antigens. There are two classes of MHC molecules: one is the MHC class I molecule presenting peptides of intracellular origin to $CD8^+$ T cell and the other is the MHC class II molecule binding extracellular-derived antigenic peptides for presenting to $CD4^+$ T cell. It has been reported that the complexity of MHC genes in the rhesus and cynomolgus macaques is higher than that in humans (Kulski et al. 2004; Watanabe et al. 2006; Gibbs et al. 2007; Otting et al. 2007, 2008; Doxiadis et al. 2011). For example, *MHC class I* configurations in macaques are usually composed of one copy of highly transcribed major *MHC-A1*gene (*Mamu-A1*or *Mafa-A1*) and several other minor *MHC-A* genes (*Mamu-A2~A7* or *Mafa-A2~A6*) in addition to several *MHC-B* genes (*Mamu-B* or *Mafa-B*) (Watanabe et al. 2006; Otting et al. 2007, 2008, 2009; Naruse et al. 2010; Doxiadis et al. 2011), whereas each one copy of *MHC-A* and *-B* genes (*HLA-A* and *-B*) can be found in human *MHC class I* locus. In addition, other *MHC* loci showing lower expression levels, i.e., *HLA-B*-like gene (*Mamu-I* or *Mafa-I*) and *HLA-G*-like non-classical gene (*Mamu-AG* or *Mafa-AG*) have been identified (Slukvin et al. 2000; Urvater et al. 2000). The extent of genetic diversity is different, in part, depending on the geographic areas, as we have previously reported for *MHC class I* genes in rhesus macaque (Naruse et al. 2010). As for the cynomolgus macaques, *MHC class I* allelic diversity was reported for Indonesian (Pendley et al. 2008; Wu et al. 2008; Kita et al. 2009; Otting et al. 2009), Malaysian (Otting et al. 2009; Aarnink et al. 2011b), Mauritian (Budde et al. 2010), Vietnamese (Wu et al. 2008; Kita et al. 2009), and Philippino (Campbell et al. 2009; Kita et al. 2009) macaques, but information about the *MHC class I* haplotype remains insufficient.

In the present study, we have analyzed *MHC class I* loci in cynomolgus macaques originated from Indonesia, Malaysia, and the Philippines to obtain information on haplotype configuration. We report here further the complex nature of *MHC class I* loci in the Old World monkey, i.e., the presence of unique haplotypes carrying two *Mafa-A1* genes.

Materials and methods

Animals

A total of 26 cynomolgus macaques from five families were the subjects. Each family was composed of one or two males with one or two females and their offspring. They were maintained in the breeding colonies in Tsukuba Primate Research Center, National Institute of Biomedical Innovation, Japan. The founders of the colonies were captured in Indonesia, Malaysia, and the Philippines. All care including blood sampling of animals were in accordance with the Guidelines for the Care and Use of Laboratory Animals published by the National Institute of Health (NIH Publication 85–23, revised 1985) and were subjected to prior approval by the local animal protection authority.

Sequencing analysis of cDNAs from *Mafa* class I genes

Total cellular RNA was extracted from whole blood by using RNAeasy (QIAGEN, Gmbh, Germany). Oligo(dT)-primed cDNA was synthesized using Transcriptor reverse transcriptase (Roche, Mannheim, Germany) according to the manufacturer's recommendations. Full-length cDNAs

Table 1 Primers used in PCR or sequencing of *Mafa* class I genes

Primer ID	Application	Direction	Sequence (5′–3′)	Position	Reference
5′ MHC_UTR	PCR	Sense	GGACTCAGAATCTCCCCAGACGCCGAG	5′ UTR	Karl et al. 2008
3′ MHC_UTR_A	PCR	Antisense	CAGGAACAYAGACACATTCAGG	3′ UTR	Karl et al. 2008
3′ MHC_UTR_B	PCR	Antisense	GTCTCTCCACCTCCTCAC	3′ UTR	Karl et al. 2008
5A long	PCR	Sense	ATGGCGCCCCGAACCCTCCTCCTG	Exon 1	Tanaka-Takahashi et al. 2007
3A	PCR	Antisense	TCACACTTTCAAGCCGTGAGAGA	Exon 7	Tanaka-Takahashi et al. 2007
5ASSP	PCR	Sense	ATGGCGCCCCGAACCCTCCTCCTGG	Exon 1	Tanaka-Takahashi et al. 2007
4R	PCR	Antisense	CCAGGTCAGTGTGATCTCCG	Exon 4	Tanaka-Takahashi et al. 2007
P000044	PCR	Sense	GATTCTCCGCAGACGCCCA	5′ UTR	Wu et al. 2008
P000023	PCR	Antisense	GGAGAACCAGGCCAGCAAT	Exon 5	Wu et al. 2008
P000076	Sequencing	Sense	GAGCAGCGACGGGACCGCA	Intron 1	Wu et al. 2008
P000060	Sequencing	Antisense	CCTGGGGCTCTCCCGGGTCA	Intron 2	Wu et al. 2008
P000096	Sequencing	Sense	TGTACTGAGTCTCCCTGATGG	Intron 2	Wu et al. 2008
P000098	Sequencing	Antisense	TTCATTCCCTCAGAGATTTT	Intron 3	Wu et al. 2008
P000055	Sequencing	Sense	CCCAGGTRCCTSTGTCCAGGA	Intron 3	Wu et al. 2008
P000281	Sequencing	Antisense	AGAGGGGAAAGTGAGGGGT	Intron 4	Wu et al. 2008

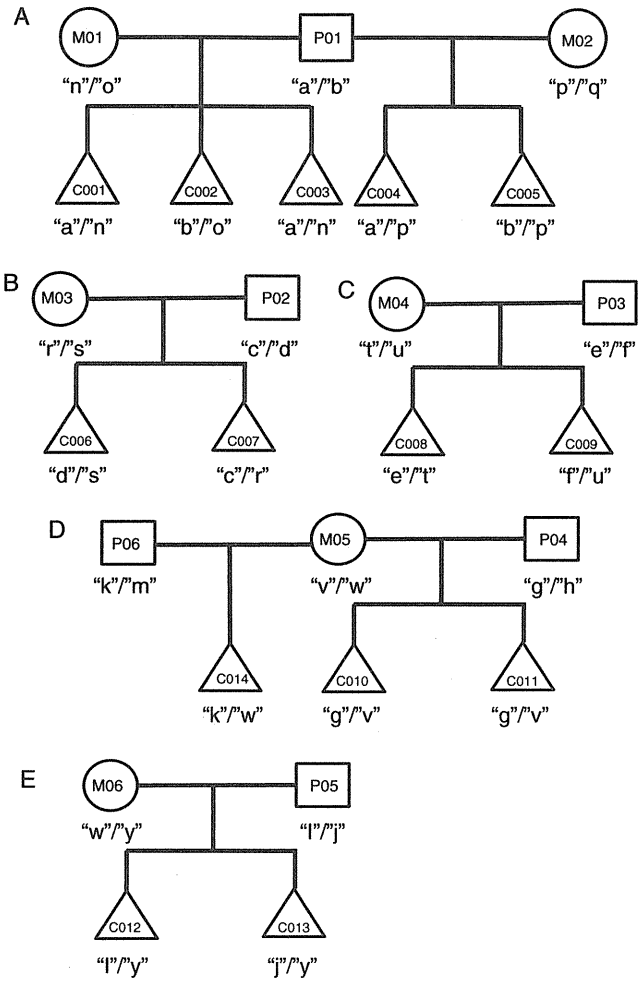

Fig. 1 Pedigree of cynomolgus macaques. The pedigrees of macaques analyzed in this study are shown. Founders were originated from Indonesia (**a**), Malaysia (**b**, **c**), and Philippines (**d**, **e**). *Open square*, *open circle*, and *open triangles* indicate father, mother, and offspring, respectively. The ID of each subject is noted in the symbol. *Mafa class I* haplotypes determined in this study are indicated under the subjects

for *Mafa* class I genes were amplified by polymerase chain reaction (PCR), as described previously (Tanaka-Takahashi et al. 2007; Naruse et al. 2010), by using locus-specific primer pairs as reported by Karl et al. (2008). Genomic gene and cDNA for *Mafa-A2* gene were analyzed according to the method described by Wu et al. (2008). The primers used in this study are listed in Table 1. To estimate the expression level of *Mafa-A* alleles, we also used an additional primer pair: MafaF (5′-TACGTGGACGA CACGCAGTT) and MafaR (5′-GGTGGGTCA CATGTGTCTTG). PCR was done under the condition of initial denaturation at 98°C for 10 s, 25 cycles of 98°C for 1 s, 64°C for 5 s, and 72°C for 20 s, followed by an additional extension at 72°C for 1 min, using Phusion Flash DNA polymerase (Finzymes, Espoo, Finland). The PCR products were cloned into pSTBlue-1 Perfectly Blunt vector (Novagen, WI, USA) according to the manufacturer's instructions and were transformed to NovaBlue Giga Singles™ competent cells (Merck Biosciences Japan, Tokyo, Japan). A total of 30 to 90 independent cDNA clones were obtained from each macaque for each locus and were sequenced on both strands by BigDye Terminator cycling system in an ABI 3730 automated sequence analyzer (Applied Biosystems, CA, USA).

Data analyses and nomenclature for *Mafa* class I allele

Nucleotide sequences of cDNA clones were aligned using the Genetyx software package (version 8.0, Genetyx Corp., Japan). When a cDNA sequence, which was represented by at least three clones, was independently obtained from at least two animals or repeatedly obtained from at least two independently prepared cDNAs from single animals, we considered it a real allele, not an artifact, and the sequences were submitted to the DNA Data Bank of Japan (DDBJ) database and to the Immuno Polymorphism Database for non-human primate MHC (http://www.ebi.ac.uk/ipd/mhc/sumit.html; Robinson et al. 2003) to obtain official nomenclature for the novel alleles of *Mafa-A* and *Mafa-B* genes. Neighbor-joining trees were constructed with Kimura's two-parameter method for a phylogenetic analysis of *Mafa-A* sequences spanning exons 2, 3, and a part of exon 4 obtained in this study by using the Genetyx software. Bootstrap values were based on 5,000 replications.

Table 2 *Mafa* class I alleles found in the cynomolgus macaques

Locus	Number of observed alleles	Number of novel alleles (%)	Number of observed alleles in macaques from different regions[a]		
			Indonesian	Malaysian	Philippino
Mafa-A	32	14 (43.7%)	9 (3), 33.3%	12 (8), 66.7%	11 (3), 27.3%
Mafa-B	46	20 (43.5%)	13 (5), 38.5%	20 (15), 75.0%	18 (1), 5.6%
Mafa-I	6	3 (50.0%)	2 (1), 50.0%	4 (3), 75.0%	2 (0), 0%
Mafa-AG	3	3 (100%)	0 (0), 0%	2 (2), 100%	1 (1), 100%
Total	87	40 (45.5%)	24 (9), 37.5%	38 (28), 73.7%	32 (5), 15.6%

[a] The number and frequency of novel alleles are indicated in parentheses

Results

Identification of *Mafa* class I alleles in cynomolgus macaques

We determined the nucleotide sequences of cDNA clones for *Mafa-A* and *-B* loci in 26 cynomolgus macaques from one family of Indonesian origin (six haplotypes), two families of Malaysian origin (eight haplotypes), and two families of Philippino origin (nine haplotypes) (Fig. 1). When the observed alleles were segregated in the family or when at least three clones with identical sequences were observed from two independent PCR for an individual, the nucleotide sequences were considered to be real and not artifacts. As shown in Table 2, 32 *Mafa-A*, 46 *Mafa-B*, 6 *Mafa-I*, and 3 *Mafa-AG* sequences were obtained in this study. Among them, 14 (43.7%), 20 (43.5%), 3 (50.0%), and 3 (100%) were novel alleles of *Mafa-A*, *Mafa-B*, *Mafa-I*, and *Mafa-AG* loci, respectively (Table 2).

Table 3 Alleles of *Mafa-A* locus identified in the cynomolgus macaques

Locus	Allele name	Novelty[a]	Accession number[b]	Origin[c]	Identical *Mamu* and/or *Mane* alleles[d]	Origin and reference of known alleles[e]
A1	*A1*001:01*		AM295828	Malaysian		Utrecht, Otting et al. 2007
A1	*A1*002:01:02*	Novel	AB569214	Indonesian		
A1	*A1*008:02*		EU392108	Philippino		Philippino, Campbell et al. 2009
A1	*A1*008:03-like*	Novel	AB647187	Philippino		
A1	*A1*018:06*		FM246489	Indonesian		Utrecht, Otting et al. 2007
A1	*A1*019:05*		AB447616	Indonesian		Indonesian, Kita et al. 2009
A1	*A1*023:01*	Novel	AB569216	Malaysian		
A1	*A1*032:05*	Novel	AB569215	Malaysian		
A1	*A1*052:02*		EU392105	Philippino	*Mamu-A1*052:01/03/06*	Philippino, Campbell et al. 2009
A1	*A1*054:01*		AB154771	Malaysian		Tsukuba, Uda et al. 2004
A1	*A1*056:02*	Novel	AB569218	Malaysian		
A1	*A1*062:05*	Novel	AB569219	Malaysian		
A1	*A1*068:02*	Novel	AB569217	Malaysian		
A1	*A1*074:02*		AB447606	Philippino		Philippino, Kita et al. 2009
A1	*A1*079:01*		AB154773	Malaysian		Tsukuba, Uda et al. 2004
A1	*A1*089:02*		EU392104	Philippino		Philippino, Campbell et al. 2009
A1	*A1*093:01*		EU392103	Philippino		Philippino, Campbell et al. 2009
A1	*A1*094:01*		EU392111	Philippino		Philippino, Campbell et al. 2009
A1	*A1*097:01*		AB447576	Indonesian	*Mamu-A1*109:01*	Indonesian, Kita et al. 2009
A1	*A1*103:01*	Novel	AB583236	Indonesian		
A1	*A1*124:01*	Novel	AB583237	Malaysian		
A2	*A2*05:13-like*	Novel	AB647189	Philippino		
A2	*A2*05:16*		AM295878	Indonesian		Utrecht, Otting et al. 2007
A2	*A2*05:34-like*	Novel	AB647190	Philippino		
A3	*A3*13:03*		EU392112	Philippino		Philippino, Campbell et al. 2009
A3	*A3*13:15*	Novel	AB583238	Malaysian		
A3	*A3*13:16*	Novel	AB583240	Indonesian		
A4	*A4*14:01*		AM295880	Indonesian		Utrecht, Otting et al. 2007
A4	*A4*14:02*		AM295881	Malaysian		Utrecht, Otting et al. 2007
A6	*A6*01:05*	Novel	AB583239	Malaysian		

[a] New alleles are indicated as novel

[b] Nucleotide sequences were submitted to a public database and given accession numbers

[c] Origin of cynomolgus macaques

[d] Identical sequences were found in *Mamu* or *Mane* alleles

[e] Origin and references in which each known allele was first reported. Utrecht and Tsukuba indicate that the alleles were found in colonies maintained in the University of Utrecht, The Netherlands, and Tsukuba primate center, Japan, respectively

Table 4 Alleles of *Mafa-B* locus identified in the cynomolgus macaques

Allele name[a]	Novelty[b]	Accession number	Origin[c]	Identical to *Mamu* and/or *Mane* alleles[d]	Origin and reference of known alleles[e]
*B*002:03*	Novel	AB569224	Indonesian, Malaysian		
*B*004:01*		EU203722	Indonesian		Indonesian, Pendley et al. 2008
*B*007:01:01*		AY958137	Philippino	*Mamu-B*007:02/03*	Mauritian, Krebs et al. 2005
*B*007:01:02*		EU392135	Philippino		Philippino, Campbell et al. 2009
*B*007:01:03*	Novel	AB569223	Indonesian		
*B*007:03*		FM212802	Philippino		Indonesian or Malaysian, Otting et al. 2009
*B*011:02*	Novel	AB569229	Malaysian		
*B*013:08*		EU392114	Indonesian, Philippino		Philippino, Campbell et al. 2009
*B*017:01*		EU392119	Philippino		Philippino, Campbell et al. 2009
*B*018:01*		AY958138	Indonesian	*Mamu-B*018:01*	Mauritian, Krebs et al. 2005
*B*030:02*		AY958134	Malaysian	*Mamu-B*030:03:01*	Mauritian, Krebs et al. 2005
*B*032:01*	Novel	AB569237	Malaysian		
*B*033:02*		EU392118	Philippino		Philippino, Campbell et al. 2009
*B*043:01*	Novel	AB569230	Malaysian	*Mamu-B*043:01*	
*B*056:01*		AY958131	Indonesian	*Mamu-B*056:01*	Mauritian, Krebs et al. 2005
*B*056:02*		EU392128	Philippino		Philippino, Campbell et al. 2009
*B*057:03*	Novel	AB569231	Malaysian	*Mamu-B*057:06*	
*B*060:04*	Novel	AB569226	Indonesian		
*B*061:01*		AB195445	Malaysian	*Mamu-B*061:04:01, Mane-B*061:01*	Tsukuba, Uda et al. 2005
*B*061:02*	Novel	AB569233	Malaysian		
*B*064:02*		FM212804	Philippino		Indonesian or Malaysian, Otting et al. 2009
*B*068:04*	Novel	AB569236	Malaysian	*Mamu-B*068:04 , Mane-B*nov078*	
*B*069:02*		FM212842	Malaysian		Indonesian or Malaysian, Otting et al. 2009
*B*074:01:02-like*	Novel	AB647188	Philippino		
*B*074:02*	Novel	AB569228	Malaysian	*Mamu-B*074:01 /02*	
*B*076:04*	Novel	AB569232	Malaysian		
*B*081:01*	Novel	AB569225	Indonesian		
*B*089:01:01*		EU392131/ FJ178820	Philippino		Philippino, Campbell et al. 2009
*B*089:01:02*		EU392125	Indonesian, Malaysian, Philippino	*Mamu-B*089:01, Mane-B*089:02*	Philippino, Campbell et al. 2009
*B*090:01*		AB195436	Malaysian		Tsukuba, Uda et al. 2005
*B*091:01*	Novel	AB569240	Malaysian	*Mamu-B*091:02*	
*B*092:01:01*	Novel	AB569227	Malaysian	*Mamu-B*092:02*	
*B*095:01*		EU392113/ AY958148	Philippino		Mauritian, Krebs et al. 2005 Philippino, Campbell et al. 2009
*B*104:03*		EU392126	Philippino		Indonesian, Pendley et al. 2008 Philippino, Campbell et al. 2009
*B*116:01*		EU392123	Philippino		Philippino, Campbell et al. 2009
*B*121:01*		AB195455	Indonesian		Tsukuba, Uda et al. 2005
*B*124:01:02*	Novel	AB569235	Malaysian		
*B*136:02*		EU203720	Indonesian,		Indonesian, Pendley et al. 2008
*B*137:03*		EU392117/ EU203723	Indonesian, Philippino		Indonesian, Pendley et al. 2008
*B*137:04*	Novel	AB569239	Malaysian		

Table 4 (continued)

Allele name[a]	Novelty[b]	Accession number	Origin[c]	Identical to *Mamu* and/or *Mane* alleles[d]	Origin and reference of known alleles[e]
*B*138:02*	Novel	AB569234	Malaysian		
*B*151:02:02*	Novel	AB569222	Indonesian		
*B*155:02*	Novel	AB569238	Malaysian		
*B*157:01*		EU392121	Philippino		Philippino, Campbell et al. 2009
*B*158:01*		EU392122	Philippino		Philippino, Campbell et al. 2009
*B*160:01*		EU606042	Philippino		-

[a] New alleles are indicated as novel

[b] Nucleotide sequences were submitted to a public database and given accession numbers

[c] Origin of cynomolgus macaques

[d] Identical sequences were found in *Mamu* or *Mane* alleles

[e] Origin and references in which each known allele was first reported. Utrecht and Tsukuba indicate that the alleles were found in colonies maintained in the University of Utrecht, The Netherlands, and Tsukuba primate center, Japan, respectively

The *Mafa-A* alleles found in this study are listed in Table 3, where 21 alleles were from the major *Mafa-A1* locus, while the remaining 11 alleles were from the minor *Mafa-A* loci, 3 from *Mafa-A2*, 3 from *Mafa-A3*, 2 from *Mafa-A4*, and 1 from *Mafa-A6* alleles (Table 3). The major *Mafa-A1*alleles were defined by the sequence similarity to the known *Mafa-A1* alleles to be given official nomenclatures by IPD, except for *Mafa-A1*008:03*-like allele, and we confirmed that the frequencies of cDNA clones for *Mafa-A1* alleles were over 10% in each macaque. Similarly, alleles of minor *Mafa-A* genes, *Mafa-A2*, *-A3*, *-A4*, and *-A6* were defined by sequence similarity to the known alleles. They, except for two novel *Mafa-A2* alleles, were also given official names by IPD. On the other hand, a total of 46 *Mafa-B* alleles (Table 4) as well as 6 *Mafa-I* and 3 *Mafa-AG* alleles (Table 5) were identified. It was found that 2 out of

Table 5 Alleles of *Mafa-AG* and *Mafa-I* locus identified in the cynomolgus macaques

Locus	Allele name[a]	Novelty[b]	Accession number[c]	Origin[d]	Identical to *Mamu* and/or *Mane* alleles[e]	Origin and reference of known alleles[e]
AG	*AG*04:03*	Novel	AB569221	Malaysian		
AG	*AG1 like-1*	Novel	AB569220	Malaysian		
AG	*AG1 like-3*	Novel	AB583241	Philippino		
I	*I*01:01:01*		EU392139	Philippino		Philippino, Campbell et al. 2009
I	*I*01:09/01:08*		AB195465/AB195464	Indonesian, Malaysian		Tsukuba, Uda et al. 2005
I	*I*01:15*		FM246493	Philippino	*Mamu-I*01:06* , *Mamu-I*01:08:01*	Indonesian or Malaysian, Otting et al. 2009
I	*I*01:15 like-1*	Novel	AB569241	Indonesian, Malaysian		
I	*I*01:15 like-2*	Novel	AB569242	Malaysian	*Mamu-I*03:01:01*, *Mamu-I*01:07:01* , *Mamu-I*01:06:05*	
I	*I*01:18 like*	Novel	AB569243	Malaysian		

[a] Official allele names were not obtained for *AG1 like-1*, *AG1 like-3*, *I*01:15 like-1*, *I*01:15 like-2*, and *I*01:18 like* due to the limited sequence information

[b] New alleles are indicated as novel

[c] Nucleotide sequences were submitted to a public database and given accession numbers

[d] Origin of cynomolgus macaques

[e] Identical sequences were found in *Mamu* or *Mane* alleles

[f] Origin and references in which each known allele was reported. Tsukuba indicates that the alleles were found in colonies maintained in the Tsukuba primate center, Japan

21 (9.5%) *Mafa-A1a* alleles and 12 out of 46 (26.1%) *Mafa-B* alleles had identical sequences to *Mamu-A1* and *Mamu-B* alleles, respectively, implying a genetic admixture of cynomolgus macaques with rhesus macaques during the evolution (Otting et al. 2007; Bonhomme et al. 2009; Otting et al. 2009). Because we determined the nucleotide sequences only for exons 2, 3, and 4, two novel *Mafa-AG* alleles and three novel *Mafa-I* alleles were not given official names. As for the geographic distribution of *Mafa* class I alleles, there was no overlapping of *Mafa-A* alleles originated from different regions (Table 3), while there were a few *Mafa-B* and *Mafa-I* alleles commonly observed in macaques from different regions (Tables 4 and 5, respectively). When we looked into the presence of novel alleles in the geographic distribution, most of the novel alleles were obtained from Malaysian macaques, while almost all of the alleles found in Philippino macaques were not novel (Table 2).

Mafa class I haplotypes identified in the family study

We could identify the *Mafa-A* and *Mafa-B* alleles composing 23 different haplotypes from the segregation studies (Table 6). It was found that one to three expressing *Mafa-A*

Table 6 *Mafa class I* haplotypes identified in the cynomolgus macaques

ID[a]	Origin[b]	Haplotype[c]	*Mafa-A1* (major)	*Mafa-A* (minor)	*Mafa-AG*	*Mafa-B* (major)	*Mafa-B* (minor)	*Mafa-I*
P01	Indonesian	"a"	A1*002:01:02	A3*13:16		B*136:02		I*01:09/01:08
		"b"	A1*103:01			B*007:01:03, B*121:01	B*151:02:02	
P02	Malaysian	"c"	A1*023:01			B*090:01	B*011:02, B*074:02	
		"d"	A1*068:02			B*043:01	B*030:02, B*057:03	I*01:15 like-2
P03	Malaysian	"e"	A1*001:01, A1*032:05		AG1 like-1	B*068:04, B*124:01:02	B*032:01, B*061:01, B*089:01:02	
		"f"	A1*079:01		AG*04:03	B*061:02, B*138:02	B*155:02	
P04	Philippino	"g"	A1*089:02	A2*05:13-like, A3*13:03		B*137:03		
		"h"	A1*008:02			B*104:03		
P05	Philippino	"i"	A1*094:01			B*007:01:02	B*160:01	
		"j"	A1*008:02		AG1 like-3	B*157:01	B*017:01, B*089:01:02, B*116:01	I*01:01:01, I*01:15
P06	Philippino	"k"	A1*08:03-like	A2*05:34-like		B*074:01:02-like		
		"m"	A1*089:02	A3*13:03		B*007:03, B*064:02	B*089:01:01	
M01	Indonesian	"n"	A1*018:06	A2*05:16, A4*14:01		B*002:03		I*01:15 like-1
		"o"	A1*097:01			B*056:01	B*089:01:02	
M02	Indonesian	"p"	A1*097:01			B*137:03	B*013:08	
		"q"	A1*019:05			B*018:01	B*004:01, B*060:04, B*081:01	
M03	Malaysian	"r"	A1*054:01			B*002:03		I*01:15 like-1
		"s"	A1*056:02	A4*14:02		B*076:04		I*01:18 like
M04	Malaysian	"t"	A1*062:05			B*069:02	B*137:04	
		"u"	A1*124:01	A3*13:15		B*091:01		
M05	Philippino	"v"	A1*074:02, A1*093:01			B*007:01:01, B*158:01		
		"w"	A1*093:01			B*007:01:02	B*160:01	
M06	Philippino	"w"	A1*093:01			B*007:01:02	B*160:01	
		"y"	A1*052:02			B*033:02, B*095:01		

[a] ID of founder animals as indicated in Fig. 1

[b] Origin of cynomolgus macaques

[c] Haplotypes were determined from studies of family as shown in Fig. 1

alleles and one to five expressing *Mafa-B* alleles consisted of *Mafa class I* haplotype, similar to the *Mamu class I* haplotypes in rhesus macaques (Naruse et al. 2010). Of particular interest was that there were two haplotypes, "e" (Malaysian founder P03) and "v" (Philippino founder M05), carrying two different *Mafa-A1* genes (Fig. 1; Table 6). Because previous studies have demonstrated that there is usually only one *Mafa-A1* allele on a chromosome (Otting et al. 2007), while the presence of two *Mamu-A1* alleles on the same haplotype was suggested in rhesus macaques (Naruse et al. 2010; Doxiadis et al. 2011), we performed further analyses.

The family studies showed that the *Mafa-A1*alleles consisting of haplotype "e", *Mafa-A1*001:01* and*Mafa-A1*032:05*, or haplotype "v", *Mafa-A1*074:02* and *Mafa-A1*093:01*, did not carry accompanying minor *Mafa-A* genes (Table 6). When we constructed a phylogenetic tree of *Mafa-A* alleles identified in this study (Fig. 2), it was found that *Mafa-A1*001:01* was mapped in the neighbor of *Mafa-A3*gene, raising a possibility that one of the two alleles on the same chromosome might be a minor *Mafa-A* allele and not the major *Mafa-A1* allele. To test the possibility, we investigate the expression level of *Mafa-A* alleles composing of haplotypes "e" and "v". For this purpose, other primer pairs were designed within the sequences completely shared by these alleles to amplify the *Mafa-A* cDNAs to avoid a possibility of affecting the efficacy of PCR by mismatches with the primer sequences. The cloning and sequencing analysis revealed that both *Mafa-A1*001:01* and *Mafa-A1*032:05* on the haplotype "e" were observed at similar frequencies among the cDNA clones of *Mafa-A* alleles in P03and C008 (Fig. 1): 29.7% and 33.3% in P03 and 22.5% and 17.5% in C008, respectively. Similarly, frequencies of haplotype "v" alleles, *Mafa-A1*074:02* and *Mafa-A1*093:01*, in cDNA clones were 59.5% and 40.5%, respectively, in M05, while those in C010 were 23.3% and 26.7% and 31.4% and 17.1% in C011, respectively. The frequencies of cDNA clones varied in different individuals presumably due to the allelic competition with the alleles of another haplotype in each individual (Fig. 1), but they were much higher than the frequencies of the minor *Mafa-A* allele (*Mafa-A3*13:03*) clones: 3.3% and 2.9% in C010 and C011, respectively. These observations indicated that two *Mafa-A* alleles were considered to be major *Mafa-A1* alleles in both haplotypes "e" and "v".

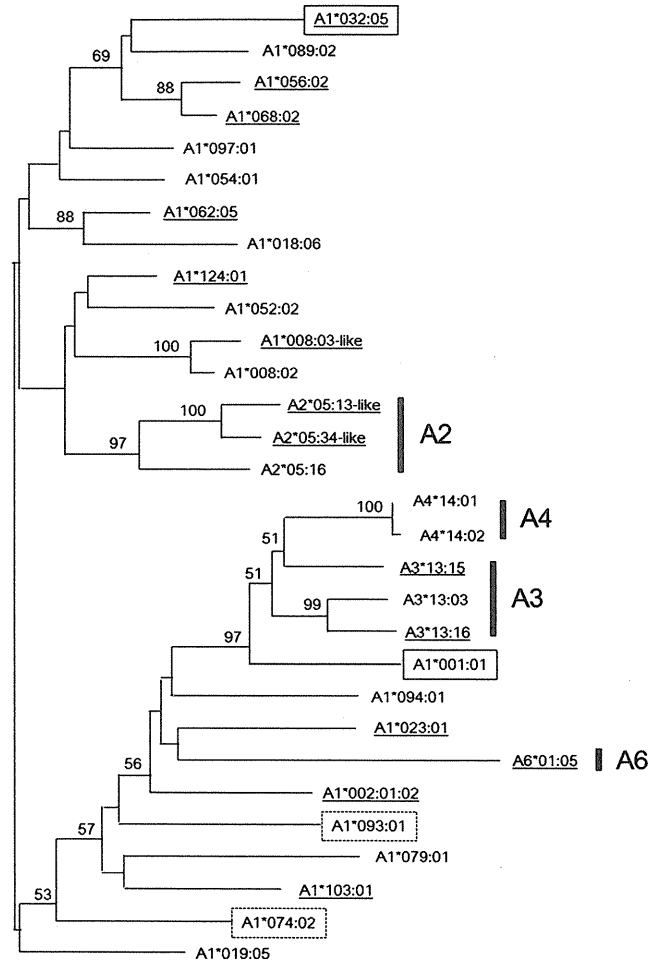

Fig. 2 Phylogenetic tree of *Mafa-A* alleles. A phylogenetic tree of the *Mafa-A* alleles detected in this study was constructed by using the neighbor-joining method with a bootstrap value of 5,000 replications. Values more than 50% are indicated as percentages. Novel alleles were *underlined*. *Mafa-A*1 alleles consisting of haplotype "e" are *boxed*, while the *stippled boxes* represent the alleles on haplotype "v". Alleles of minor *Mafa-A* genes, *Mafa-A2*, *A3*, *A4*, and *A6*, are also indicated

Discussion

Native cynomolgus macaques are widespread throughout the islands of Southeast Asia into mainland Asia. They are mainly found in Indonesia, Malaysia, and the Philippines, then Burma, India, Vietnam, Cambodia, Laos, and Thailand (Lang 2006). It was suggested that the founding population of Mauritian macaques was introduced from Indonesia (Pendley et al. 2008; Campbell et al. 2009). More than 40% of *Mafa* class I alleles observed in this study were novel, even though there have been many reports on the analysis of *Mafa class I* genes, demonstrating that the diversity of MHC in the cynomolgus macaques still needs to be investigated. When we considered the origin of founders, 73.7% (28/38) were novel in alleles found in Malaysian macaques, while only 15.6% (5/32) were novel alleles in Philippino macaques (Table 2). The geographic distribution of novel alleles may be due to the fact that the Malaysian macaques had not been extensively analyzed before (Otting et al. 2007;

Pendley et al. 2008; Kita et al. 2009). In the present study, *B*089:01:02* was found in individuals among Indonesian, Malaysian, and Philippino macaques in different *Mafa-B* haplotypes (Table 6). Likewise, *B*137:03* was found in Indonesian and Malaysian macaques (Table 4). In addition, shared alleles among the cynomolgus macaques, rhesus macaques, and pig-tailed macaques (*Macaca nemestrina*) were noted (Tables 3, 4, and 5). These observations indicated that the diversity of *MHC class I* genes is similar not only in the cynomolgus macaque population but also among the Old World monkeys, suggesting that the *MHC class I* polymorphisms might be generated before the divergence of Old World monkeys and/or there were admixtures of the Old World monkeys.

In this study, we determined the haplotype structure of *Mafa* class I locus by family studies and a total of 23 haplotypes were identified. Among them, haplotypes "i" and "w" carried identical *Mafa-B* alleles but different *Mafa-A* alleles (Table 6), suggesting that there were haplotypes originated by a recombination between the *Mafa-A* and *Mafa-B* loci. We showed that the *Mafa class I* haplotypes were usually composed of one to three *Mafa-A* alleles and one to five *Mafa-B* alleles, similar to the *Mamu class I* haplotypes, of which usually one *MHC-A1* gene and a few (one to three) *MHC-B* genes were highly transcribed (Otting et al. 2007, 2008; Naruse et al. 2010; Doxiadis et al. 2011). As for the *MHC-A* locus in the cynomolgus macaques, highly transcribed *Mafa-A1*gene and other minor *Mafa-A* genes, such as *Mafa-A2*, *-A3*, *-A4*, and *-A6* could be detected. It was reported that 87% of cynomolgus macaques had at least one *Mafa-A2* alleles (Wu et al. 2008). However, only 3 out of 23 (13.0%) haplotypes carried a *Mafa- A2* allele in this study (Table 6). We could not exclude a possibility that the strategy of our study might not be sufficient to detect the *Mafa-A* genes with low expression and/or the alleles with mismatches at the primer site, based on the number of clones within a PCR sample. Such a possibility is unlikely because we used the primer pairs which could cover the known *Mafa-A2* alleles, although there might be novel *Mafa-A2* alleles having different sequences at the primer binding sites. Therefore, we might underestimate the complexity of *Mafa* class I alleles in this study. High-throughput pyrosequencing methods may be a useful strategy to avoid the possibility of missing alleles, as described by several investigators (Wiseman et al. 2009; Budde et al. 2010; Aarnink et al. 2011b). In addition, because it was reported that the cell surface expression of Mamu class I molecule was varied depending on the locus and allelic structure (Rosner et al. 2010), locus- and allele-dependent expression of Mafa class I molecule at the cell surface will be required.

The most important finding in this study was that we demonstrated evidence for the presence of haplotypes carrying two major *MHC-A1* genes on the same chromosome from the family studies and additional cloning studies. Interestingly, we and others have reported similar phenomena in rhesus macaques (Naruse et al. 2010; Doxiadis et al. 2011). In addition, several haplotypes carried multiple major *Mafa-B1* alleles (Table 6), similar to the *Mamu-B1* locus (Otting et al. 2008; Doxiadis et al. 2011). The *raison d'etre* of multiple major *MHC class I* genes/alleles on the same chromosome may be that they play an immunological role as the "double lock strategy" (Doxiadis et al. 2011) in which the double *MHC-A1* alleles of high transcription level might be favorable to present peptide to CD8+ T cells. However, there is another unique haplotype which carries no *MHC-A1*allele in cynomolgus macaques (Otting et al. 2007) and maybe in rhesus macaques (Doxiadis et al. 2011). These observations suggested that the diversity of MHC in the Old World monkey is far more complicated than in humans.

In summary, we investigated 26 cynomolgus macaques from five families for the diversity of *MHC class I* alleles and haplotypes. A total of 87 alleles were identified, of which 40 were novel. There were 23 different haplotypes, and two of them carried two *MHC-A1* genes, demonstrating further the complexity of *MHC class I* locus in the Old World monkey.

Acknowledgments We thank Ms. Yukiko Ueda for her technical assistance. We acknowledge Dr. Natasja de Groot and Dr. Nel Otting for assigning nomenclature of *Mafa class I* alleles. This work was supported in part by research grants from the Ministry of Health, Labor and Welfare, Japan; the Japan Health Science Foundation; the program of Founding Research Centers for Emerging and Reemerging Infection Disease; the program of Research on Publicly Essential Drugs and Medical Devices; and Grant-in-Aids for scientific research from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT), Japan. This work was also supported by a program of support for women researchers from the Tokyo Medical and Dental University.

References

Aarnink A, Dereuddre-Bosquet N, Vaslin B, Le Grand R, Winterton P, Apoil PA, Blancher A (2011a) Influence of the MHC genotype on the progression of experimental SIV infection in the Mauritian cynomolgus macaque. Immunogenetics 63:267–274

Aarnink A, Apoil PA, Takahashi I, Osada N, Blancher (2011b) Characterization of MHC class I transcripts of a Malaysian cynomolgus macaque by high-throughput pyrosequencing and EST libraries. Immunogenetics. doi:10.1007/s00251-011-0550-8

Bonhomme M, Cuartero S, Blancher A, Crouau-Roy B (2009) Assessing natural introgression in 2 biomedical model species, the rhesus macaque (*Macaca mulatta*) and the long-tailed macaque (*Macaca fascicularis*). J Hered 100:158–169

Budde ML, Wiseman RW, Karl JA, Hanczaruk B, Simen BB, O'connor DH (2010) Characterization of Mauritian cynomolgus macaque major histocompatibility complex class I haplotypes by high-resolution pyrosequencing. Immunogenetics 62:773–780

Burwitz BJ, Pendley CJ, Greene JM, Detmer AM, Lhost JJ, Karl JA, Piaskowski SM, Rudersdorf RA, Wallace LT, Bimber BN, Loffredo JT, Cox DG, Bardet W, Hildebrand W, Wiseman RW, O'Connor SL, O'Connor DH (2009) Mauritian cynomolgus macaques share two exceptionally common major histocompatibility complex class I alleles that restrict simian immunodeficiency virus-specific CD8+ T cells. J Virol 83:6011–6019

Campbell KJ, Detmer AM, Karl JA, Wiseman RW, Blasky AJ, Hughes AL, Bimber BN, O'Connor SL, O'Connor DH (2009) Characterization of 47 MHC class I sequences in Filipino cynomolgus macaques. Immunogenetics 61:177–187

Doxiadis GG, de Groot N, Otting N, Blokhuis JH, Bontrop RE (2011) Genomic plasticity of the MHC class I A region in rhesus macaques: extensive haplotype diversity at the population level as revealed by microsatellites. Immunogenetics 63:73–83

Gibbs RA, Rogers J, Katze MG et al (2007) Evolutionary and biomedical insights from the rhesus macaque genome. Science 316:222–234

Karl JA, Wiseman RW, Campbell KJ, Blasky AJ, Hughes AL, Ferguson B, Read DS, O'Connor DH (2008) Identification of MHC class I sequences in Chinese-origin rhesus macaques. Immunogenetics 60:37–46

Kita YF, Hosomichi K, Kohara S, Itoh Y, Ogasawara K, Tsuchiya H, Torii R, Inoko H, Blancher A, Kulski K, Shiina T (2009) MHC class I A loci polymorphism and diversity in three Southeast Asian populations of cynomolgus macaques. Immunogenetics 61:635–648

Kobasa D, Jones SM, Shinya K, Kash JC, Copps J, Ebihara H, Hatta Y, Kim JH, Halfmann P, Hatta M, Feldmann F, Alimonti JB, Fernando L, Li Y, Katze MG, Feldmann H, Kawaoka Y (2007) Aberrant innate immune response in lethal infection of macaques with the 1918 influenza virus. Nature 445:319–323

Krebs KC, Jin Z, Rudersdorf R, Hughes AL, O'Connor DH (2005) Unusually high frequency MHC class I alleles in Mauritian origin cynomolgus macaques. J Immunol 175:5230–5239

Kulski JK, Anzai T, Shiina T, Inoko H (2004) Rhesus macaque class I duplicon structures, organization, and evolution within the alpha block of the major histocompatibility complex. Mol Biol Evol 21:2079–2091

Lang KC (2006) http://pin.primate.wisc.edu/factsheets/entry/long-tailed_macaque

Lawler JV, Endy TP, Hensley LE, Garrison A, Frinz EA, Lesar M, Baric RS, Kulesh DA, Norwood DA, Wasieloski LP, Ulrich MP, Slezak TR, Vitalis E, Hugging JW, Jahrling PB, Paragas J (2006) Cynomolgus macaque as an animal model for severe acute respiratory syndrome. PLoS Med 3:677–686

Loffredo JT, Bean AT, Beal DR, León EJ, May GE, Piaskowski SM, Furlott JR, Reed J, Musani SK, Rakasz EG, Friedrich TC, Wilson NA, Allison DB, Watkins DI (2008) Patterns of CD8+ immunodominance may influence the ability of Mamu-B*08-positive macaques to naturally control simian immunodeficiency virus SIVmac239 replication. J Virol 82:1723–1738

Matano T, Kobayashi M, Igarashi H, Takeda A, Nakamura H, Kano M, Sugimoto C, Mori K, Iida A, Hirata T, Hasegawa M, Yuasa T, Miyazawa M, Takahashi Y, Yasunami M, Kimura A, O'Connor DH, Watkins DI, Nagai Y (2004) Cytotoxic T lymphocyte-based control of simian immunodeficiency virus replication in a preclinical AIDS vaccine trial. J Exp Med 199:1709–1718

Mee ET, Berry N, Ham C, Sauermann U, Maggiorella MT, Martinon F, Verschoor EJ, Heeney JL, Le Grand R, Titti F, Almond N, Rose NJ (2009) Mhc haplotype H6 is associated with sustained control of SIVmac251 infection in Mauritian cynomolgus macaques. Immunogenetics 61:327–339

Naruse TK, Chen Z, Yanagida R, Yamashita T, Saito Y, Mori K, Akari H, Yasutomi Y, Miyazawa M, Matano T, Kimura A (2010) Diversity of MHC class I genes in Burmese-origin rhesus macaques. Immunogenetics 62:601–611

Otting N, deVos-Rouweler AJM, Heijmans CMC, de Groot NG, Doxiadis GG, Bontrop RE (2007) MHC class I A region diversity and polymorphism in macaque species. Immunogenetics 59:367–375

Otting N, Heijmans CM, van der Wiel M, de Groot NG, Doxiadis GG, Bontrop RE (2008) A snapshot of the Mamu-B genes and their allelic repertoire in rhesus macaques of Chinese origin. Immunogenetics 60:507–514

Otting N, Doxiadis GG, Bontrop RE (2009) Definition of Mafa-A and -B haplotypes in pedigreed cynomolgus macaques (*Macaca fascicularis*). Immunogenetics 61:745–753

Pendley CJ, Becker EA, Karl JA, Blasky AJ, Wiseman RW, Hughes AL, O'Connor SL, O'connor DH (2008) MHC class I characterization of Indonesian cynomolgus macaques. Immunogenetics 60:339–351

Robinson J, Waller MJ, Parham P, de Groot N, Bontrop R, Kennedy LJ, Stoehr P, Marsh SGE (2003) IMGT/HLA and IMGT/MHC: sequence databases for the study of the major histocompatibility complex. Nucl Acid Res 31:311–314

Rosner C, Kruse PH, Lübke T, Walter L (2010) Rhesus macaque MHC class I molecules show differential subcellular localization. Immunogenetics 62:149–158

Slukvin II, Lunn DD, Watkins DI, Golos TG (2000) Placental expression of the nonclassical MHC class I molecule Mamu-AG at implantation in the rhesus monkey. Proc Natl Acad Sci USA 97:9104–9109

Tanaka-Takahashi Y, Yasunami M, Naruse T, Hinohara K, Matano T, Mori K, Miyazawa M, Honda M, Yasutomi Y, Nagai Y, Kimura A (2007) Reference strand-mediated conformation analysis-based typing of multiple alleles in the rhesus macaque MHC class I Mamu-A and Mamu-B loci. Electrophoresis 28:918–924

Tsukamoto T, Dohki S, Ueno T, Kawada M, Takeda A, Yasunami M, Naruse T, Kimura A, Takiguchi M, Matano T (2008) Determination of a major histocompatibility complex class I restricting simian immunodeficiency virus Gag241-249 epitope. AIDS 22:993–998

Uda A, Tanabayashi K, Yamada YK, Akari H, Lee YJ, Mukai R, Terao K, Yamada A (2004) Detection of 14 alleles derived from the MHC class I A locus in cynomolgus monkeys. Immunogenetics 56:155–163

Uda A, Tanabayashi K, Fujita O, Hotta A, Terao K, Yamada A (2005) Identification of the MHC class I B locus in cynomolgus monkeys. Immunogenetics 57:189–197

Urvater JA, Otting N, Loehrke JH, Rudersdorf R, Slukvin II, Piekarcyk MS, Golos TG, Hughes AL, Bontrop RE, Watkins DI (2000) Mamu-I: a novel primate MHC class I B-related locus with unusually low variability. J Immunol 164:1386–1398

Watanabe A, Shiina T, Shimizu S, Hosomichi K, Yanagiya K, Kita YF, Kimura T, Soeda E, Torii R, Ogasawara K, Kulski JK, Inoko H (2006) A BAC-based contig map of the cynomolgus macaque (*Macaca fascicularis*) major histocompatibility complex genomic region. Genomics 89:402–412

Wiseman RW, O'Connor DH (2007) Major histocompatibility complex-defined macaques in transplantation research. Transplant Rev 21:17–25

Wiseman RW, Wojcechowskyj JA, Greene JM, Blasly AJ, GoponT SomaT, FriedrichT C, O'Connor SL, O'Connor DH (2007)

Simian immunodeficiency virus SIV mac239 infection of major histocompatibility complex-identical cynomolgus macaques from Mauritius. J Virol 81:349–361

Wiseman RW, Karl JA, Bimber BN, O'Leary CE, Lank SM, Tuscher JJ, Detmer AM, Bouffard P, Levenkova N, Turcotte CL, Szekeres E Jr, Wright C, Harkins T, O'Connor DH (2009) Major histocompatibility complex genotyping with massively parallel pyrosequencing. Nat Med 15:1322–1326

Wu J, Bassinger S, Montoya GD, Chavez L, Jones CE, Holder-Lockyer B, Masten B, Williams TM, Prilliman KR (2008) Allelic diversity within the high frequency Mamu-A2*05/Mane-A2*05(Mane-A*06)/Mafa-A2*05 family of macaque MHC-A loci. Tissue Antigens 72:29–38

JVI
Journals.ASM.org

Impact of Vaccination on Cytotoxic T Lymphocyte Immunodominance and Cooperation against Simian Immunodeficiency Virus Replication in Rhesus Macaques

Hiroshi Ishii,[a,b] Miki Kawada,[b] Tetsuo Tsukamoto,[b] Hiroyuki Yamamoto,[a] Saori Matsuoka,[a] Teiichiro Shiino,[a] Akiko Takeda,[a] Makoto Inoue,[c] Akihiro Iida,[c] Hiroto Hara,[c] Tsugumine Shu,[c] Mamoru Hasegawa,[c] Taeko K. Naruse,[d] Akinori Kimura,[d] Masafumi Takiguchi,[e] and Tetsuro Matano[a,b]

AIDS Research Center, National Institute of Infectious Diseases, Tokyo, Japan[a]; Institute of Medical Science, University of Tokyo, Tokyo, Japan[b]; DNAVEC Corporation, Tsukuba, Japan[c]; Department of Molecular Pathogenesis, Medical Research Institute, Tokyo Medical and Dental University, Tokyo, Japan[d]; and Center for AIDS Research, Kumamoto University, Kumamoto, Japan[e]

Cytotoxic T lymphocyte (CTL) responses play a central role in viral suppression in human immunodeficiency virus (HIV) infections. Prophylactic vaccination resulting in effective CTL responses after viral exposure would contribute to HIV control. It is important to know how CTL memory induction by vaccination affects postexposure CTL responses. We previously showed vaccine-based control of a simian immunodeficiency virus (SIV) challenge in a group of Burmese rhesus macaques sharing a major histocompatibility complex class I haplotype. $Gag_{206-216}$ and $Gag_{241-249}$ epitope-specific CTL responses were responsible for this control. In the present study, we show the impact of individual epitope-specific CTL induction by prophylactic vaccination on postexposure CTL responses. In the acute phase after SIV challenge, dominant $Gag_{206-216}$-specific CTL responses with delayed, naive-derived $Gag_{241-249}$-specific CTL induction were observed in $Gag_{206-216}$ epitope-vaccinated animals with prophylactic induction of single $Gag_{206-216}$ epitope-specific CTL memory, and vice versa in $Gag_{241-249}$ epitope-vaccinated animals with single $Gag_{241-249}$ epitope-specific CTL induction. Animals with $Gag_{206-216}$-specific CTL induction by vaccination selected for a $Gag_{206-216}$-specific CTL escape mutation by week 5 and showed significantly less decline of plasma viral loads from week 3 to week 5 than in $Gag_{241-249}$ epitope-vaccinated animals without escape mutations. Our results present evidence indicating significant influence of prophylactic vaccination on postexposure CTL immunodominance and cooperation of vaccine antigen-specific and non-vaccine antigen-specific CTL responses, which affects virus control. These findings provide great insights into antigen design for CTL-inducing AIDS vaccines.

Human immunodeficiency virus (HIV) infection induces chronic, persistent viral replication leading to AIDS onset in humans. Virus-specific cytotoxic T lymphocyte (CTL) responses play a central role in the resolution of acute peak viremia (3, 4, 13, 22, 28) but mostly fail to contain viral replication in the natural course of HIV infection. Vaccination resulting in more effective CTL responses after viral exposure than in natural HIV infections would contribute to HIV control (30, 33). CTL memory induction by prophylactic vaccination may lead to efficient secondary CTL responses, but naive-derived primary CTL responses specific for viral nonvaccine antigens can also be induced after viral exposure. It is important to know how CTL memory induction by vaccination affects these postexposure CTL responses.

Cumulative studies on HIV-infected individuals have shown association of HLA genotypes with rapid or delayed AIDS progression (5, 14, 31, 34). 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 (1, 8, 23, 24). Indian rhesus macaques possessing certain major histocompatibility complex class I (MHC-I) alleles, such as *Mamu-A*01*, *Mamu-B*08*, and *Mamu-B*17*, tend to show simian immunodeficiency virus (SIV) control (19, 25, 36). This implies possible HIV control by induction of particular effective CTL responses (2, 7, 12, 16, 27).

Recent trials of prophylactic T-cell-based vaccines in macaque AIDS models have indicated the possibility of reduction in post-challenge viral loads (6, 15, 17, 21, 35). We previously developed a prophylactic AIDS vaccine consisting of a DNA prime and a boost with a Sendai virus (SeV) vector expressing SIVmac239 Gag (SeV-Gag) (20). Our trial showed vaccine-based control of an SIVmac239 challenge in a group of Burmese rhesus macaques sharing the MHC-I haplotype *90-120-Ia* (21). Animals possessing *90-120-Ia* dominantly elicited Mamu-A1*043:01 (GenBank accession number AB444869)-restricted $Gag_{206-216}$ (IINEEAADWDL) epitope-specific and Mamu-A1*065:01 (AB444921)-restricted $Gag_{241-249}$ (SSVDEQIQW) epitope-specific CTL responses after SIV challenge and selected for viral *gag* mutations, GagL216S (leading to a leucine [L]-to-serine [S] substitution at amino acid [aa] 216 in Gag) and GagD244E (aspartic acid [D]-to-glutamic acid [E] at aa 244), resulting in escape from CTL recognition with viral fitness costs in the chronic phase (9, 26). Vaccinees possessing *90-120-Ia* failed to control a challenge with a mutant SIV carrying these two CTL escape mutations, indicating that $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses play a crucial role in the vaccine-based control of wild-type SIVmac239 replication

Received 5 September 2011 **Accepted** 31 October 2011
Published ahead of print 9 November 2011
Address correspondence to Tetsuro Matano, tmatano@nih.go.jp.

doi:10.1128/JVI.06226-11

TABLE 1 Animals analyzed in this study

Group	No. of animals	Vaccination[a]	SIV-specific CTL response postboost
I	6	None	None
II	5	Gag (pCMV-SHIVdEN DNA prime, SeV-Gag boost)	Gag-specific CTL
III	6	$Gag_{241-249}$-specific (p$Gag_{236-250}$-EGFP-N1 DNA prime, SeV-$Gag_{236-250}$-EGFP boost)	$Gag_{241-249}$-specific CTL
IV	5	$Gag_{206-216}$-specific (p$Gag_{202-216}$-EGFP-N1 DNA prime, SeV-$Gag_{202-216}$-EGFP boost)	$Gag_{206-216}$-specific CTL

[a] All animals were challenged with SIVmac239.

(10). Furthermore, in an SIVmac239 challenge experiment with *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 postchallenge (32).

Thus, it is hypothesized that induction of single $Gag_{206-216}$ or $Gag_{241-249}$ epitope-specific CTL responses by vaccination may result in different patterns of CTL immunodominance and viral replication after SIV challenge. In the present study, we analyzed the impact of prophylactic vaccination inducing single $Gag_{206-216}$ epitope-specific CTL responses on SIV control in *90-120-Ia*-positive macaques and compared the results with those of vaccination inducing single $Gag_{241-249}$ epitope-specific CTL responses. This analysis revealed differences in CTL responses and patterns of viral control after SIV challenge between these vaccinated groups, indicating significant effects of prophylactic vaccination on postexposure CTL immunodominance and cooperation of vaccine antigen-specific and non-vaccine antigen-specific CTL responses.

MATERIALS AND METHODS

Animal experiments. Animal experiments were conducted through the Cooperative Research Program at Tsukuba Primate Research Center, National Institute of Biomedical Innovation, with the help of the Corporation for Production and Research of Laboratory Primates. Blood collection, vaccination, and virus challenge were performed under ketamine anesthesia. All animals were maintained in accordance with the Guideline for Laboratory Animals of the National Institute of Infectious Diseases.

Five Burmese rhesus macaques (*Macaca mulatta*) possessing the MHC-I haplotype *90-120-Ia* (26) (group IV) received a DNA-prime/SeV-boost vaccine eliciting $Gag_{206-216}$-specific CTL responses followed by an SIVmac239 challenge and were compared with three groups (I, II, and III) of *90-120-Ia*-positive animals reported previously (10, 32) (Table 1). Group I animals ($n = 6$) received no vaccination, while group II animals ($n = 5$) received a DNA-prime/SeV-boost vaccine eliciting Gag-specific CTL responses. The DNA, CMV-SHIVdEN, used for the vaccination was constructed from a simian/human immunodeficiency virus ($SHIV_{MD14YE}$) molecular clone DNA with *env* and *nef* deleted (29) and has the genes encoding SIVmac239 Gag, Pol, Vif, and Vpx; SIVmac239–HIV-1 chimeric Vpr; and HIV-1 Tat and Rev (21). In group II animals, CTL responses were undetectable after DNA prime but Gag-specific CTL responses became detectable after SeV-Gag boost. Group III animals ($n = 6$) received a DNA-prime/SeV-boost vaccine eliciting $Gag_{241-249}$-specific CTL responses. A p$Gag_{236-250}$-EGFP-N1 DNA and an SeV-$Gag_{236-250}$-EGFP vector, both expressing an SIVmac239 $Gag_{236-250}$ (IAGTTSSVDEQ IQWM)-EGFP fusion protein, were used for the group III vaccination. After the SeV-$Gag_{236-250}$-EGFP boost, group III animals induced $Gag_{241-249}$-specific CTL responses; the animals showed no $Gag_{236-250}$-specific $CD4^+$ T-cell responses but elicited SeV/EGFP-specific $CD4^+$ T-cell responses (32). For the group IV vaccination, A p$Gag_{202-216}$-EGFP-N1 DNA and an SeV-$Gag_{202-216}$-EGFP vector, both expressing an SIVmac239 $Gag_{202-216}$ (IIRDIINEEAADWDL)-EGFP fusion protein, were used (Fig. 1). Approximately 3 months after the boost, all animals were challenged intravenously with 1,000 50% tissue culture infective doses of SIVmac239 (11). In our previous study (32), the unvaccinated and the control-vaccinated

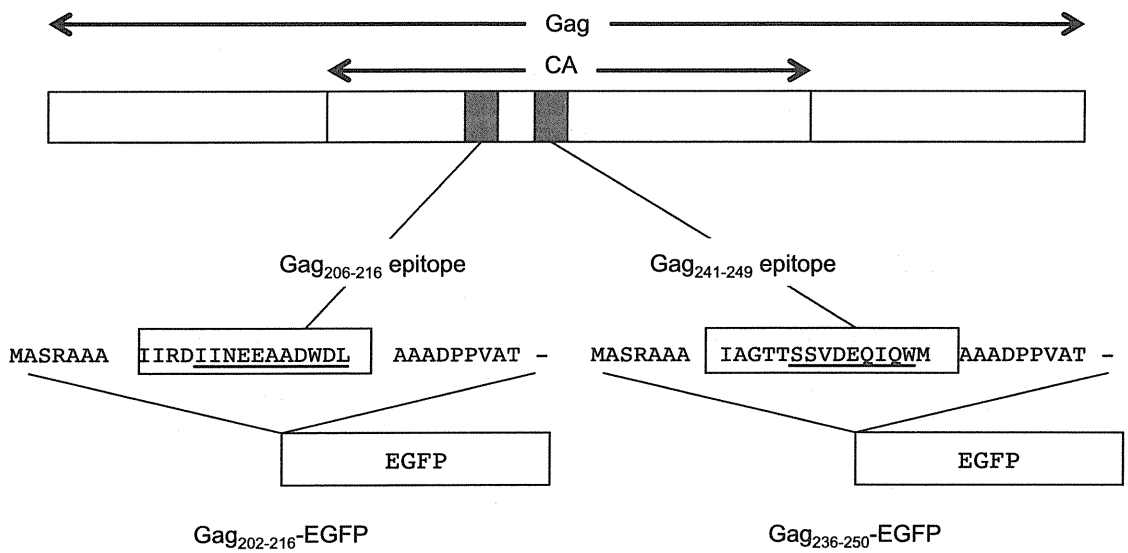

FIG 1 Schema of the cDNA constructs encoding $Gag_{202-216}$-EGFP and $Gag_{236-250}$-EGFP fusion proteins. A DNA fragment that encodes a 31-mer peptide (boxes) including the $Gag_{202-216}$ or $Gag_{236-250}$ sequence (underlining) was introduced into the 5′ end of the EGFP cDNA.

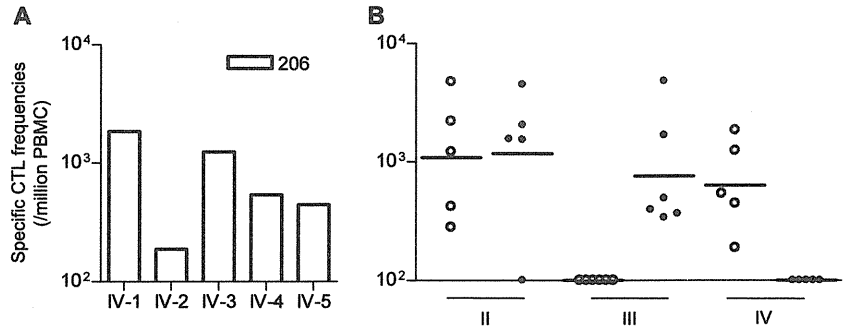

FIG 2 $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses after prophylactic vaccination. (A) $Gag_{206-216}$-specific $CD8^+$ T-cell frequencies 1 week after SeV-$Gag_{202-216}$–EGFP boost in group IV macaques (open boxes). (B) $Gag_{206-216}$-specific (open circles) and $Gag_{241-249}$-specific (closed circles) $CD8^+$ T-cell frequencies 1 week after boost in group II (green), III (blue), and IV (red) macaques. The bars indicate the geometric mean of each group. No animal showed detectable Gag-specific CTL responses before the boost.

animals receiving a DNA and an SeV expressing EGFP showed no significant differences in viral loads after SIV challenge.

Analysis of antigen-specific CTL 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 (21). Peripheral blood mononuclear cells (PBMCs) were cocultured with autologous herpesvirus papioimmortalized B-lymphoblastoid cell lines pulsed with 1 μM SIVmac239 $Gag_{206-216}$ (IINEEAADWDL), $Gag_{241-249}$ (SSVDEQIQW), or $Gag_{367-381}$ (ALKEALAPVPIPFAA) peptide for $Gag_{206-216}$-specific, $Gag_{241-249}$-specific, or $Gag_{367-381}$-specific stimulation. Intracellular IFN-γ staining was performed with a CytofixCytoperm kit (BD, Tokyo, Japan) and fluorescein isothiocyanate-conjugated anti-human CD4 (BD), peridinin chlorophyll protein-conjugated anti-human CD8 (BD), allophycocyanin (APC)-Cy7-conjugated anti-human CD3 (BD), and phycoerythrin (PE)-conjugated anti-human IFN-γ (Biolegend, San Diego, CA) monoclonal antibodies. Specific T-cell levels were calculated by subtracting nonspecific IFN-γ T-cell frequencies from those after peptide-specific stimulation. Specific T-cell levels lower than 100 per million PBMCs were considered negative.

Sequencing of the viral genome. Plasma RNA was extracted using the High Pure viral RNA kit (Roche Diagnostics, Tokyo, Japan). Fragments corresponding to nucleotides from 1231 to 2958 (containing the entire *gag* region) in the SIVmac239 genome (GenBank accession number M33262) were amplified by nested reverse transcription (RT)-PCR. The PCR products were sequenced using dye terminator chemistry and an automated DNA sequencer (Applied Biosystems, Tokyo, Japan).

Statistical analysis. Statistical analyses were performed using R software (R Development Core Team). Differences in geometric means of plasma viral loads were examined by one-way analysis of variance (ANOVA) and Tukey-Kramer's multiple-comparison test. Plasma viral loads at week 3 were examined for differences between group III and groups II and IV by analysis of covariance (ANCOVA) with week 5 viral loads as a covariate.

RESULTS

CTL responses after prophylactic vaccination. We previously reported the efficacy of vaccination eliciting whole Gag-specific or single $Gag_{241-249}$ epitope-specific CTL memory against SIVmac239 challenge (10, 32). In the present study, we examined the efficacy of prophylactic induction of single $Gag_{206-216}$ epitope-specific CTL memory against SIVmac239 challenge and compared the results with those of the previous experiments.

Five Burmese rhesus macaques possessing MHC-I haplotype *90-120-Ia* received a DNA-prime/SeV-boost vaccine eliciting single $Gag_{206-216}$ epitope-specific CTL responses. A plasmid DNA (p$Gag_{202-216}$-EGFP-N1) and an SeV (SeV-$Gag_{202-216}$-EGFP) vector, both expressing an SIVmac239 $Gag_{202-216}$-EGFP fusion pro-

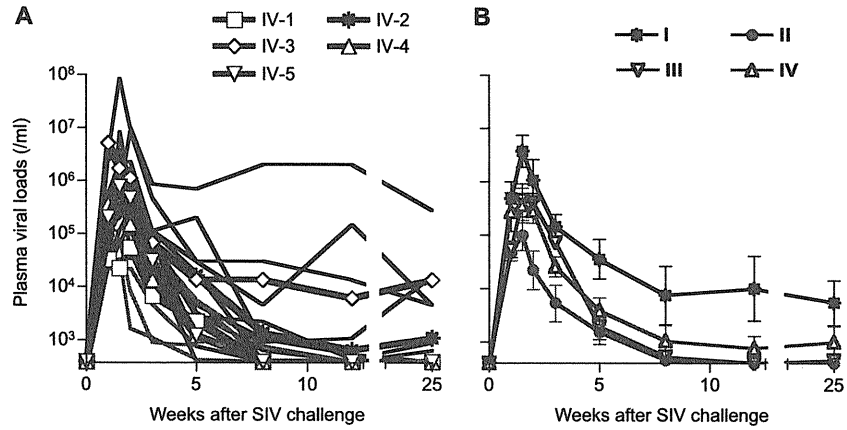

FIG 3 Plasma viral loads after SIVmac239 challenge. The plasma viral loads in group I, group II, group III, and group IV animals were determined as described previously (21). The lower limit of detection was approximately 4×10^2 copies/ml. (A) Changes in plasma viral loads (SIV *gag* RNA copies/ml plasma) after challenge. (B) Changes in geometric means of plasma viral loads after challenge. Groups II and III (but not group IV) showed significantly lower set point viral loads than group I ($P = 0.0390$ between groups I and II, $P = 0.0404$ between groups I and III, and $P > 0.05$ between groups I and IV at week 25 by one-way ANOVA and Tukey-Kramer's multiple-comparison test).

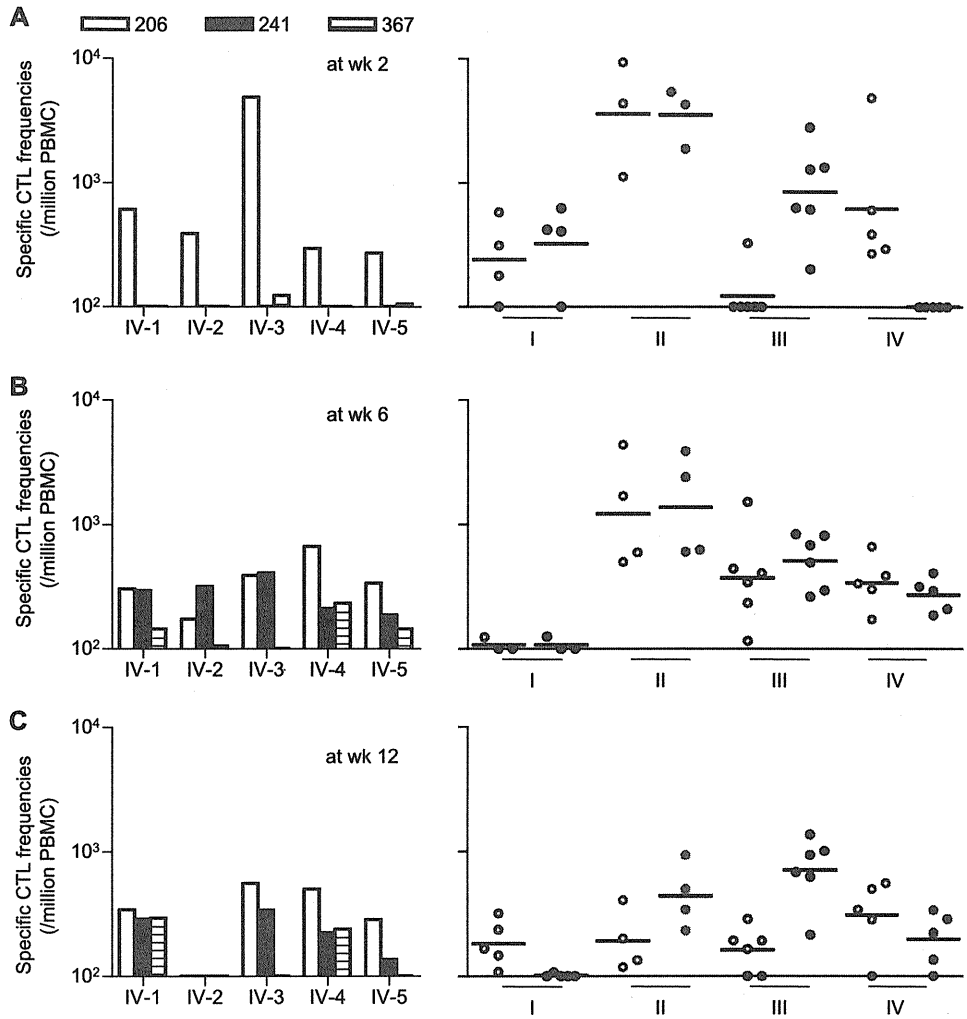

FIG 4 $Gag_{206\text{-}216}$-specific and $Gag_{241\text{-}249}$-specific CTL responses after SIVmac239 challenge. CTL responses at week 2 (A), week 6 (B), and week 12 (C) are shown. In the graphs on the left, $Gag_{206\text{-}216}$-specific (open boxes), $Gag_{241\text{-}249}$-specific (closed boxes), and $Gag_{367\text{-}381}$-specific (striped boxes) $CD8^+$ T-cell frequencies in group IV macaques are shown. On the right, $Gag_{206\text{-}216}$-specific (open circles) and $Gag_{241\text{-}249}$-specific (closed circles) $CD8^+$ T-cell frequencies in group I (black), II (green), III (blue), and IV (red) macaques are shown. The bars indicate the geometric mean of each group. Samples from macaques I-1, I-6, II-1, and II-3 at week 2; macaques I-1, I-2, I-6, and II-5 at week 6; and macaques I-1 and II-5 at week 12 were unavailable for this analysis. Statistical analyses among four groups at week 12 revealed significant differences in $Gag_{241\text{-}249}$-specific CTL levels (I and III, $P < 0.0001$; I and II, and III and IV, $P < 0.01$; I and IV, II and III, and II and IV, $P > 0.05$ by one-way ANOVA and Tukey-Kramer's multiple-comparison test) but not in $Gag_{206\text{-}216}$-specific CTL levels ($P > 0.05$ by one-way ANOVA).

tein, were used for the vaccination (Fig. 1). We confirmed $Gag_{206\text{-}216}$-specific CTL responses 1 week after SeV-$Gag_{202\text{-}216}$-EGFP boost in all five animals (Fig. 2A). As expected, no $Gag_{241\text{-}249}$-specific CTL responses were detected in these animals. No $Gag_{202\text{-}216}$-specific $CD4^+$ T-cell responses were detected in the animals except for one (IV-5) showing marginal levels of responses (data not shown).

Plasma viral loads after SIV challenge. We compared these five animals (referred to as group IV) with other groups (I, II, and III) of *90-120-Ia*-positive macaques reported previously (Table 1). Group I animals ($n = 6$) received no vaccination, group II ($n = 5$) received a DNA-prime/SeV-boost vaccine eliciting whole Gag-specific CTL responses, and group III ($n = 6$) received a DNA-prime/SeV-boost vaccine eliciting single $Gag_{241\text{-}249}$ epitope-specific CTL responses. Both $Gag_{206\text{-}216}$-specific and $Gag_{241\text{-}249}$-specific CTL responses were detectable after SeV-Gag boost in four of five group II animals except for one animal (II-3), in which $Gag_{206\text{-}216}$-specific, but not $Gag_{241\text{-}249}$-specific, CTL responses were detected. In all group III animals, $Gag_{241\text{-}249}$-specific CTL responses were confirmed, while no $Gag_{206\text{-}216}$-specific CTL responses were detected after SeV-$Gag_{236\text{-}250}$-EGFP boost (Fig. 2B).

After SIVmac239 challenge, all animals were infected and showed plasma viremia during the acute phase. Plasma viremia was maintained in five of six unvaccinated animals in group I but became undetectable in one animal (I-2) at week 12. In contrast, all animals in groups II and III contained SIV replication with significantly reduced plasma viral loads compared to group I at the set point. In group IV, however, vaccine efficacy was not so clear; while three out of five animals contained SIV replication, the remaining two (IV-2 and IV-3) failed to control viral replication with persistent plasma viremia (Fig. 3).

Gag-specific CTL responses after SIV challenge. We then measured $Gag_{206\text{-}216}$-specific and $Gag_{241\text{-}249}$-specific CTL responses after SIVmac239 challenge by detection of peptide-

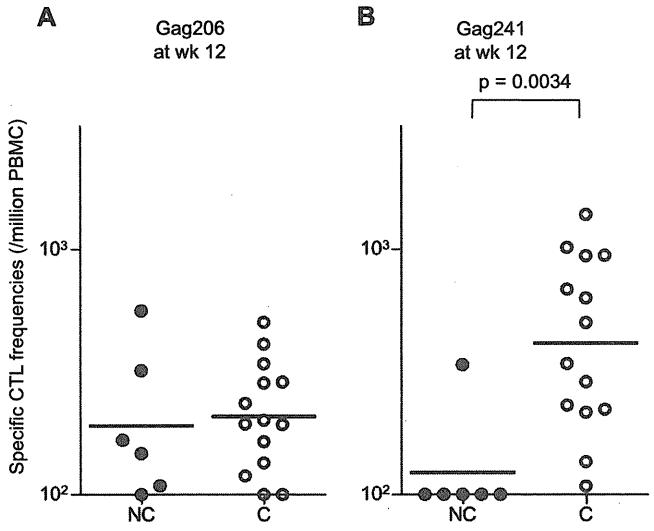

FIG 5 Comparison of $Gag_{206\text{-}216}$-specific or $Gag_{241\text{-}249}$-specific CTL responses in noncontrollers and controllers at week 12. (A) $Gag_{206\text{-}216}$-specific $CD8^+$ T-cell frequencies in noncontrollers (NC; closed circles) and controllers (C; open circles). (B) $Gag_{241\text{-}249}$-specific $CD8^+$ T-cell frequencies in noncontrollers and controllers. $Gag_{241\text{-}249}$-specific CTL levels in controllers were significantly higher than those in noncontrollers ($P = 0.0034$ by Mann-Whitney test). The bars indicate the geometric mean of each group. Data on a noncontroller (I-1) and a controller (II-5) were unavailable.

specific IFN-γ induction. At week 2 (Fig. 4A), most animals in groups I and II elicited both $Gag_{206\text{-}216}$-specific and $Gag_{241\text{-}249}$-specific CTL responses, whereas group III animals induced $Gag_{241\text{-}249}$-specific CTL responses dominantly. Remarkably, all animals in group IV showed efficient $Gag_{206\text{-}216}$-specific CTL responses without detectable $Gag_{241\text{-}249}$-specific CTL responses at week 2. These results indicate dominant $Gag_{206\text{-}216}$-specific CTL responses with delayed induction of $Gag_{241\text{-}249}$-specific CTL responses postchallenge in group IV animals with prophylactic $Gag_{206\text{-}216}$-specific CTL induction, and vice versa in group III animals.

At week 6 (Fig. 4B), efficient $Gag_{206\text{-}216}$-specific and $Gag_{241\text{-}249}$-specific CTL responses were observed in all vaccinated animals in groups II, III, and IV, but not in group I. $Gag_{206\text{-}216}$-specific and $Gag_{241\text{-}249}$-specific CTL responses were induced equivalently even in groups III and IV. We also examined subdominant $Gag_{367\text{-}381}$ epitope-specific CTL responses, which were undetectable at week 2 but became detectable at week 6 in most group IV animals (Fig. 4, graphs on left). At week 12 (Fig. 4C), however, different CTL immunodominance patterns were observed among the groups. $Gag_{241\text{-}249}$-specific CTL levels were higher than $Gag_{206\text{-}216}$-specific levels in groups II and III but were reduced in groups I and IV. Interestingly, comparison between the animals with persistent viremia (referred to as noncontrollers) and those controlling SIV replication (referred to as controllers) revealed significant differences in $Gag_{241\text{-}249}$-specific CTL levels, but not in $Gag_{206\text{-}216}$-specific levels, at week 12 ($P = 0.0034$ by Mann-Whitney test) (Fig. 5).

Selection of a CTL escape mutation. Next, we examined viral genome *gag* sequences at weeks 5 and 12 after challenge to determine whether CTL escape mutations were selected in these animals (Table 2). At week 5, a mutation leading to an L-to-S substitution at the 216th residue in Gag (L216S) was selected in all the group II animals. This GagL216S change results in escape from $Gag_{206\text{-}216}$-specific CTL recognition, as described previously (21). All the group IV animals with $Gag_{206\text{-}216}$-specific CTL induction also showed rapid selection of this CTL escape mutation at week 5. Analysis at week 3 found the GagL216S mutation dominant in two (II-2 and II-5) group II and two (IV-1 and IV-3) group IV animals (data not shown). However, animals in group III showed no *gag* mutations at week 5, except for one animal (III-5) selecting a mutation leading to an L-to-F substitution at the 216th residue. Later, at week 12, the $Gag_{206\text{-}216}$-specific CTL escape mutation, GagL216S, was selected even in group III animals. No animals showed mutations around the $Gag_{241\text{-}249}$ epitope-coding region even at week 12. These results indicate that selection of this $Gag_{206\text{-}216}$-specific CTL escape mutation may be accelerated by prophylactic vaccination inducing $Gag_{206\text{-}216}$-specific CTL responses. On the other hand, in group III animals with single $Gag_{241\text{-}249}$ epitope-specific CTL induction, selection of a $Gag_{206\text{-}216}$-specific CTL escape mutation was delayed but was observed before selection of a $Gag_{241\text{-}249}$-specific CTL escape mutation, suggesting strong selective pressure by delayed $Gag_{206\text{-}216}$-specific CTL responses after SIV challenge.

In order to see the effect of rapid selection of the $Gag_{206\text{-}216}$-specific CTL escape mutation on SIV control, we compared plasma viral loads at weeks 3 and 5 between groups II and IV (referred to as group II+IV) with rapid selection of the GagL216S

TABLE 2 Selection of a CTL escape mutation

Group	Macaque ID	Amino acid change for Gag residues[b]: 206–216 Wk 5	206–216 Wk 12	241–249 Wk 5	241–249 Wk 12
I	I-1	None	ND	None	ND
	I-2[a]	None	L216S	None	None
	I-3	None	L216S	None	None
	I-4	None	None	None	None
	I-5	None	None	None	None
	I-6	None	None	None	None
II	II-1[a]	L216S	ND	None	ND
	II-2[a]	L216S	ND	None	ND
	II-3[a]	L216S	ND	None	ND
	II-4[a]	L216S	ND	None	ND
	II-5[a]	L216S	ND	None	ND
III	III-1[a]	None	L216S	None	None
	III-2[a]	None	L216S	None	None
	III-3[a]	None	NA	None	NA
	III-4[a]	None	NA	None	NA
	III-5[a]	L216F	L216S	None	None
	III-6[a]	None	L216S	None	None
IV	IV-1[a]	L216S	L216S	None	None
	IV-2	L216S	L216S	None	None
	IV-3	L216S	L216S	None	None
	IV-4[a]	L216S	L216S	None	None
	IV-5[a]	L216S	NA	None	NA

[a] Animals that controlled SIV replication at week 12 (controllers).
[b] Plasma viral gag genome mutations were examined at weeks 5 and 12. Amino acid substitutions in $Gag_{206\text{-}216}$ and $Gag_{241\text{-}249}$ epitope regions are shown. L216S results in viral escape from $Gag_{206\text{-}216}$-specific CTL recognition. It remains undetermined whether L216F results in CTL escape. ND, not determined; NA, not determined because Gag fragments were unable to be amplified from plasma RNA.

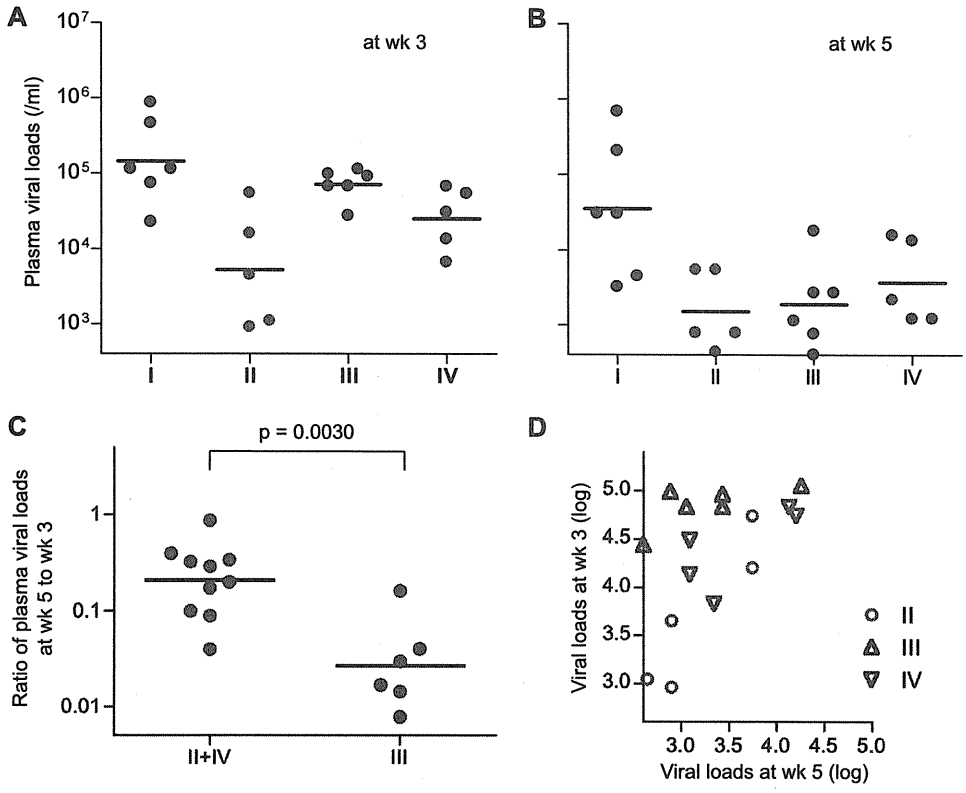

FIG 6 Comparison of plasma viral loads at weeks 3 and 5 among four groups. (A) Plasma viral loads at week 3 in group I, II, III, and IV animals. (B) Plasma viral loads at week 5 in group I, II, III, and IV animals. (C) Comparison of ratios of plasma viral loads at week 5 to week 3 in group II+IV animals and group III animals. The ratios in group III were significantly lower than those in group II+IV ($P = 0.0030$ by Mann-Whitney test). The bars indicate the geometric mean of each group. (D) Scatter plots between plasma viral loads at weeks 3 and 5 in group II, III, and IV animals.

mutation and group III without the mutation at week 5 (Fig. 6). Ratios of plasma viral loads at week 5 to week 3 in group III were significantly lower than those in group II+IV ($P = 0.0030$ by Mann-Whitney test) (Fig. 6C). To confirm this result, we examined the difference in week 3 viral loads between groups III and II+IV by ANCOVA, with week 5 viral loads as a covariate. This analysis revealed that week 3 viral loads controlled for by week 5 viral loads were significantly higher in group III than those in group II+IV (Fig. 6D and Table 3); i.e., the decline in viral loads from week 3 to week 5 was significantly sharper in group III than in group II+IV, possibly reflecting viral escape from suppressive pressure by $Gag_{206\text{-}216}$-specific CTL responses in the latter group during this period (from week 3 to week 5).

DISCUSSION

In the present study, we analyzed the impact of vaccination inducing single $Gag_{206\text{-}216}$ epitope-specific CTL memory on postchallenge CTL responses and SIV control in *90-120-Ia*-positive macaques and then compared the results with those of vaccination inducing single $Gag_{241\text{-}249}$ epitope-specific CTL responses. Our results indicate that these prophylactic vaccinations result in different patterns of $Gag_{206\text{-}216}$-specific and $Gag_{241\text{-}249}$-specific CTL immunodominance and cooperation after SIVmac239 challenge.

Unvaccinated *90-120-Ia*-positive macaques (group I) showed both $Gag_{206\text{-}216}$-specific and $Gag_{241\text{-}249}$-specific CTL responses after SIV challenge. In group IV animals with prophylactic induc-

TABLE 3 ANCOVA on week 3 viral loads with week 5 viral loads as a covariate between groups III and II+IV

ANOVA	Parameter	SS[a]	df[b]	MS[c]	*F*	*P* value
Homogeneity of slopes of regression	Group × slope	0.304	1	0.304	2.099	0.173
	Residual	1.735	12	0.145		
	Total	2.038	13	0.157		
Difference in week 3 viral loads with week 5 viral loads as a covariate between groups III and II+IV	Effect and group	1.106	1	1.106	7.052	0.020
	Residual	2.038	13	0.157		
	Total	3.144	14	0.225		

[a] SS, sum of squares.
[b] df, degrees of freedom.
[c] MS, mean squares.

tion of single $Gag_{206-216}$ epitope-specific CTL responses, $Gag_{206-216}$-specific CTL responses were induced dominantly but $Gag_{241-249}$-specific CTL responses were undetectable at week 2. In contrast, $Gag_{241-249}$-specific CTL responses were induced dominantly at week 2 in group III. Both groups showed $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses equivalently at week 6. It may be difficult to compare these results with those in group II animals inducing whole Gag antigen-specific CTL and $CD4^+$ T-cell responses before challenge; the group II animals elicited $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses equivalently at week 2. Our results indicate that prophylactic vaccination results in dominant induction of vaccine antigen-specific CTL responses and may delay CTL responses specific for viral antigens other than vaccine antigens (referred to as nonvaccine antigens) after viral exposure.

A significant difference between groups III and IV is the pattern of selection of CTL escape mutation. All group IV animals showed rapid selection of a $Gag_{206-216}$-specific CTL escape mutation, while most group III animals showed no *gag* mutation at week 5 but selection of the $Gag_{206-216}$-specific CTL escape mutation later, at week 12. Thus, prophylactic vaccination may affect the patterns of viral genome diversification, possibly accelerating selection of CTL escape mutations. Interestingly, $Gag_{241-249}$-specific CTL mutations were not detected even at week 12 in group III animals, although a previous study observed not only the $Gag_{206-216}$-specific CTL escape mutation (GagL216S), but also a $Gag_{241-249}$-specific CTL escape mutation (GagD244E) in the chronic phase of SIV infection in *90-120-Ia*-positive macaques (9). These results indicate that delayed, naive-derived $Gag_{206-216}$-specific CTL responses, as well as preceding $Gag_{241-249}$-specific CTL responses, exert strong suppressive pressure on SIV replication in group III animals, implying cooperation between vaccine antigen-specific and non-vaccine antigen-specific CTL responses for virus control.

Rapid selection of the $Gag_{206-216}$-specific CTL escape mutation (GagL216S) in group II and delayed selection of this mutation without a detectable $Gag_{241-249}$-specific CTL escape mutation (GagD244E) in group III suggest that the virus with GagL216S (SIVmac239Gag216S) replicates more efficiently than the virus with GagD244E (SIVmac239Gag244E) under both $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses. Our previous competition assay did not find a significant difference in viral fitness between these mutant viruses. Possibly, escape of SIVmac239Gag216S from $Gag_{206-216}$-specific CTL pressure may be more efficient than that of SIVmac239Gag244E from $Gag_{241-249}$-specific CTL pressure.

Our analysis revealed that the decline of plasma viral loads from week 3 to week 5 in group II+IV with rapid selection of the GagL216S mutation was significantly less than that in group III without the mutation at week 5, possibly reflecting viral escape from suppressive pressure by $Gag_{206-216}$-specific CTL responses in the former groups around weeks 3 to 5. Even the comparison between groups II and III, both showing dominant $Gag_{241-249}$-specific CTL responses at week 2, revealed a significantly sharper decline in the latter ($P = 0.0087$). Thus, our results suggest three patterns of $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL cooperation for virus control after SIVmac239 challenge. First, as observed in group II, dominantly induced $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses both work against wild-type SIV replication around week 2, but then a mutant virus escaping from the former CTL responses is selected, and the responses work against this mutant virus replication. Second, as observed in group III, dominantly induced $Gag_{241-249}$-specific CTL responses work against wild-type SIV replication around week 2 and then contribute to virus control, together with delayed, naive-derived $Gag_{206-216}$-specific CTL responses. Third, as observed in group IV, dominantly induced $Gag_{206-216}$-specific CTL responses work against wild-type SIV replication around week 2, but then a mutant virus escaping from $Gag_{206-216}$-specific CTL responses is selected, and delayed, naive-derived $Gag_{241-249}$-specific CTL responses instead work against this mutant virus replication. Viral loads at week 3 in group III looked higher than those in group IV, implying that $Gag_{206-216}$-specific CTL responses may exert a stronger suppressive effect on SIV replication in the acute phase than $Gag_{241-249}$-specific CTL responses. However, viral loads at week 5 in group III looked lower than those in group IV, and the comparison between the two groups showed significantly less decline in the latter ($P = 0.0303$). It is speculated that the third pattern observed in group IV is prone to failure in virus control. Indeed, two of five animals in group IV failed to control SIV replication. Even if vaccines are designed to express multiple antigens, of the vaccine-induced CTLs generated, only several epitope-specific cells may recognize the incoming HIV because of viral diversity and host MHC polymorphisms (18), and cooperation of these vaccine antigen-specific and non-vaccine antigen-specific CTL responses would be required for viral control. Thus, our results may imply a rationale of inducing escape-resistant, epitope-specific CTL memory by prophylactic AIDS vaccines.

In summary, this study showed dominant induction of vaccine antigen-specific CTL responses and delay in non-vaccine antigen-specific CTL responses in the acute phase of SIV infection, clearly describing the impact of prophylactic vaccination on CTL immunodominance and cooperation after virus exposure. Our results indicate that the patterns of cooperation of vaccine antigen-specific and non-vaccine antigen-specific CTL responses affect virus control and selection of CTL escape mutations. These findings provide great insights into antigen design in the development of a CTL-inducing AIDS vaccine.

ACKNOWLEDGMENTS

This work was supported by grants-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology; grants-in-aid from the Ministry of Health, Labor, and Welfare; and a grant from the Takeda Science Foundation, Japan.

We thank F. Ono, K. Oto, K. Komatsuzaki, M. Hamano, Y. Emoto, H. Akari, and Y. Yasutomi for their assistance in animal experiments.

REFERENCES

1. **Altfeld M, et al.** 2003. Influence of HLA-B57 on clinical presentation and viral control during acute HIV-1 infection. AIDS 17:2581–2591.
2. **Berger CT, et al.** 2011. High functional avidity CTL responses to HLA-B-restricted Gag-derived epitopes associate with relative HIV control. J. Virol. 85:9334–9345.
3. **Borrow P, Lewicki H, Hahn BH, Shaw GM, Oldstone MB.** 1994. Virus-specific CD8 cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. J. Virol. 68:6103–6110.
4. **Goulder PJ, Watkins DI.** 2004. HIV and SIV CTL escape: implications for vaccine design. Nat. Rev. Immunol. 4:630–640.
5. **Goulder PJ, Watkins DI.** 2008. Impact of MHC class I diversity on immune control of immunodeficiency virus replication. Nat. Rev. Immunol. 8:619–630.
6. **Hansen SG, et al.** 2009. Effector memory T cell responses are associated

with protection of rhesus monkeys from mucosal simian immunodeficiency virus challenge. Nat. Med. 15:293–299.
7. **Julg B, et al.** 2010. Enhanced anti-HIV functional activity associated with Gag-specific CD8 T-cell responses. J. Virol. 84:5540–5549.
8. **Kaslow RA, et al.** 1996. Influence of combinations of human major histocompatibility complex genes on the course of HIV-1 infection. Nat. Med. 2:405–411.
9. **Kawada M, et al.** 2006. Involvement of multiple epitope-specific cytotoxic T-lymphocyte responses in vaccine-based control of simian immunodeficiency virus replication in rhesus macaques. J. Virol. 80:1949–1958.
10. **Kawada M, et al.** 2008. Gag-specific cytotoxic T-lymphocyte-based control of primary simian immunodeficiency virus replication in a vaccine trial. J. Virol. 82:10199–10206.
11. **Kestler HW III, et al.** 1991. Importance of the nef gene for maintenance of high virus loads and for development of AIDS. Cell 65:651–662.
12. **Kiepiela P, et al.** 2007. CD8+ T-cell responses to different HIV proteins have discordant associations with viral load. Nat. Med. 13:46–53.
13. **Koup RA, et al.** 1994. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. J. Virol. 68:4650–4655.
14. **Leslie A, et al.** 2010. Additive contribution of HLA class I alleles in the immune control of HIV-1 infection. J. Virol. 84:9879–9888.
15. **Letvin NL, et al.** 2006. Preserved CD4+ central memory T cells and survival in vaccinated SIV-challenged monkeys. Science 312:1530–1533.
16. **Li F, et al.** 2011. Mapping HIV-1 vaccine induced T-cell responses: bias towards less-conserved regions and potential impact on vaccine efficacy in the Step study. PLoS One 6:e20479.
17. **Liu J, et al.** 2009. Immune control of an SIV challenge by a T-cell-based vaccine in rhesus monkeys. Nature 457:87–91.
18. **Liu Y, McNevin JP, Holte S, McElrath MJ, Mullins JI.** 2011. Dynamics of viral evolution and CTL responses in HIV-1 infection. PLoS One 6:e15639.
19. **Loffredo JT, et al.** 2008. Patterns of CD8 immunodominance may influence the ability of Mamu-B*08-positive macaques to naturally control simian immunodeficiency virus SIVmac239 replication. J. Virol. 82: 1723–1738.
20. **Matano T, Kano M, Nakamura H, Takeda A, Nagai Y.** 2001. Rapid appearance of secondary immune responses and protection from acute CD4 depletion after a highly pathogenic immunodeficiency virus challenge in macaques vaccinated with a DNA prime/Sendai virus vector boost regimen. J. Virol. 75:11891–11896.
21. **Matano T, et al.** 2004. Cytotoxic T lymphocyte-based control of simian immunodeficiency virus replication in a preclinical AIDS vaccine trial. J. Exp. Med. 199:1709–1718.
22. **Matano T, et al.** 1998. Administration of an anti-CD8 monoclonal antibody interferes with the clearance of chimeric simian/human immunodeficiency virus during primary infections of rhesus macaques. J. Virol. 72: 164–169.
23. **Migueles SA, et al.** 2000. HLA B*5701 is highly associated with restriction of virus replication in a subgroup of HIV-infected long term nonprogressors. Proc. Natl. Acad. Sci. U. S. A. 97:2709–2714.
24. **Miura T, et al.** 2009. HLA-B57/B*5801 human immunodeficiency virus type 1 elite controllers select for rare gag variants associated with reduced viral replication capacity and strong cytotoxic T-lymphocyte [corrected] recognition. J. Virol. 83:2743–2755.
25. **Mothé BR, et al.** 2003. Expression of the major histocompatibility complex class I molecule Mamu-A*01 is associated with control of simian immunodeficiency virus SIVmac239 replication. J. Virol. 77:2736–2740.
26. **Naruse TK, et al.** 2010. Diversity of MHC class I genes in Burmese-origin rhesus macaques. Immunogenetics 62:601–611.
27. **Ndhlovu ZM, et al.** 2011. Mosaic HIV-1 Gag antigens can be processed and presented to human HIV-specific CD8+ T cells. J. Immunol. 186: 6914–6924.
28. **Schmitz JE, et al.** 1999. Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. Science 283:857–860.
29. **Shibata R, et al.** 1997. Infection and pathogenicity of chimeric simian-human immunodeficiency viruses in macaques: determinants of high virus loads and CD4 cell killing. J. Infect. Dis. 176:362–373.
30. **Streeck H, et al.** 2009. Human immunodeficiency virus type 1-specific CD8+ T-cell responses during primary infection are major determinants of the viral set point and loss of CD4+ T cells. J. Virol. 83:7641–7648.
31. **Tang J, et al.** 2002. Favorable and unfavorable HLA class I alleles and haplotypes in Zambians predominantly infected with clade C human immunodeficiency virus type 1. J. Virol. 76:8276–8284.
32. **Tsukamoto T, et al.** 2009. Impact of cytotoxic-T-lymphocyte memory induction without virus-specific CD4+ T-cell help on control of a simian immunodeficiency virus challenge in rhesus macaques. J. Virol. 83: 9339–9346.
33. **Turnbull EL, et al.** 2009. Kinetics of expansion of epitope-specific T cell responses during primary HIV-1 infection. J. Immunol. 182:7131–7145.
34. **Wang YE, et al.** 2009. Protective HLA class I alleles that restrict acute-phase CD8+ T-cell responses are associated with viral escape mutations located in highly conserved regions of human immunodeficiency virus type 1. J. Virol. 83:1845–1855.
35. **Wilson NA, et al.** 2006. Vaccine-induced cellular immune responses reduce plasma viral concentrations after repeated low-dose challenge with pathogenic simian immunodeficiency virus SIVmac239. J. Virol. 80: 5875–5885.
36. **Yant LJ, et al.** 2006. The high-frequency major histocompatibility complex class I allele Mamu-B*17 is associated with control of simian immunodeficiency virus SIVmac239 replication. J. Virol. 80:5074–5077.

frontiers in MICROBIOLOGY

MINI REVIEW ARTICLE
published: 04 January 2012
doi: 10.3389/fmicb.2011.00267

CTL escape and viral fitness in HIV/SIV infection

Sayuri Seki[1,2] and Tetsuro Matano[1,2]*

[1] AIDS Research Center, National Institute of Infectious Diseases, Tokyo, Japan
[2] The Institute of Medical Science, The University of Tokyo, Tokyo, Japan

Edited by:
Akio Adachi, The University of Tokushima Graduate School, Japan

Reviewed by:
Hirofumi Akari, Kyoto University, Japan
Yasuko Yokota, National Institute of Infectious Diseases, Japan

***Correspondence:**
Tetsuro Matano, AIDS Research Center, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan.
e-mail: tmatano@nih.go.jp

Cytotoxic T lymphocyte (CTL) responses exert a suppressive effect on HIV and simian immunodeficiency virus (SIV) replication. Under the CTL pressure, viral CTL escape mutations are frequently selected with viral fitness costs. Viruses with such CTL escape mutations often need additional viral genome mutations for recovery of viral fitness. Persistent HIV/SIV infection sometimes shows replacement of a CTL escape mutation with an alternative escape mutation toward higher viral fitness. Thus, multiple viral genome changes under CTL pressure are observed in the chronic phase of HIV/SIV infection. HIV/SIV transmission to HLA/MHC-mismatched hosts drives further viral genome changes including additional CTL escape mutations and reversions under different CTL pressure. Understanding of viral structure/function and host CTL responses would contribute to prediction of HIV evolution and control of HIV prevalence.

Keywords: HIV, SIV, MHC, cytotoxic T lymphocyte, escape mutation, viral fitness, capsid

INTRODUCTION

Virus-specific $CD8^+$ cytotoxic T lymphocyte (CTL) responses play a central role in the control of HIV and simian immunodeficiency virus (SIV) replication (Borrow et al., 1994; Koup et al., 1994; Matano et al., 1998; Jin et al., 1999; Schmitz et al., 1999; Goulder and Watkins, 2008). CTLs recognize viral antigen-derived peptides (epitopes) presented by major histocompatibility class I (MHC-I) molecules on the surface of viral-infected cells. Under the CTL pressure, viral mutations in and around epitope-coding regions which result in viral escape from CTL recognition are frequently selected with the cost of viral fitness (Phillips et al., 1991; Borrow et al., 1997; Goulder et al., 1997; Price et al., 1997). Thus, analysis of structural and functional constraints in viral proteins could facilitate determination of effective CTLs that can limit viral escape options, contributing to immunogen design in development of CTL-inducing AIDS vaccines.

We previously developed an AIDS vaccine using a Sendai virus vector expressing Gag (SeV-Gag), which induces Gag-specific CTL responses efficiently. Our analysis showed vaccine-based control of a SIVmac239 challenge in a group of Burmese rhesus macaques possessing the MHC-I haplotype *90-120-Ia* (Matano et al., 2004; Kawada et al., 2008). $Gag_{206–216}$ (IINEEAADWDL) epitope-specific CTL responses exert a suppressive effect on SIV replication and select for a CTL escape mutation, GagL216S, leading to a leucine (L)-to-serine (S) substitution at the 216th amino acid (aa) in Gag capsid (CA) with viral fitness costs (Kobayashi et al., 2005). Our studies starting with this finding revealed viral genome changes in persistent SIV infection, providing insights into HIV/SIV evolution.

LOSS OF VIRAL FITNESS BY ESCAPE MUTATIONS AND ITS RECOVERY BY COMPENSATORY MUTATIONS

In contrast to the SIVmac239 challenge experiment, *90-120-Ia*-positive vaccinees failed to control a challenge with another pathogenic SIV strain, SIVsmE543-3 (Hirsch et al., 1997), which has the same $Gag_{206–216}$ amino acid sequence with SIVmac239. SIVsmE543-3 has a different amino acid (glutamate [E]) from SIVmac239 (aspartate [D]) at Gag residue 205, and this GagD205E change resulted in escape from $Gag_{206–216}$-specific CTL recognition, leading to failure in control of SIVsmE543-3 replication in *90-120-Ia*-positive vaccinees (Moriya et al., 2008).

Theoretically, $Gag_{206–216}$-specific CTL responses can select for either GagD205E or GagL216S mutation. SIVmac239-infected *90-120-Ia*-positive macaques, however, select the latter GagL216S mutation but not GagD205E in a year postchallenge. This suggests a possibility that the GagD205E substitution in SIVmac239 results in larger reduction of viral fitness than GagL216S. Indeed, our analysis *in vitro* revealed much lower replicative ability of the virus with this GagD205E substitution, SIVmac239Gag205E, compared to the wild-type SIVmac239 (Inagaki et al., 2010). On LuSIV cells, which contain a luciferase indicator gene under the control of the SIVmac239 long terminal repeat, SIVmac239Gag205E infection showed significantly lower luciferase activity compared to wild-type SIVmac239, indicating suppression of the early phase of this mutant virus replication.

Further passage of SIVmac239Gag205E-infected culture supernatants *in vitro* found an additional mutation, GagV340M, resulting in a valine (V)-to-methionine (M) substitution at the 340th aa in Gag. Interestingly, SIVmac239 has V while SIVsmE543-3 has M at the Gag residue 340. SIVmac239Gag205E340M showed similar replication kinetics with wild-type SIVmac239, indicating compensation for loss of viral fitness in SIVmac239Gag205E by addition of the GagV340M substitution. Thus, CTL escape mutations resulting in loss of viral fitness could be selected with compensatory mutations. **Figure 1** is a schema indicating the interaction between escape and compensatory mutations.

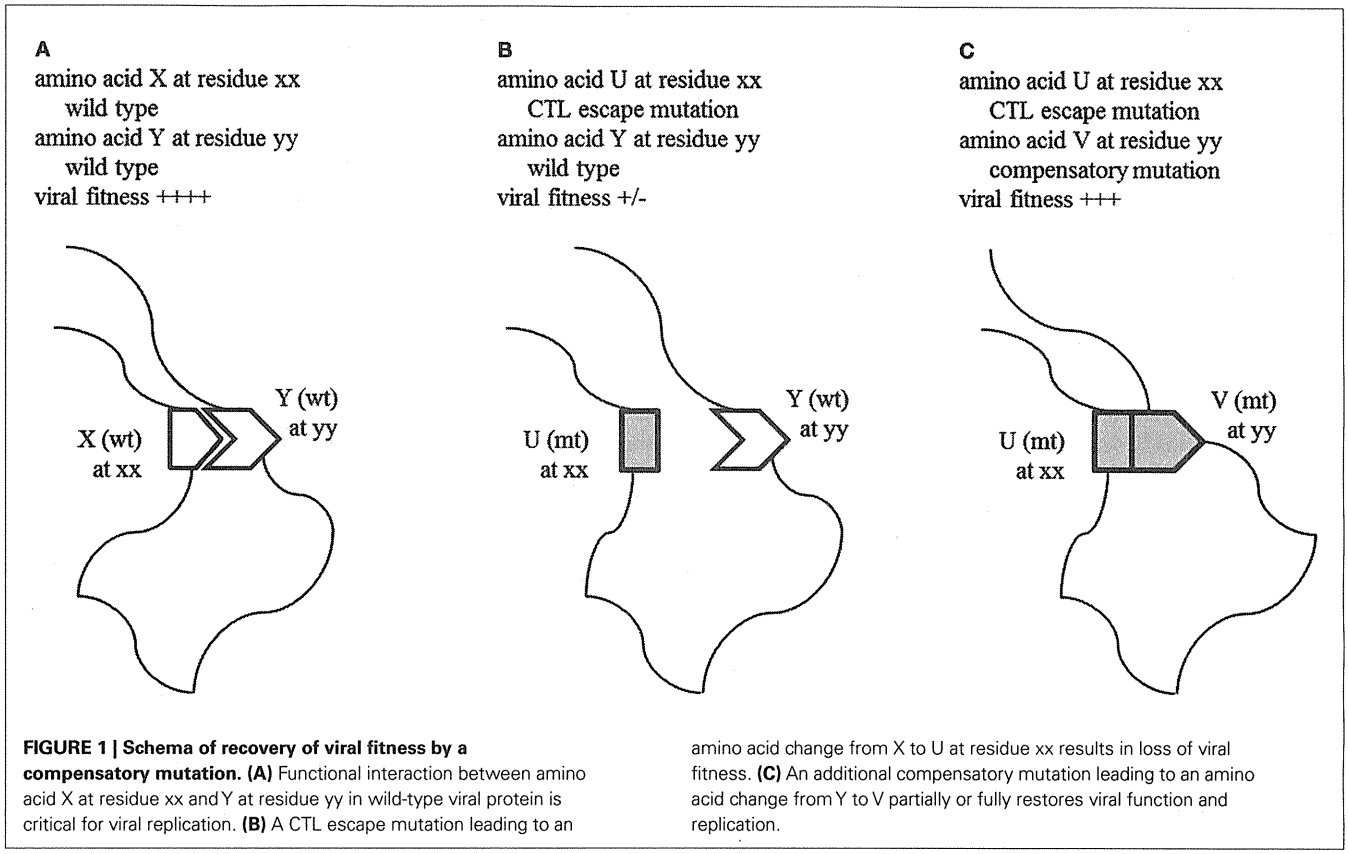

FIGURE 1 | Schema of recovery of viral fitness by a compensatory mutation. (A) Functional interaction between amino acid X at residue xx and Y at residue yy in wild-type viral protein is critical for viral replication. **(B)** A CTL escape mutation leading to an amino acid change from X to U at residue xx results in loss of viral fitness. **(C)** An additional compensatory mutation leading to an amino acid change from Y to V partially or fully restores viral function and replication.

GAG CA INTERMOLECULAR INTERACTION

The Gag CA is comprised of the N-terminal (NTD) and the C-terminal domains (CTD) (Momany et al., 1996; Gamble et al., 1997; Berthet-Colominas et al., 1999). Modeling of CA monomer structure showed that the Gag 205th residue is located in the helix 4 of CA NTD and the 340th is in the loop between helices 10 and 11 of CTD. A possibility of intramolecular contact between Gag residues 205 and 340 is not supported by this modeling. However, CA molecules are known to form hexamer lattice in mature virions (Ganser et al., 1999; Li et al., 2000; Ganser-Pornillos et al., 2007, 2008; Pornillos et al., 2009). Modeling of CA hexamer structure revealed that the Gag 205th residue is located in close proximity to the 340th of the adjacent CA molecule. The molecular model of CA hexamers incorporating the GagD205E substitution suggested shortening of the distance between Gag205 and Gag340 residues, which appeared compensated by GagV340M substitution. Thus, there may be intermolecular interaction between Gag residues 205 and 340 in CA hexamers. This is consistent with our results obtained by viral core stability assay. The core stability was reduced by the GagD205E substitution but recovered by the GagV340M substitution. Loss of viral fitness by GagD205E and its recovery by GagV340M implies a structural constraint for functional interaction between CA NTD and CTD involved in the formation of CA hexamers. In addition to previous reports on intramolecular compensation for loss of viral fitness by CTL escape mutations (Friedrich et al., 2004a; Crawford et al., 2007), our results present evidence indicating intermolecular compensation.

REPLACEMENT OF A CTL ESCAPE MUTATION WITH AN ALTERNATIVE ESCAPE MUTATION TOWARD HIGHER VIRAL FITNESS

As stated above, SIVmac239-infected *90-120-Ia*-positive macaques usually select the $Gag_{206–216}$-specific CTL escape mutation, GagL216S, but not GagD205E in a year postchallenge. After that, however, we found that the GagD205E mutation together with GagV340M became dominant instead of GagL216S in a *90-120-Ia*-positive macaque (Inagaki et al., 2010). In this macaque, neither GagD205E nor GagV340M was detected until week 123 after SIVmac239 challenge, but both became detectable at week 137 and were dominant at week 150. In contrast, the GagL216S mutation dominant until week 123 was undetectable at week 150. Thus, in this animal, SIVmac239Gag216S, whose replicative ability is lower than wild-type SIVmac239 but higher than SIVmac239Gag205E, became dominant under $Gag_{206–216}$-specific CTL pressure in the early phase, while in the later phase, this mutant virus was replaced with SIVmac239Gag205E340M, whose replicative ability is similar with the wild-type. This indicates replacement of a CTL escape mutation with an alternative escape mutation toward higher viral fitness in the chronic phase, implying persistent $Gag_{206–216}$-specific CTL pressure for more than 2 years after selection of the CTL escape mutation.

MULTIPLE VIRAL GENOME CHANGES UNDER CTL PRESSURE

In another study (Kawada et al., 2006), we observed accumulation of multiple CTL escape mutations in viral genomes in SIV-infected macaques. SeV-Gag-vaccinated animals possessing