Ⅱ．　研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Naruse TK, Chen Z, Yanagida R, Yamashita T, Saito Y, Mori K, Akari H, Yasutomi Y, Miyazawa M, Matano T, Kimura A.	Diversity of MHC class I genes in Burmese-origin rhesus macaques.	Immunogenetics	62	601-611	2010
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Ⅲ.　研究成果の刊行物・別刷

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ORIGINAL PAPER

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

Taeko K. Naruse · Zhiyong Chen · Risa Yanagida · Tomoko Yamashita · Yusuke Saito · Kazuyasu Mori · Hirofumi Akari · Yasuhiro Yasutomi · Masaaki Miyazawa · Tetsuro Matano · Akinori Kimura

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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.

Keywords Rhesus macaque · MHC · *Mamu-A* · *Mamu-B* · *Mamu-I* · Haplotype

T. K. Naruse · Z. Chen · T. Yamashita · A. Kimura (✉)
Department of Molecular Pathogenesis,
Medical Research Institute, Tokyo Medical and Dental University,
1-5-45 Yushima, Bunkyo-ku,
Tokyo 113-8510, Japan
e-mail: akitis@mri.tmd.ac.jp

R. Yanagida · Y. Saito · A. Kimura
Laboratory of Genome Diversity,
Graduate School of Biomedical Science,
Tokyo Medical and Dental University,
Tokyo 113-8510, Japan

K. Mori
AIDS Research Center, National Institute of Infectious Diseases,
Tokyo 162-8640, Japan

H. Akari
Primate Research Institute, Kyoto University,
Inuyama 484-8506, Japan

Y. Yasutomi
Tsukuba Primate Research Center,
National Institute of Biomedical Innovation,
Ibaraki 305-0843, Japan

M. Miyazawa
Department of Immunology,
Kinki University School of Medicine,
Osaka-Sayama 589-8511, Japan

T. Matano
International Research Center for Infectious Diseases,
The Institute of Medical Science, The University of Tokyo,
Tokyo 108-8639, Japan

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 $Gag_{241\text{-}249}$ 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 RNAiso 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′-GGACTCAGAAT CTCCCCAGACGCCGAG) and 3'MHC_UTR_A (5′-CAG GAACAYAGACACATTCAGG) for *Mamu-A* locus and 5'MHC_UTR and 3'MHC_UTR_B (5'-GTCTCTCCAC CTCCTCAC) 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 *Mafa* or *Mane* 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	*Mafa-A1*004:02*
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	*Mafa-A1*052:02*
A1	A1*056:02		AB477384	C	6	
A1	A1*056:02:02	Novel	AB444935		3	
A1	A1*065:01		AB444921	C	6	*Mafa-A1*065:04*
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	*Mafa-A1*096:01*
A1	A1*108:01	Novel	AB444925		1	
A1	A1*109:01	Novel	AB444902		7	*Mafa-A1*097:01*
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 *Mafa* or *Mane* 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	*Mane-A2*05:18*
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	*Mane-A2*05:03:01*
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 Gentetyx 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*

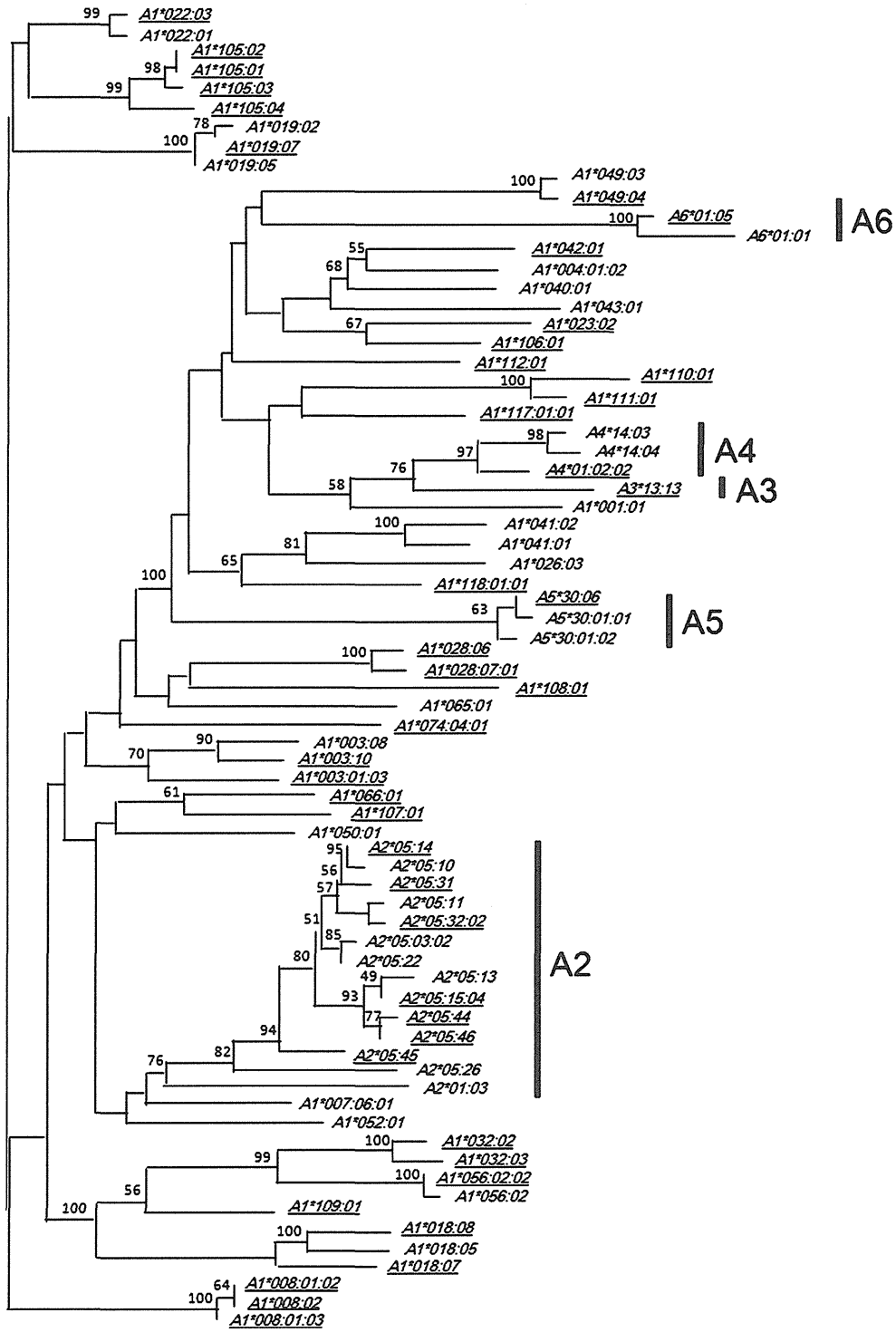

Fig. 1 Phylogenetic tree of *Mamu-A* alleles detected in this study. The tree was constructed using neighbor-joining method with bootstrap values of 5,000 replications. The values are indicated as percentages and those values less than 50% are not shown. *Mamu-A1* 001:01* was included in the analysis 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 *Mafa* or *Mane* 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	*Mafa-B*105:01*
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	*Mafa-B*050:01*
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	*Mafa-B*012:01*
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	*Mane-B*061:01*
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 *Mafa* or *Mane* 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	*Mafa-B*110:01*
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	*Mane-B*105:01*
B	B*124:01:01	Novel	AB540203		10	*Mane-B*124:01*
B	B*142:01:01	Novel	AB542050		1	*Mafa-B*023:02*
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	*Mafa-I*01:11*
I	I*01:06:08	Novel	AB477416		12	
I	I*01:06:09	Novel	AB541976		3	*Mane-I*01:01:02*
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 *Mafa* or *Mane* 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

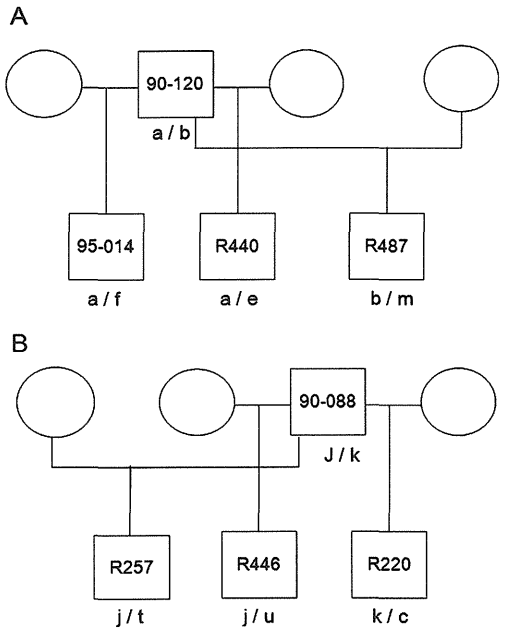

Fig. 2 Segregation of *Mamu* class I haplotypes in the pedigrees of macaques. Pedigree information and haplotype information are indicated along with ID of macaques. A. *Mamu* class I haplotypes of *a* and *b* in the parent (90-120) were segregated to its offspring 95-014, R440, and R487. B. *Mamu* class 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 $Gag_{241-249}$ (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 $Gag_{241-249}$ epitope is overlapped with a HLA-B*5701-restricted HIV-1 $Gag_{240-249}$ epitope, TW10 (TSTLQEQIAW), 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-orign 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

Nami Iwamoto[a], Tetsuo Tsukamoto[a], Miki Kawada[a], Akiko Takeda[a], Hiroyuki Yamamoto[a], Hiroaki Takeuchi[a] and Tetsuro Matano[a,b]

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-Ia*, 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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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.

[a]International Research Center for Infectious Diseases, The Institute of Medical Science, The University of Tokyo, and [b]AIDS Research Center, National Institute of Infectious Diseases, Tokyo, Japan.

Correspondence to Tetsuro Matano, International Research Center for Infectious Diseases, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan.

Tel: +81 3 6409 2078; fax: +81 3 6409 2076; e-mail: matano@ims.u-tokyo.ac.jp

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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 $Gag_{206-216}$ (IINEEAADWDL) epitope-specific and $Gag_{241-249}$ (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 $Gag_{206-216}$-specific and $Gag_{241-249}$-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 $Gag_{241-249}$ epitope fused with enhanced green fluorescent protein (EGFP), this single epitope vaccination resulted in control of SIVmac239 replication with dominant induction of $Gag_{241-249}$-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 (SIVmac239Gag216S244E247L 312V373T) 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), GagA312 V (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 SIV-mac239-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 $Gag_{241-249}$ epitope-specific CTL responses (referred to as DNA/SeV-$Gag_{236-250}$-EGFP vaccine) and contained SIVmac239 challenge as reported previously [25]. In this vaccine protocol, animals were primed with 5 mg of $pGag_{236-250}$-EGFP-N1 DNA expressing a $Gag_{236-250}$-EGFP fusion protein, followed by a boost with 6×10^9 CIU of F-deleted SeV expressing the $Gag_{236-250}$-EGFP fusion protein (SeV-$Gag_{236-250}$-EGFP). Macaque R04-015 in group II received a prophylactic prime-boost vaccine eliciting $Gag_{206-216}$ epitope-specific and $Gag_{241-249}$ epitope-specific CTL responses (referred to as DNA/SeV-$Gag_{202-216}$-EGFP and DNA/SeV-$Gag_{236-250}$-EGFP vaccine); this animal was primed with $pGag_{202-216}$-EGFP-N1 and $pGag_{236-}$

(a)

Group	Macaques	Vaccination	SIVmac239 challenge	SIV-G64723mt superchallenge	Anti-SIV NAb
I	R06-015	DNA/SeV-Gag	week 0	week 40	Undetectable
	R03-014	DNA/SeV-Gag	week 0	week 53	Undetectable
	R03-012	DNA/SeV-Gag	week 0	week 53	Undetectable
	R02-002	DNA/SeV-Gag	week 0	week 156	Undetectable
	R02-003	DNA/SeV-Gag	week 0	week 208	Undetectable
II	R04-015	DNA/SeV-Gag202-216-EGFP & DNA/SeV-Gag236-250-EGFP	week 0	week 116	Undetectable
	R04-016	DNA/SeV-Gag236-250-EGFP	week 0	week 116	Undetectable
	R06-007	DNA/SeV-Gag236-250-EGFP	week 0	week 123	Undetectable

(b)

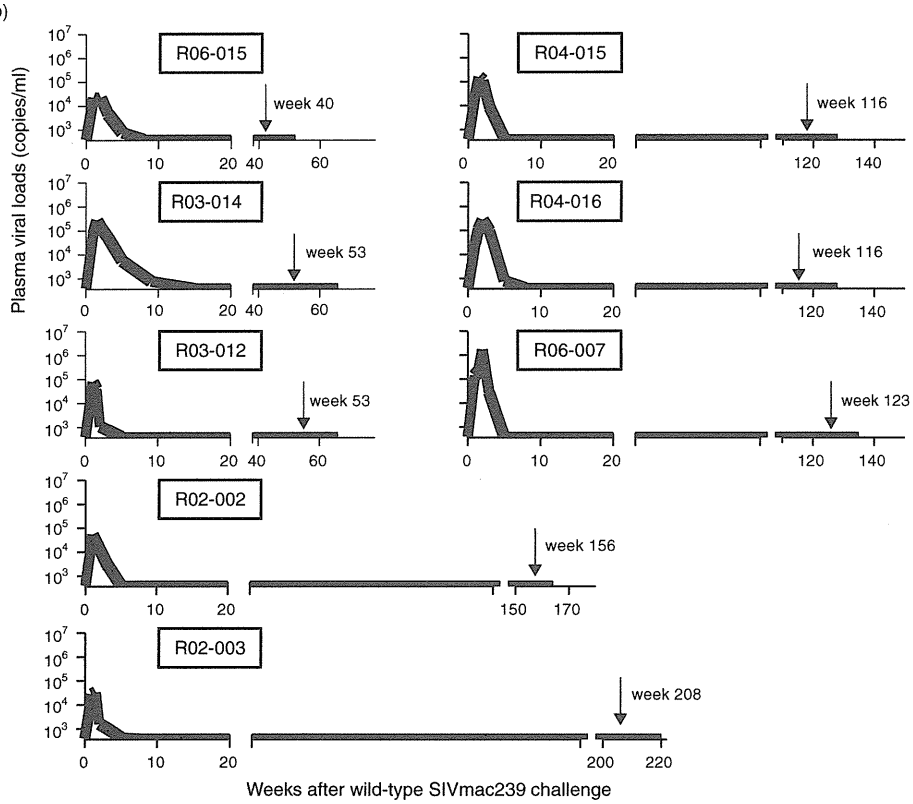

Fig. 1. Plasma viral loads in simian immunodeficiency virus controllers. (a) The list of rhesus macaques used in this study. All are *90-120-Ia*-positive. SIVmac239-specific neutralizing antibody (anti-SIV NAb) responses just before the mutant SIV superchallenge were undetectable. (b) Plasma viral loads (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-$Gag_{202-216}$-EGFP and SeV-$Gag_{236-250}$-EGFP. Both p$Gag_{202-216}$-EGFP-N1 and SeV-$Gag_{202-216}$-EGFP express a $Gag_{202-216}$-EGFP fusion protein [33]. These vaccinated animals were challenged intravenously with 1000 50% tissue culture infective doses ($TCID_{50}$) of SIVmac239 [34] approximately 3 months after the boost and were superchallenged intravenously with 1000 $TCID_{50}$ 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 $1:10^3$ $TCID_{50}$/cell or with SIV-G64723mt at MOI of $1:10^2$ $TCID_{50}$/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 TGTTCCTG 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 $Gag_{206-216}$, $Gag_{241-249}$, or $Gag_{367-381}$ peptides for $Gag_{206-216}$-specific, $Gag_{241-249}$-specific, or $Gag_{367-381}$-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 frequencies 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 $TCID_{50}$ virus replication as described previously [26]. Serial two-fold dilutions of heat-inactivated plasma were prepared in quadruplicate and mixed with 10 $TCID_{50}$ of SIVmac239. In each culture, 5 μl of virus was incubated with 5 μl of plasma for 45 min and was added to 5×10^4 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-$Gag_{202-216}$-EGFP and DNA/SeV-$Gag_{236-250}$-EGFP and two (R04-016 and R06-007) vaccinated with DNA/SeV-$Gag_{236-250}$-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*