu-A* molecule that could efficiently present a SIV-derived Gag₂₄₁₋₂₄₉ peptide to cytotoxic T cells from the vaccinated macaques (Tsukamoto et al. 2008).

The aim of present study was to define the allelic polymorphisms and haplotype diversity of the *Mamu* class I gene from Burmese-origin macaques.

Materials and methods

Animals

A total of 100 rhesus macaques from breeding colonies maintained in Japan were enrolled. Founders of colonies were captured in Myanmar or Laos, and the colonies were separately maintained. Macaque colonies were classified into seven groups based on their paternal lineages (90-120, 90-010, 90-030, 90-088, 89-002, 89-075, and 91-010F1) (Tanaka-Takahashi et al. 2007). The animal 91-010F1 was an offspring of 89-075.

Sequencing analysis of cDNAs from *Mamu* class I genes

Total cellular RNA was extracted from B lymphoblastoid cell lines established from the macaques by using RNeasy reagent (TaKaRa, Shiga, Japan). Oligo (dT)-primed cDNA was synthesized using Transcriptor reverse high fidelity transcriptase (Roche, Mannheim, Germany) according to the manufacturer's recommendations. Full-length cDNAs for *Mamu* class I genes were amplified by polymerase chain reaction (PCR) using locus-specific primer pairs, as described previously (Tanaka-Takahashi et al. 2007), with a modification of primer pairs to those reported by Karl et al. (Karl et al. 2008): 5'MHC_UTR (5'-GGACTCAGAATCTCCCCAGACGCCGAG) and 3'MHC_UTR_A (5'-CAGGAACAYAGACACATTCAGG) for *Mamu-A* locus and 5'MHC_UTR and 3'MHC_UTR_B (5'-GTCTCTCCACCTCCTCAC) for *Mamu-B*, *-I* loci, using Phusion Flash DNA polymerase (Finzymes, Espoo, Finland). The PCR

Table 1 *Mamu* class I alleles found in Burmese-origin macaques

Loci	Number of analyzed macaques	Number of observed alleles	Novel alleles (number, %)	Known alleles (number, %)
Mamu-A	100	73	44 60.2	29 39.8
Mamu-B	93	83	45 54.2	38 45.8
Mamu-I	93	15	8 53.3	7 46.7
Others (AG, F)	93	2	0 -	2 100
Total		173	97 56.1	76 43.9

Table 2 Alleles of *Mamu-A* locus identified in Burmese-origin macaques

Locus	Allele name	Novelty ^a	Accession Number ^b	Shared allele ^c	Number of animals	Identity to <i>Mafa</i> or <i>Mane</i> alleles ^d
A1	A1*003:01:03	Novel	AB496714		1	
A1	A1*003:08		AB444903	C	7	
A1	A1*003:10	Novel	AB444904		1	
A1	A1*004:01:02		AB444866	C	19	<i>Mafa-A1*004:02</i>
A1	A1*007:06:01	Novel	AB540211		2	
A1	A1*008:01:02	Novel	AB430443		11	
A1	A1*008:01:03	Novel	AB496711		1	
A1	A1*008:02	Novel	AB477383		2	
A1	A1*015:01		AB551785		2	
A1	A1*018:05		AB444927	I	1	
A1	A1*018:07	Novel	AB444928		11	
A1	A1*018:08	Novel	AB444926		6	
A1	A1*019:02		AB444900	C	2	
A1	A1*019:05		AB444901	C	1	
A1	A1*019:07	Novel	AB444899		2	
A1	A1*022:01		AB444895	C	1	
A1	A1*022:03	Novel	AB444894		7	
A1	A1*023:02	Novel	AB444874		4	
A1	A1*026:03		AB477385	C	1	
A1	A1*028:06	Novel	AB444924		1	
A1	A1*028:07:01	Novel	AB444923		3	
A1	A1*032:02	Novel	AB444933		13	
A1	A1*032:03	Novel	AB444934		4	
A1	A1*040:01		(AM295910)		1	
A1	A1*041:01		AB444931	C	1	
A1	A1*041:02		(EU429608)	C	1	
A1	A1*042:01	Novel	AB444868	C	2	
A1	A1*043:01		AB444869	C	7	
A1	A1*049:03		AB444880	C	2	
A1	A1*049:04	Novel	AB444881		2	
A1	A1*050:01		AB444889	C	7	
A1	A1*052:01		AB444890	C	3	<i>Mafa-A1*052:02</i>
A1	A1*056:02		AB477384	C	6	
A1	A1*056:02:02	Novel	AB444935		3	
A1	A1*065:01		AB444921	C	6	<i>Mafa-A1*065:04</i>
A1	A1*066:01	Novel	AB444888		14	
A1	A1*074:04:01	Novel	AB540213		1	
A1	A1*105:01	Novel	AB444898		1	
A1	A1*105:02	Novel	AB444896		11	
A1	A1*105:03	Novel	AB496716		2	
A1	A1*105:04	Novel	AB496709		1	
A1	A1*106:01	Novel	AB444875		1	
A1	A1*107:01	Novel	AB444887		9	<i>Mafa-A1*096:01</i>
A1	A1*108:01	Novel	AB444925		1	
A1	A1*109:01	Novel	AB444902		7	<i>Mafa-A1*097:01</i>
A1	A1*110:01	Novel	AB444884		4	
A1	A1*111:01	Novel	AB444886		1	
A1	A1*112:01	Novel	AB496717		1	
A1	A1*117:01:01	Novel	AB540212		2	

Table 2 (continued)

Locus	Allele name	Novelty ^a	Accession Number ^b	Shared allele ^c	Number of animals	Identity to <i>Mafa</i> or <i>Mane</i> alleles ^d
A1	A1*118:01:01	Novel	AB540214		1	
A2	A2*01:03	Novel	AB444917		15	
A2	A2*05:03:02		AB444910	C	2	
A2	A2*05:10		AB444907	I	2	
A2	A2*05:11		AB444909	I	7	
A2	A2*05:13		(AM295927)	C	1	
A2	A2*05:14		(AM295928)	C	1	
A2	A2*05:15:04	Novel	AB444914		3	
A2	A2*05:22		AB444911	C	1	<i>Mane-A2*05:18</i>
A2	A2*05:26		AB496715	C	2	
A2	A2*05:31	Novel	AB444908		2	
A2	A2*05:32:02	Novel	AB444920		2	
A2	A2*05:44	Novel	AB444912		1	
A2	A2*05:45	Novel	AB444915		2	
A2	A2*05:46	Novel	AB444913		4	<i>Mane-A2*05:03:01</i>
A3	A3*13:13	Novel	AB496712		4	
A4	A4*01:02:02	Novel	AB444879		3	
A4	A4*14:03		AB444876	C, I	15	
A4	A4*14:04		AB444878	C	1	
A5	A5*30:01:01		(AM295945)	C	1	
A5	A5*30:01:02		AB444882	C	1	
A5	A5*30:06	Novel	AB444883		2	
A6	A6*01:01		AB444938	C	1	
A6	A6*01:05	Novel	AB444937		4	

^a New alleles are indicated as novel

^b Nucleotide sequences were submitted to public database and can be obtained with the indicated accession number. The accession numbers in the parentheses indicated that the Mamu class I sequences were identical to those numbers which had been deposited previously by other investigators.

^c Alleles found in Burmese-origin macaques were shared with macaques originated from the other region. C Chinese-origin macaques, I Indian-origin macaques

^d Identical sequences found in *Mafa* or *Mane* alleles

program was composed of the following steps: denaturation at 98°C for 10 s; 25 cycles at 98°C for 1 s, 63°C for 5 s, 72°C for 20 s; and additional extension at 72°C for 1 min. The PCR products were cloned into pSTBlue-1 Perfectly Blunt vector (Novagen, WI, USA) according to the manufacturer's instructions. Both strands from 30 to 90 independent cDNA clones obtained from each macaque for each locus were sequenced by BigDye Terminator cycling system and analyzed in an ABI 3730 automated sequence analyzer (Applied Biosystems, CA, USA).

Data analyses and nomenclature for *Mamu* class I alleles

Nucleotide sequences of cDNAs were analyzed and aligned using Genetyx Ver. 8 software package (Genetyx Corp., Japan). When at least three clones from independent PCR or from different individuals showed identical sequences, we submitted the sequences to DNA Data Bank of Japan database and to the Immuno Polymorphism Database for

nonhuman primate MHC (<http://www.ebi.ac.uk/ipd/mhc/submit.html>; Robinson et al. 2003) to obtain official nomenclature for novel alleles of *Mamu-A* and *-B* genes. Phylogenetic analysis of *Mamu-A* sequences corresponding to exon 2, 3 and a part of exon 4 obtained in this study was done by using Genetyx Ver. 8 software package. *Mamu-A1* 001:01* was included in the analysis as a reference. Neighbor-joining trees were constructed with the Kimura 2 parameter method. Bootstrap values were based on 5,000 replications.

Results

Identification of *Mamu* class I alleles in Burmese-origin macaques

We analyzed cDNA clones obtained by RT-PCR for *Mamu-A* locus and *Mamu-B* locus (Table 1). When at least three

clones with identical sequences were obtained from two independent PCR for an individual or from at least two individuals, the nucleotide sequences were considered to be real and not artifacts. We identified 73 different *Mamu-A* sequences in 100 individuals. Among them, 44 (60.2%) were novel, whereas the other 29 (39.8%) were identical to those reported mainly from Chinese- or Indian-origin macaques (Table 2). In addition, 50 sequences were from

Mamu-A1, while 14, 1, 3, 3, and 2 sequences were from *Mamu-A2*, *-A3*, *-A4*, *-A5*, and *-A6*, respectively (Table 2). A neighbor-joining analysis showed that the sequences from the same minor *Mamu-A* genes were clustered with each other (Fig. 1).

On the other hand, 83 *Mamu-B* alleles and 15 *Mamu-I* alleles were observed in 93 individuals. Among them, 45 (54.2%) and 8 (53.3%) were novel *Mamu-B* and *Mamu-I*

Fig. 1 Phylogenetic tree of *Mamu-A* alleles detected in this study. The tree was constructed using neighbor-joining method with bootstrap values of 5,000 replications. The values are indicated as percentages and those values less than 50% are not shown. *Mamu-A1* 001:01* was included in the analysis as a reference. The *Mamu-A* sequences with official nomenclature found in Burmese macaques are indicated, and novel alleles of *Mamu-A* genes are underlined. Clustering of alleles of minor *Mamu-A* genes, *Mamu-A2*, *-A3*, *-A4*, *-A5*, and *-A6* genes, are indicated by vertical bars

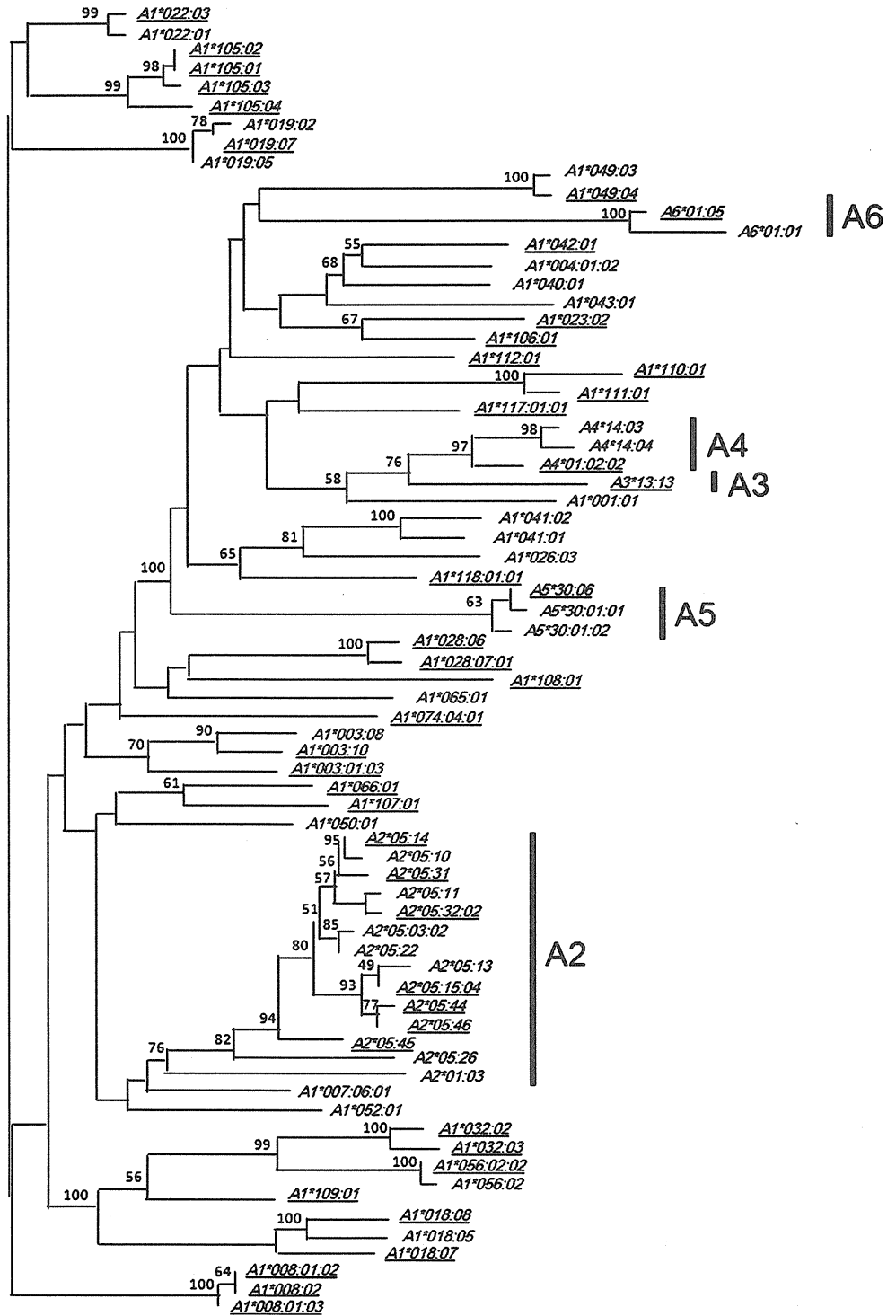


Table 3 Alleles of *Mamu-B* locus identified in Burmese-origin macaques

Locus	Allele name	Novelty ^a	Accession Number ^b	Shared allele ^c	Number of animals	Identity to <i>Mafa</i> or <i>Mane</i> alleles ^d
B	B*001:01:01		AB477408	I	12	
B	B*001:01:02		(AM902529)	C	6	
B	B*002:01		(U41833)	I	5	
B	B*003:01		(U41825)	C, I	2	
B	B*004:01		AB477405	I	11	
B	B*005:02		AB535753	I	14	
B	B*007:02		AB477409	C, I	33	
B	B*007:03		AB477412	C, I	1	
B	B*007:04:02	Novel	AB540183		2	
B	B*013:01		(AM902539)	C	1	
B	B*013:02:01	Novel	AB540185		1	
B	B*014:01		(AM902540)	C	1	<i>Mafa-B*105:01</i>
B	B*015:02		(AM902542)	C	1	
B	B*015:03:01	Novel	AB540186		2	
B	B*016:02:01	Novel	AB477395		9	
B	B*017:01		(AF199358)	I	2	
B	B*017:03		(AM902533)	C	8	
B	B*021:02		(AM902536)	C	1	
B	B*023:01		(AM902530)	C	2	
B	B*024:01		(AJ556881)	C, I	3	
B	B*026:02		AB477402	I	8	
B	B*028:02:01		(AM902532)	C	1	
B	B*029:03:01	Novel	AB540191		1	
B	B*036:03:01	Novel	AB477388		4	
B	B*037:01		AB477401	I	6	<i>Mafa-B*050:01</i>
B	B*038:01		(AJ556889)	I	1	
B	B*038:02:01	Novel	AB477391		3	
B	B*039:01		AB477411	C, I	12	
B	B*040:01:01	Novel	AB535751		8	
B	B*043:01		AB477403	C, I	14	
B	B*044:06:01	Novel	AB540205		1	
B	B*045:07:01	Novel	AB477389		5	<i>Mafa-B*012:01</i>
B	B*046:03:01	Novel	AB477397		2	
B	B*046:15		(EU915284)	I	1	
B	B*046:18:01	Novel	AB477398		2	
B	B*046:19:01	Novel	AB540193		1	
B	B*051:06:01	Novel	AB477387		2	
B	B*051:07:01	Novel	AB540206		1	
B	B*054:02:01	Novel	AB540194		5	
B	B*056:03:01	Novel	AB540195		2	
B	B*056:04:01	Novel	AB540207		2	
B	B*059:01		(AM902563)	C	1	
B	B*060:01		(EU669870)	I	1	
B	B*060:03		(EU934766)	I	1	
B	B*060:04:01	Novel	AB477394		4	
B	B*061:02		(AM902564)	C	3	
B	B*061:03	Novel	AB430442		7	
B	B*061:04:01	Novel	AB540196		10	<i>Mane-B*061:01</i>
B	B*063:02:01	Novel	AB540210		3	

Table 3 (continued)

Locus	Allele name	Novelty ^a	Accession Number ^b	Shared allele ^c	Number of animals	Identity to <i>Mafa</i> or <i>Mane</i> alleles ^d
B	B*063:02:02	Novel	AB540197		4	
B	B*063:04:01	Novel	AB477399		2	
B	B*063:05:01	Novel	AB540204		2	
B	B*066:01		AB477406	I	28	
B	B*066:02:01	Novel	AB540198		1	
B	B*068:04		(AM902571)	C	10	
B	B*069:01		(AF519898)	C, I	1	
B	B*069:06:01	Novel	AB540209		1	
B	B*069:07:01	Novel	AB540208		2	
B	B*070:02		(AM902575)	C	1	
B	B*071:01		(AJ489330)	I	2	
B	B*071:02:01	Novel	AB540199		1	
B	B*073:01		AB477404	C	4	
B	B*073:02:01	Novel	AB540200		1	
B	B*074:02		(AF219484)	C	1	
B	B*077:02		AB477410	C	1	<i>Mafa-B*110:01</i>
B	B*082:01		(EF580160)	C	1	
B	B*082:05:01	Novel	AB477396		5	
B	B*082:06:01	Novel	AB540201		2	
B	B*083:01		(EF580161)	C	2	
B	B*083:02:01	Novel	AB542052		1	
B	B*085:03:01	Novel	AB540202		5	
B	B*089:01		(EF580172)	C	11	
B	B*091:03	Novel	AB551786		2	
B	B*092:02:01	Novel	AB477386		7	
B	B*092:03:01	Novel	AB542053		1	
B	B*101:01:01	Novel	AB477400		3	
B	B*102:01:01	Novel	AB477392		10	
B	B*105:01:01	Novel	AB540184		1	<i>Mane-B*105:01</i>
B	B*124:01:01	Novel	AB540203		10	<i>Mane-B*124:01</i>
B	B*142:01:01	Novel	AB542050		1	<i>Mafa-B*023:02</i>
B	B*156:01:01	Novel	AB540192		1	
B	B*162:01:01	Novel	AB477390		3	
B	B*163:01:01	Novel	AB542051		2	
I	I*01:06:01		(EF580176)	C	2	
I	I*01:06:05		(EU934767)	I	4	
I	I*01:06:07		(FN396419)		1	<i>Mafa-I*01:11</i>
I	I*01:06:08	Novel	AB477416		12	
I	I*01:06:09	Novel	AB541976		3	<i>Mane-I*01:01:02</i>
I	I*01:06:10	Novel	AB541977		1	
I	I*01:07:01		AB477420	I	7	
I	I*01:08:01		(FJ009194)	I	13	
I	I*01:08:02		(GQ471888)	I	4	
I	I*01:09:01	Novel	AB477415		1	
I	I*01:18		(EF580175)	C	1	
I	I*01:20:02	Novel	AB477414		2	
I	I*01:22:01	Novel	AB477417		7	
I	I*01:23:01	Novel	AB477418		8	
I	I*01:24:01	Novel	AB477413		2	

Table 3 (continued)

Locus	Allele name	Novelty ^a	Accession Number ^b	Shared allele ^c	Number of animals	Identity to <i>Mafa</i> or <i>Mane</i> alleles ^d
F	F*01:03			I	3	
AG	AG*03:01:01			I	1	

^a New alleles are indicated as novel

^b Nucleotide sequences were submitted to public database and can be obtained with the indicated accession number. The accession numbers in the parentheses indicated that the Mamu class I sequences were identical to those numbers which had been deposited previously by other investigators.

^c Alleles found in Burmese-origin macaques were shared with macaques originated from the other region. C Chinese-origin macaques, I Indian-origin macaques

^d Identical sequences found in *Mafa* or *Mane* alleles

alleles, respectively. The other *Mamu-B* and *Mamu-I* sequences were identical to those reported from Chinese- and/or Indian-origin macaques (Table 3).

Mamu class I haplotypes observed in Burmese-origin macaques

From the cDNA analyses of genetically related macaques, we could identify the *Mamu-A* and *Mamu-B* sequences comprising 13 different haplotypes from seven paternal lineages (haplotype 'w' was shared by 89-075 and its offspring 91-

010F1) and eight other haplotypes in the colonies; the *Mamu* class I haplotype consisted of one to three expressing *Mamu-A* genes and one to five expressing *Mamu-B* (including *Mamu-I*) genes, confirming that the number of expressed *Mamu* class I genes varied with the haplotype (Table 4). Examples of family pedigrees are shown in Fig. 2. Although usually only one *Mamu-A1* allele could be identified in the haplotypes, the 90-120-a haplotype carried two different *Mamu-A1* alleles, which was confirmed by the analysis of family pedigree (Fig. 2a). In addition, *Mamu-B*001* alleles were tightly linked to a *Mamu-B*007* allele (Table 4).

Table 4 *Mamu* class I haplotypes identified in Burmese-origin macaques

Founder Lineage ^a	Haplotype	Major Mamu-A (A1)	Minor Mamu-A	Mamu-B
90-120	a	A1*043:01, A1*065:01		B*061:03, B*068:04, B*089:01
	b	A1*018:08	A2*05:31	B*036:03:01, B*037:01, B*043:01, B*162:01:01,
90-010	d	A1*032:02		B*004:01, B*102:01:01
	e	A1*066:01		B*005:02, B*040:01:01
90-030	g	A1*105:02	A2*05:11	B*066:01
	h	A1*004:01:02	A4*14:03	B*043:01, B*092:02:01
90-088	j	A1*008:01:02		B*007:02, B*039:01
	k	A1*018:08	A2*05:45	B*001:01:01, B*007:02
89-002	p	A1*018:07	A2*01:03, A4*14:03	B*001:01:01, B*007:02
	q	A1*107:01		B*016:02:01
91-010F1	s	A1*003:08		B*023:01, I*01:08:01
	w	A1*022:03	A4*01:02:02	B*001:01:02, B*007:02, B*017:03
89-075	w	A1*022:03	A4*01:02:02	B*001:01:02, B*007:02, B*017:03
	v	A1*109:01	A3*13:13	B*054:02:01, B*061:04:01, B*063:02:02, B*068:04, B*124:01:01
R428	i	A1*050:01	A2*05:11	B*066:01
R360	o	A1*028:07:01		B*056:04:01, B*066:01
R236	r	A1*049:03	A2*05:22	B*001:01:02, B*007:02, B*017:03
95-014	f	A1*066:01	A2*05:14, A5*30:01:01	B*005:02
R487	m	A1*018:08	A2*05:31	B*026:02, B*045:07:01, B*051:06:01
R252	t	A1*032:03	A2*05:14, A5*30:01:01	B*005:02
R446	u	A1*004:01:02		B*026:02, B*043:01, B*073:01
R220	c	A1*050:01		B*063:02:01, B*066:01

^a ID of founder in which each Mamu class I haplotype was found

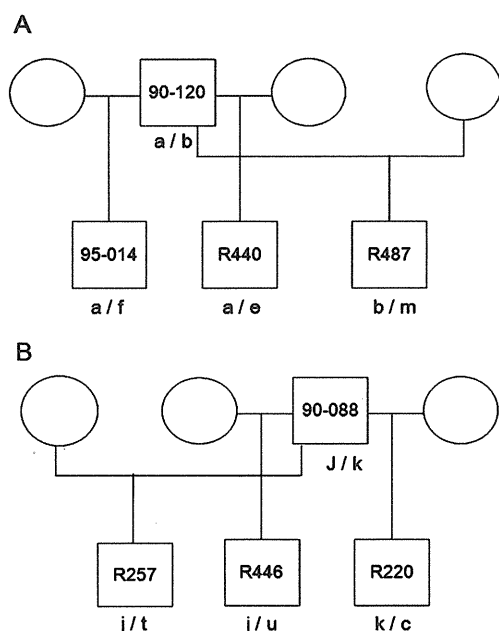


Fig. 2 Segregation of *Mamu* class I haplotypes in the pedigrees of macaques. Pedigree information and haplotype information are indicated along with ID of macaques. **A.** *Mamu* class I haplotypes of *a* and *b* in the parent (90-120) were segregated to its offspring 95-014, R440, and R487. **B.** *Mamu* class I haplotypes of *j* and *k* in the parent (90-088) were segregated to R257, R446, and R220. The *Mamu* class I alleles composing the indicated haplotypes are listed in Table 4

Discussion

The rhesus macaque is widely used in the experimental design for developing a vaccine against HIV. Indian-origin macaques are well characterized as a model system and it has been reported that there are several “elite controller” alleles such as *Mamu-A*001* and *Mamu-B*017*, with which most macaques showed lower viral loads after SIVmac239 challenge (Friedrich et al. 2004). In this study, we did not observe *Mamu-A1*001* in Burmese-origin macaques, while we previously reported that a group of animals carrying the MHC class I haplotype 90120a (‘a’ haplotype designated in this study, Table 4) showed vaccine-based control of SIVmac239 replication (Matano et al. 2004). This haplotype contains *Mamu-A*065:01* (previously noted as *Mamu-A*90120-5*) allele, and cytotoxic T lymphocyte (CTL) responses specific for an SIVmac239 Gag₂₄₁₋₂₄₉ (SSVDEQIQW) epitope restricted by this *Mamu-A1* allele are responsible for the SIV control in the vaccinated macaques carrying the 90120a haplotype (Kawada et al. 2008). Interestingly, the SIV Gag₂₄₁₋₂₄₉ epitope is overlapped with a HLA-B*5701-restricted HIV-1 Gag₂₄₀₋₂₄₉ epitope, TW10 (TSTLQEQLAW), and TW10-specific CTL responses have also been indicated to exert strong suppression on HIV-1 replication resulting in lower viral loads (Tsukamoto et al. 2008; Goulder and Watkins 2008).

Among 73 *Mamu-A* sequences detected in this study, only four sequences were reported to be found in the

Indian-origin macaques. In clear contrast, 25 *Mamu-A* sequences were also found in the Chinese-origin macaques, implying that the genetic background of Burmese-origin macaques might be closer to Chinese-origin macaques than to Indian-origin macaques. However, 27 and 25 *Mamu-B* sequences were identical to those reported in Chinese- and Indian-origin macaques, respectively, demonstrating that Burmese-origin macaques represent a mixture of geographically distinct Chinese- and Indian-origin macaque populations. In addition, more than half of *Mamu* class I alleles found in this study were novel, indicating that the regional difference in MHC allelic distribution exists similar to that in human HLA. Because the habitat of Burmese-origin rhesus macaques is overlapped in part with the habitat of crab-eating macaques (*Cynomolgus rhesus*, *Macaca fascicularis*) and Southern pig-tailed macaques (*Macaca nemestrina*), it is interesting to investigate whether the identical sequences to *Mamu* class I alleles would be frequently found in *Mafa* or *Mane* class I alleles. As shown in Tables 2 and 3, about 10% of *Mamu* class I alleles had identical sequences to equivalent *Mafa* or *Mane* class I alleles, as has been observed in the other macaque populations (Campbell et al. 2009; Otting et al. 2009), demonstrating that the frequency of shared MHC class I alleles was relatively constant in different populations of macaques.

The *Mamu* locus is known to be composed of multiple copies of polymorphic DNA sequences (Daza-Vamenta et al. 2004; Kulski et al. 2004); for example, *Mamu-A* locus encodes for a major and highly transcribed *Mamu-A1* and other minor *Mamu-A2*, *-A3*, *-A4*, *-A5*, *A6*, and *-A7* with relatively low transcription (Otting et al. 2004, 2007). In this study, we identified two different *Mamu-A1* alleles on one haplotype, *Mamu-A1*043:01* and *Mamu-A1*065:01* on the haplotype 90120-a, which was confirmed by the segregation study of 90-120 family (Fig. 2a). In the phylogenetic tree of *Mamu-A* sequences, *Mamu-A1*043:01* and *Mamu-A1*065:01* alleles were classified into the *Mamu-A1* allele group (Fig. 1). These data showed the presence of *Mamu-A* haplotype carrying multiple major *Mamu-A1*, albeit that it might be a rare exception.

On the other hand, we deduced that some *Mamu-A1* alleles could not be well amplified by the PCR primer pair used in this study. For instance, *Mamu-A1*065:01* in the “a” haplotype (90-120 lineage, Table 4) and *Mamu-A1*003:08* in the “s” haplotype (91-010F1 lineage, Table 4) could not be well amplified with the primer-set of 5’MHC_UTR and 3’MHC_UTR_A. On the contrary, *Mamu-A1*004:01:02* in the “h” haplotype (90-030 lineage, Table 4) and *Mamu-A1*10:701* in the “q” haplotype (89-002 lineage, Table 4) were amplified more efficiently with this primer pair than the other primer pair reported previously (Tanaka-Takahashi et al. 2007). These observations raised a possibility that there might be further copy

number variations in the *Mamu* class I loci. It appears that a higher number of highly transcribed and expressed MHC alleles on a haplotype would be desirable, when the immunological role in antigen presentation after viral infection is considered, because the multiple MHC alleles will enable one to present more number of antigenic peptides. However, the presence of highly transcribed and expressed multiple MHC alleles could lead to multiple holes in the antigen recognition through elimination of T cells recognizing self-antigenic peptides or foreign antigenic peptides mimicking self-antigens. In this regard, it should be noted that the transcription levels of *Mamu-B* alleles, as estimated by the number of clones isolated from each macaque, were not so different from one another. We found that several *Mamu-B* alleles on the specific haplotypes, such as “b” haplotype (90-120 lineage) and “v” haplotype (89-075 lineage), showed similar transcription levels, although their expression levels might be moderate. However, because Rosner et al. reported that cell surface expression of Mamu molecules encoded by several *Mamu-B* alleles was weak at the similar expression level to that of *Mamu-A4* (Ronser et al. 2010), there might be a group of minor *Mamu-B*, indicating that further analyses will be required to decipher the complexity of *Mamu-B* locus.

It is worth noting that we observed a link between *B*001:01* and *B*007:02* in four different haplotypes (Table 4). It was reported that *B*001:01* and *B*007* were common in Indian- and Chinese-origin macaques and that a haplotype including these alleles, *Mamu-B*001*, *B*07*, and *B*030:02*, was frequently found in both populations (Otting et al. 2008). However, that *Mamu-B*030:02* or related allele was not found in Burmese-origin macaques suggested that the distance between *Mamu-B*001* and *B*07* was closer than that to *Mamu-B*030:02*.

In this study, we sequenced 30–90 clones for each locus obtained from each macaque. As has been described (Karl et al. 2008; Otting et al. 2007, 2004), picking up from 16 to 88 clones was enough to detect major *Mamu* class I alleles, for example, *Mamu-A1* alleles. Therefore, we hoped to isolate the major *Mamu-A1* alleles from all individuals in this study. On the other hand, there were only nine out of 21 haplotypes carrying a *Mamu-A2* allele in this study, although Bassinger et al. (2008) reported that 75% of Chinese-origin macaques carried at least one *Mamu-A2* allele. We could not exclude a possibility that our cDNA cloning strategy might be insufficient to detect *Mamu-A* genes with low expression, such as *Mamu-A2*. Alternatively, *Mamu-A* haplotypes not carrying *Mamu-A2* might be prevalent in Burmese-origin macaques. In addition, the number of *Mamu-I* alleles detected in this study was much less than that of *Mamu-B* alleles, which is consistent with the results in a previous report (Urvater et al. 2000).

In conclusion, we characterized the diversity of *Mamu* class I genes in the Burmese macaques, which showed, only in part, a similarity to Chinese- and Indian-origin macaques. Because the *Mamu-A1* gene is responsible for exerting the classical antigen presentation function (Chu et al. 2007; Sidney et al. 2000), characterization of the *Mamu-A* and *Mamu-B* alleles in Burmese-origin macaques will provide us with essential information in designing the vaccination experiments against SIV.

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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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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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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₋

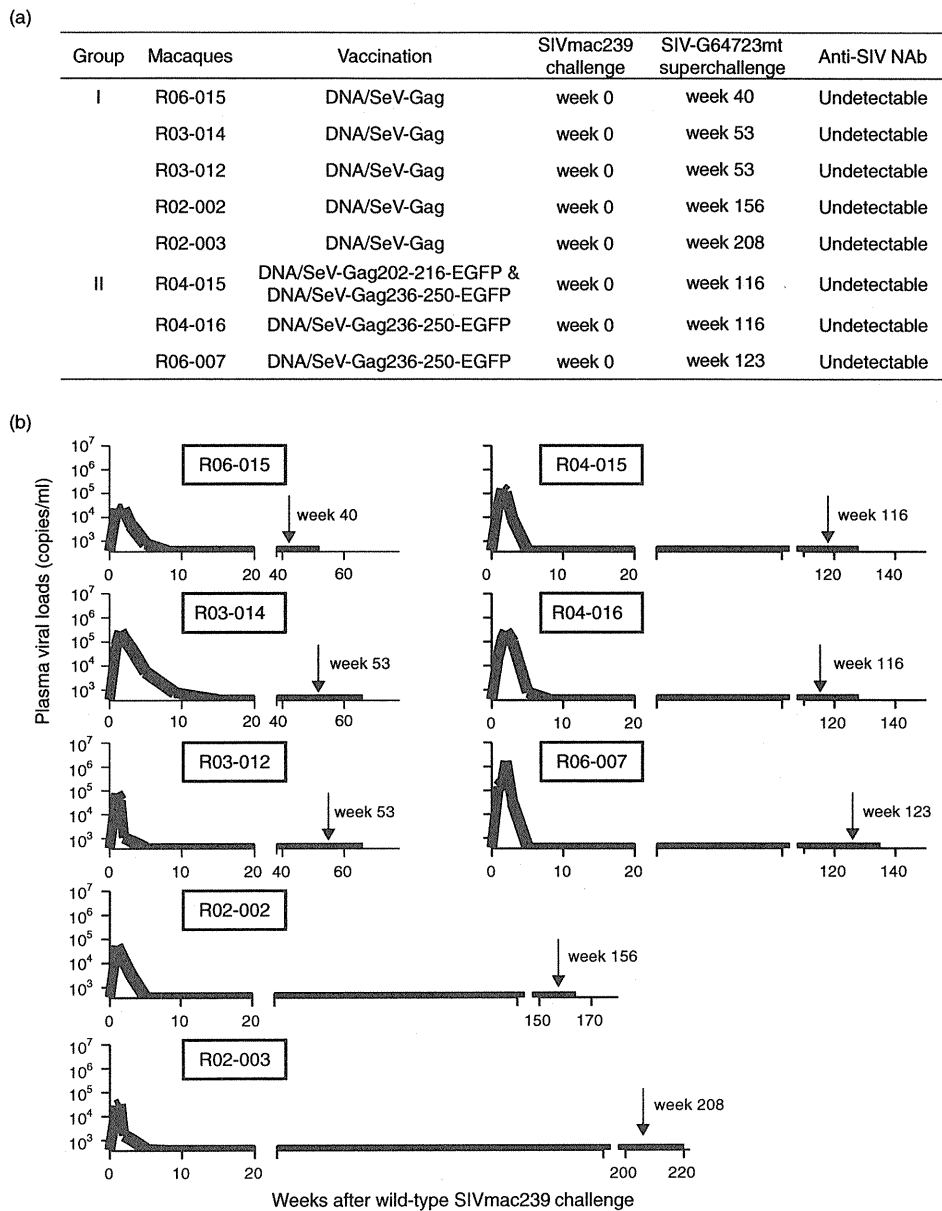


Fig. 1. Plasma viral loads in simian immunodeficiency virus controllers. (a) The list of rhesus macaques used in this study. All are *90-120-Ia*-positive. SIVmac239-specific neutralizing antibody (anti-SIV NAb) responses just before the mutant SIV superchallenge were undetectable. (b) Plasma viral loads (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 TGTTCTCTGTTTCCACCACTA) and probes (GCATTCACGCA GAAGAGAAAGTGAAACA-Flu and LCRed-ACTGAGGAAGCAAAAACAGATAGTGCAGAGA) (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 CytotfixCytoperm 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*

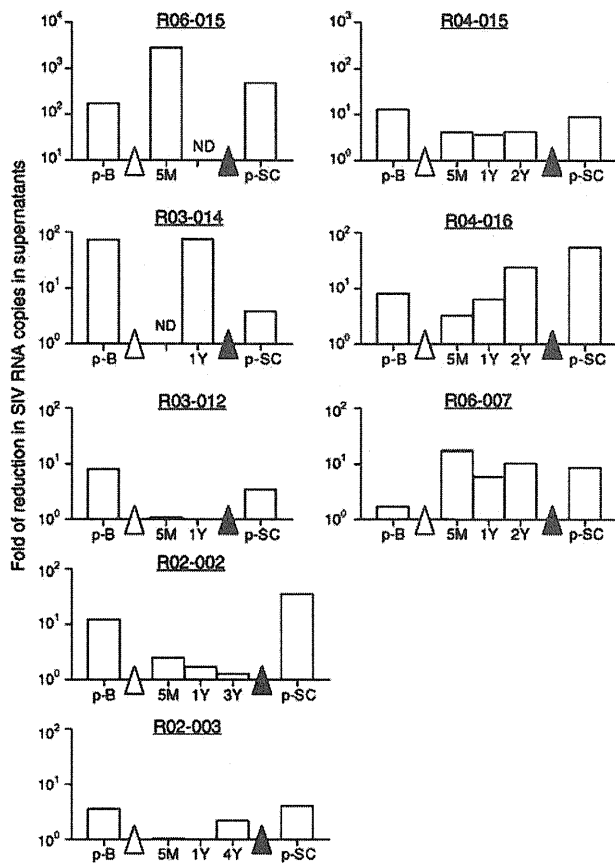


Fig. 2. Anti-SIVmac239 efficacy *in vitro* of CD8⁺ cells in simian immunodeficiency virus controllers. PBMC-derived CD8⁻ (target) cells infected with SIVmac239 were cultured alone or cocultured with autologous PBMC-derived CD8⁺ (effector) cells at several time points at an *E*:*T* ratio of 1:4. The ratios 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

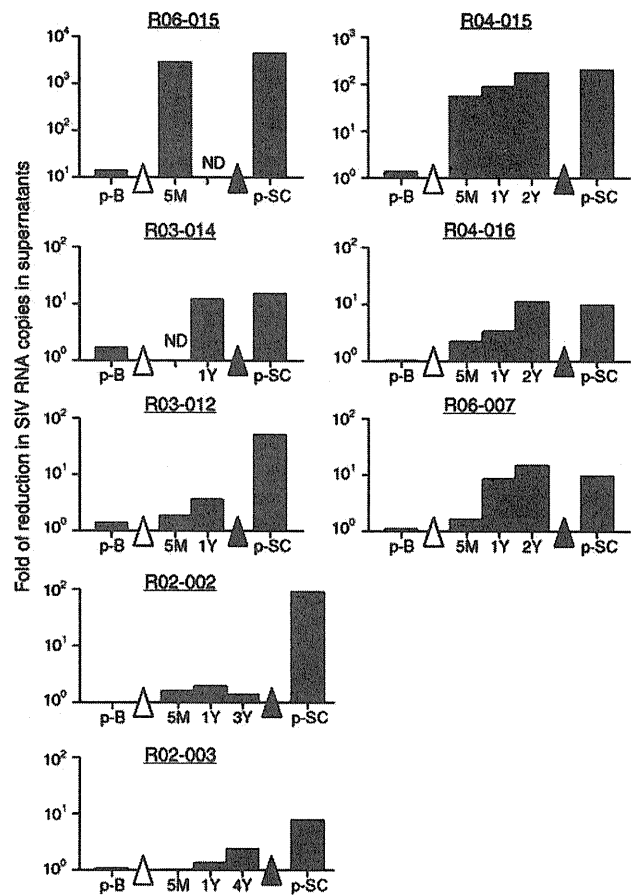


Fig. 3. Anti-SIV-G64723mt efficacy *in vitro* of CD8⁺ cells in simian immunodeficiency virus controllers. PBMC-derived CD8⁻ cells infected with SIV-G64723mt were cultured alone or cocultured with autologous PBMC-derived CD8⁺ cells at several time points at an *E*:*T* ratio of 1:4. The ratios of viral 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