human RAET1B in complex with NKG2D (Radaev et al. 2001) from the Molecular Modeling Database (MMCB No. 18231) was used as the reference. Polymorphic sites were mapped on the 3-D structure model of macaque RAET1E by using the Cn3D 4.1 program (http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml).

**Table 1** Identified alleles of the ULBP4 gene in rhesus and cynomolgus

| Species | Aliele name | Accession no. | Reference animal | Identical sequence |
|---|---|---|---|---|
| Rhesus macaque | *Mamu-ULBP4*1.1* | AB568525 | R228, R367 | |
| | *Mamu-ULBP4*1.2* | AB568533 | R492, R396, R465 | |
| | *Mamu-ULBP4*2* | AB568526 | R283, R384, R328, R337 | |
| | *Mamu-ULBP4*3* | AB568527 | R346, R361, R396, R379, R408 | |
| | *Mmau-ULBP4*4* | AB568528 | R320, R490, R321, R465, R367, R446, R328, R234, R237, R314 | |
| | *Mamu-ULBP4*5* | AB568529 | R430, R453, R325, R477, R439, R360, R379, R446, R355 | |
| | *Mamu-ULBP4*6* | AB568530 | R437, R350, | |
| | *Mamu-ULBP4*7.1* | AB568531 | R325, R384, R491, R333, R337 | |
| | *Mamu-ULBP4*7.2* | AB568544 | R477 | |
| | *Mamu-ULBP4*8* | AB568532 | R408, R454, R241, R342, R316 | |
| | *Mamu-ULBP4*9.1* | AB568534 | R312, R314 | |
| | *Mamu-ULBP4*9.2* | AB568535 | R333 | |
| | *Mamu-ULBP4*10* | AB568536 | R316 | |
| | *Mamu-ULBP4*11* | AB568537 | R241 | |
| | *Mamu-ULBP4*12* | AB568538 | R342 | |
| | *Mamu-ULBP4*13* | AB568539 | R491 | |
| | *Mamu-ULBP4*14* | AB568540 | R495 | *Mafa-ULBP4*1.1* |
| | *Mamu-ULBP4*15* | AB568541 | R350 | |
| | *Mamu-ULBP4*16* | AB568542 | R492 | |
| | *Mamu-ULBP4*17* | AB568543 | R495 | |
| | *Mamu-ULBP4*18* | AB568545 | R454 | |
| | *Mamu-ULBP4*19* | AB568546 | R321 | |
| | *Mamu-ULBP4*20* | AB568547 | R355 | |
| | *Mamu-ULBP4*21* | AB571025 | R437 | |
| | *Mamu-ULBP4*22* | AB571026 | R439 | |
| Crab-eating macaque | *Mafa-ULBP4*1.1* | AB578934 | M01, P01, P02, C001, C003, C004, C005, C006 | *Mamu-ULBP4*14* |
| | *Mafa-ULBP4*1.2* | AB578935 | M02, C004 | |
| | *Mafa-ULBP4*2* | AB578936 | P04, M06, C010, C011, C013 | |
| | *Mafa-ULBP4*3* | AB578938 | M03, C007 | |
| | *Mafa-ULBP4*4* | AB578939 | M03, C006 | |
| | *Mafa-ULBP4*5* | AB578940 | P04, P05, M05, M06, C012, C013 | |
| | *Mafa-ULBP4*6* | AB578941 | M05, C010, C011 | |
| | *Mafa-ULBP4*7.1* | AB578942 | M01, C002 | |
| | *Mafa-ULBP4*7.2* | AB578943 | P03, C008 | |
| | *Mafa-ULBP4*8* | AB578944 | P03, M04, C008, C009 | |
| | *Mafa-ULBP4*9* | AB578945 | P01, C001, C002 | |
| | *Mafa-ULBP4*10* | AB578946 | M04, C009 | |
| | *Mafa-ULBP4*11* | AB578947 | P02, C007 | |
| | *Mafa-ULBP4*12* | AB578948 | M02, C005 | |

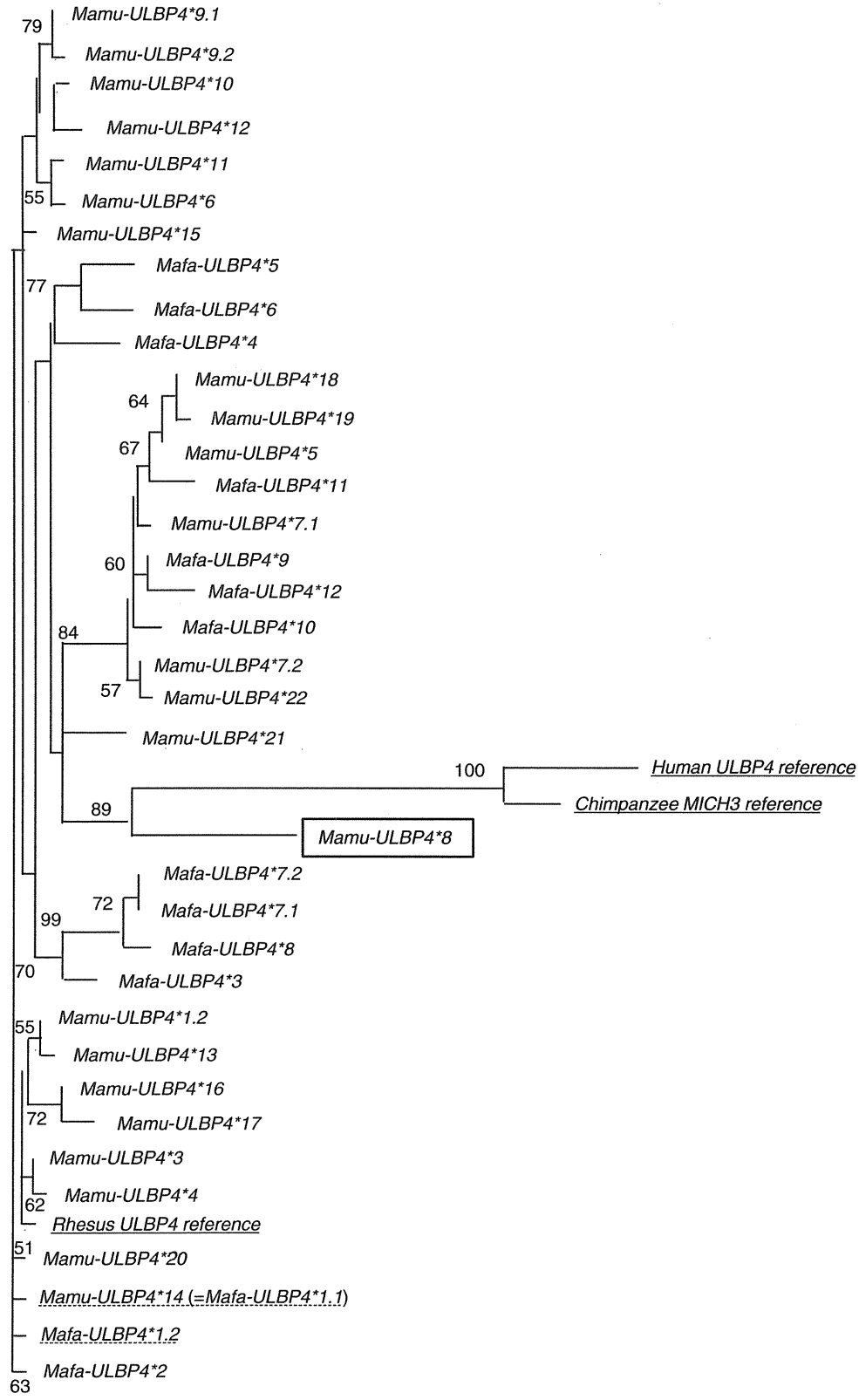


**Fig. 1** Phylogenetic tree of *Mamu-* and *Mafa-ULBP4/RAET1E* alleles. A phylogenetic tree of *ULBP4/RAET1E* sequences spanning from exons 2 to 3, obtained in this study, was constructed by using the neighbor-joining method with bootstrap values of 5,000 replications. Values are indicated as percentages, and only those with more than 50% are shown. Sequences of human *ULBP4/RAET1E* (AY252119), chimpanzee *MICH3* (AY032638), and rhesus *ULBP4/RAET1E* (NC007861) were *underlined* and included in the analysis as reference sequences. Alleles represented with *broken underlines* had identical amino acid sequences predicted from the nucleotide sequences. The allele containing an in-frame termination codon was *boxed*

## Results

### *ULBP4/RAET1E* polymorphisms in the rhesus macaque

In the rhesus macaque genome (Gibbs et al. 2007), there are two paralogous genes for *ULBP4/RAET1E*, one of which appears to be functional, whereas the other is a pseudogene because it contains a large deletion containing the most part of exons 2, 3, and 4. Therefore, we designed primer pairs to amplify the region containing exons 2 and 3, which encode for α1 and α2 domains of ULBP4/RAET1E molecule, respectively, from the functional *ULBP4/RAET1E*. By using the primer pair, we obtained *ULBP4/RAET1E* sequences from 38 individuals of rhesus macaque. Because one or two sequences were obtained from each individual, the sequences were considered to be alleles of *ULBP4/RAET1E*. They were classified into 25 different alleles, designated as *Mamu-ULBP4*1.1* to *Mamu-ULBP4*22*, submitted to DDBJ, and given accession numbers (Table 1). The allele names with different numbers indicate that they are different in predicted amino acid sequences, whereas the alleles with the same deduced amino acid sequences but different nucleotide sequences, such as *Mamu-ULBP4*1.1* and *Mamu-ULBP4*1.2*, are designated as subtypes. None of the sequences obtained in this study was identical to the reference sequence, NC007861, which was previously deposited to the GenBank database as the rhesus *ULBP4/RAET1E*. On the other hand, when the sequences were aligned referring the human *ULBP4/RAET1E*, one rhesus allele (*Mamu-ULBP4*8*) was found to contain a nonsense mutation at codon 29, which would make the ULBP4/RAET1E molecule non-functional.

### *ULBP4/RAET1E* polymorphisms in the crab-eating macaque

By using the primer pair designed for the rhesus *ULBP4/RAET1E*, we could amplify the *ULBP4/RAET1E* sequences from 24 individuals of the crab-eating macaque. Sequencing analysis revealed 14 different *ULBP4/RAET1E* alleles, and inheritance of each allele was confirmed by family studies. The identified allele sequences were submitted to DDBJ, given accession numbers, and designated as *Mafa-ULBP4*1.1* to *Mafa-ULBP4*12* (Table 1). The nucleotide sequences from exons 2 to 3 of *Mamu-ULBP4*14* were identical to those of *Mafa-ULBP4*1.1* and differed by only one nucleotide in intron 2 from those of *Mafa-ULBP4*1.2*. In addition, a neighbor-joining analysis performed by using nucleotide sequences spanning from exons 2 to 3 showed that the alleles of rhesus and crab-eating macaques were not separately clustered from each other (Fig. 1).

### Comparative analysis of *ULBP4/RAET1E*

The alignment of *ULBP4/RAET1E* sequences from rhesus and crab-eating macaques with those from humans and chimpanzees showed that the macaque genes were homologous to the human gene by more than 90% and were equally diverged (Fig. 2). In addition, rhesus and crab-

```
                                                                                50
                  Human :GHSLCFNFTI KSLSRPGQPW CEAQVFLNKN LFLQYNSDNN MVKPLGLLGK KVYATSTwge
             Chimpanzee :---------- -L-------- ---------- ---------- ---------- --N-------
          Rhesus macaque :A--------- --W------- ------M-K- -----D—S- ---------- --N-----Q-
      Crab-eating macaque :A--------- --W------- ------M--- -F---D—SN ---------- --N-----Q-

                                                                    100
                  Human :ltqtlgevgr dlrmllcdIK PQIKTSDPST LQVEMFCQRE AERCTGASWQ FATNGEKSLL
             Chimpanzee :---M------ ------L--- ------G--- ---------- ---------- --I-------
          Rhesus macaque :---K------ ------L-V- ------GP-- -----L---- ---------- -TI----C--
      Crab-eating macaque :---------- -LM---L-V- P-----G--- L----L---- ---------- -TI----C--

                                                          150
                  Human :FDAMNMTWTV INHEAskike twkkdrglek yfrklskgdc dhwlreflgh weampEPTV
             Chimpanzee :---------- ---------- ---------- ---------- ---------- --------Q
          Rhesus macaque :F----M---- ----AS---- ---K------ ------M--- N------L-- Q-A-----Q
      Crab-eating macaque :---------- -----S---- ---------- ------M--- N-WLR----- Q------TQ
```

**Fig. 2** Alignment of deduced amino acid sequences of α1 and α2 domains of ULBP4/RAET1E. Amino acid sequences were deduced from the nucleotide sequences of *ULBP4/RAET1E* or *MICH3* from humans (AY252119), chimpanzees (AY032638), rhesus macaques (NC007861), and crab-eating macaques (AY032639). *Numbers* above the sequences represent the amino acid positions in mature protein. *Dashes* indicate identical sequences. Sequences for the predicted α helix structure were indicated by *small italicized characters*. Positions of polymorphic sites in the *human*, *rhesus macaque*, and *crab-eating macaque* were *underlined*

**Table 2** Single nucleotide polymorphisms of ULBP4 gene among human and Old World monkeys

| | Number of alleles | Exon 2 | | Intron 2 | Exon 3 | |
|---|---|---|---|---|---|---|
| | | Polymorphism | Non-synonymous change (%) | Polymorphism | Polymorphism | Non-synonymous change (%) |
| Human | 5 | 2 | 2 (100%) | 3 | 3 | 3 (100%) |
| Rhesus macaque | 25 | 9 | 5 (55.6%) | 22 | 22 | 14 (63.6%) |
| Crab-eating macaque | 14 | 17 | 9 (52.9%) | 18 | 16 | 9 (56.3%) |

eating macaques showed a higher degree of polymorphism in the analyzed region, namely, exon 2, intron 2, and exon 3, than in humans (Table 2). All polymorphisms found in exons of human *ULBP4/RAET1E* were non-synonymous, whereas a considerable part of the polymorphisms were synonymous in the Old World monkeys. On the other hand, the polymorphic sites in the rhesus macaque (positions 29, 46, 59, 64, 79, 88, 112, 121, 126, 135, 136, 144, 157, 158, 161, 168, 171, and 173) and the crab-eating macaque (positions 32, 39, 40, 59, 72, 73, 79, 91, 112, 136, 163, 164, 165, 171, 178, and 179) were shared at five positions (59, 79, 112, 136, and 171) by each other, whereas only one position (position 112) was shared with polymorphic sites in humans (positions 53, 99, 112, and 113) (Fig. 2). In addition, a termination at position 29 was found in a rhesus macaque allele *Mamu-ULBP4*8*; a single amino acid deletion caused by deletions of a total of three nucleotides was found in a crab-eating macaque allele *Mafa-ULBP4*6* [i.e., TGGCTCAGG sequences corresponding to codons 163–165 were changed to TGCTCA, which may be due to two different deletions at codons 163 (from TGG to TG) and 165 (from AGG to A)], whereas such polymorphisms were not observed in humans. These findings suggest that a selection pressure to generate and maintain the polymorphic sites might be considerably different between the lineages of humans and the Old World monkeys.

## Discussion

It has been suggested that the ancestral gene for the ULBP/REAT molecule of placental mammals was originally diverged and duplicated in each species after an emigration from the MHC region (Kondo et al. 2010). In humans, MHC genes (*HLA* genes) are clustered and mapped on the short arm of chromosome 6, 6p21.3, whereas the *ULBP/RAET1* genes are located on the long arm of chromosome 6, 6q25.1. As for the *MHC* genes in the macaque, it was previously reported that rhesus macaque MHC, e.g., BAT1 gene, was localized to chromosome 6q24 by using fiber-fluorescence in situ hybridization (Huber et al. 2003) and cynomolgus (crab-eating) macaque MHC, e.g., Mafa-A and Mafa-B genes, was cytogenetically mapped to chromosome 6p13 (Liu et al. 2007), although the rhesus macaque MHC is mapped on the short arm of chromosome 4 in the draft genome sequence database of rhesus macaques (Gibbs et al. 2007); e.g., Mamu-A and BAT1 were mapped from positions 29, 517, 308 to 29, 520, 221 and from 31, 164, 822 to 31, 175, 032, respectively, on chromosome 4 (data were obtained from the UCSC Genome Browser at http://genome.ucsc.edu/cgi-bin/hgGateway). The discrepancy between the cytogenetic mapping and the assignment in draft genome sequence should be resolved in the future. On the other hand, it is interesting to note that each member of the *ULBP/RAET1* gene family, except for *ULBP6*, is completely or partially duplicated in the rhesus genome. As for the *ULBP4/RAET1E*, two related sequences, LOC695031 (NC007861) and LOC694265, have been identified as orthologs of human *ULBP4/RAET1E*. On the other hand, the configuration of *ULBP/RAET1* loci in the crab-eating macaque genome remained unknown. Because LOC694265 was a pseudogene lacking most part of the coding exons, we designed PCR primers by referring the NC007861 sequence. By using the designed primers, we could successfully amplify *ULBP4/RAET1E* alleles from both rhesus and crab-eating macaques.

In this study, we identified a total of 25 and 14 alleles from rhesus and crab-eating macaques, respectively. One of the rhesus macaque alleles had identical sequences to one of the crab-eating macaque alleles, and the phylogenetic analysis demonstrated that the *ULBP4/RAET1E* alleles were widely diverged. None of the alleles identified in this study were identical to the previously reported sequence NC007861, which was derived from an individual of Indian rhesus macaque. Given that we analyzed rhesus macaques of Burmese origin in this study, and allele distribution of MHC-related polymorphic genes are well known to be largely dependent on the habitat regions, the extent of diversity and variation in *ULBP4/RAET1E* may be further expanded.

It was demonstrated that the diversity of *ULBP4/RAET1E* in the Old World monkeys was much higher than that of human *ULBP4/RAET1E*. It is possible that the genes in the *ULBP/RAET1* locus, in particular, *ULBP4/RAET1E* and *ULBP/RAET1*s, might be highly polymorphic in the

Old World monkeys. We therefore investigated ten unrelated rhesus macaque subjects, in which we had detected 16 *ULBP4/RAET1E* alleles for polymorphisms in the adjacent *ULBP/RAET1* genes. We found one *ULBP1/RAET1I* allele, seven *ULBP2/RAET1H* alleles, and one *ULBP3/RAET1N* allele in these subjects. The observation suggested that *ULBP4/RAET1E* was highly polymorphic as compared to the adjacent *ULBP/RAET1* genes.

We revealed a high degree of polymorphism in the *ULBP4/RAET1E* of the rhesus and crab-eating macaques, although about half of the polymorphisms were synonymous changes (Table 2). Albeit the expression of the ULBP4/RAET1E molecule is known to be involved in the recognition of tumor cells by the NKG2D receptor (Cao et al. 2008; Kong et al. 2009), the functional significance of the polymorphisms in the extracellular domain of the ULBP4/RAET1E molecules remained unknown. To investigate a possible role of the polymorphisms, we have created a 3-D structure model of rhesus ULBP4/RAET1E molecule by using the structure data of human ULBP3/RAET1N in complex with NKG2D (Radaev et al. 2001) as the reference. As shown in Fig. 3, only one polymorphic site at 173 was on the surface of the α helix pointing to the NKG2D receptor, five sites at 59, 136, 144, 161, and 165 were positioned outside the α helix, and only two sites at 32 and 91 were mapped on the β sheet in the groove. The other polymorphic sites were on the β sheet outside of the groove or were not on the surface of the α helix. In addition, expression of *ULBP4/RAET1E* is predominantly found in the skin and tumor tissues and not induced by viral infection in normal cells (Chalupny et al. 2003; Eagle et al. 2006). These observations suggest that the polymorphisms are unlikely to be involved in the differential presentation of characteristic small molecules bound by the ULBP4/RAET1E molecules, as found in the presentation of antigenic peptides by the MHC molecules. Nevertheless, highly prevalent polymorphisms leading to amino acid replacements suggest that a selection pressure had operated on the configuration of diversity in *ULBP4/RAET1E*.

Of particular interest in this study was the rhesus macaque allele *Mamu-ULBP4*8*, which was supposed to contain a stop codon in the exon 2 coding sequence that would truncate the most part of the molecule. This is the first report of a non-functional *ULBP/RAET1* allele in primates; however, a similar situation was reported for another NKG2D ligand gene, *MIC*. For example, a specific human *MIC* haplotype linked to HLA-B*048 consists of non-functional *MIC* genes, in which *MICA* was deleted and *MICB* contained a termination codon (Ota et al. 2000); the non-functional *MIC* haplotype is widely distributed in the East Asian populations (Komatsu-Wakui et al. 2001). It is interesting to note that there are two distinct and polymorphic genes for *MIC* in the rhesus macaque, *MICA* (previously designated as *MIC1* and *MIC3*) and *MICB* (previous *MIC2*); however, they are not considered to be orthologous to the human *MICA* and *MICB* genes, respectively (Seo et al. 1999, 2001; Doxiadis et al. 2007; Averdam et al. 2007). Because members of the MIC and ULBP/RAET1 molecules are structurally related (Li et al. 2002), there is a functional redundancy in the recognition by NKG2D, and thus, the presence of a null allele had been allowed during the evolution of primates.

In the present study, we demonstrated the *ULBP4/RAET1E* allelic polymorphisms not only in the rhesus macaque but also in the crab-eating macaque. Although the localization of *ULBP4/RAET1E* in the crab-eating macaque

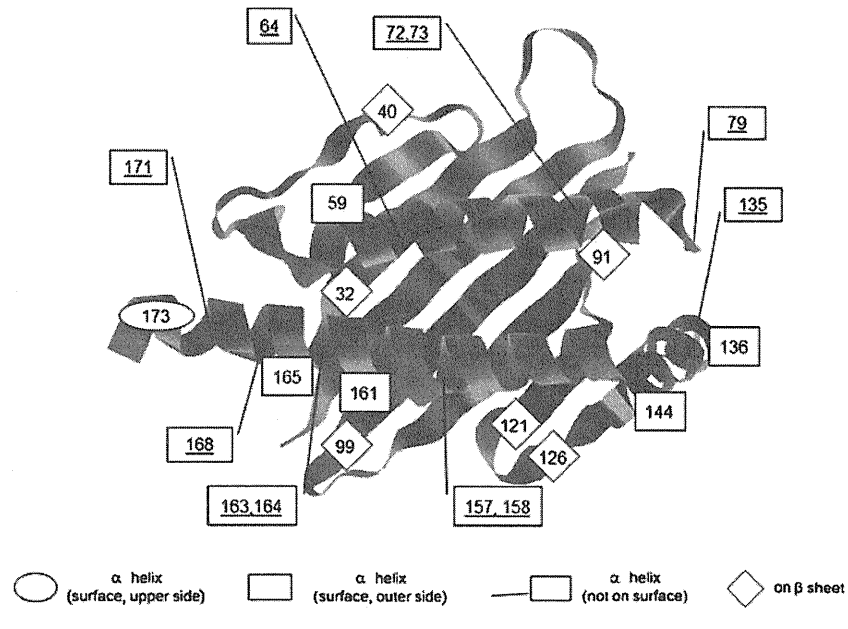


**Fig. 3** Mapping of polymorphic sites on the structure model of the macaque ULBP4/RAET1E molecule. Polymorphic sites found in the Old World monkeys were mapped on the 3-D structure model of ULBP4/RAET1E. Residues on the upper and outer sides of the α helix structure were indicated by a *circle* and *squares*, respectively. Residues not found on the surface of the α helix were *underlined*, and those on the β sheet structure were represented by *rhombi*

genome is unknown, a homology search showed that a *Mafa-MICH3* gene (AY032639) was homologous to *Mafa-ULBP4/RAET1E* because the nucleotide sequences of *Mafa-ULBP4*1.1* showed a 96% homology to *Mafa-MICH3*. Similarly, nucleotide sequences of a chimpanzee gene, *Patr-MICH3* (AY032638), showed a 94% homology to the rhesus *ULBP4/RAET1E*. These findings strongly suggest that *MICH3* in the crab-eating macaque and chimpanzee is orthologous to *ULBP4/RAET1E* in the human and rhesus macaque.

In conclusion, we revealed a large diversity of *ULBP4/RAET1E* in two related species of the Old World monkey. Because there were extremely large polymorphisms in the extracellular domain of the ULBP4/RAET1E molecule in the Old World monkey, which was larger than that in the human, the functional impact of the polymorphisms and its significance in the evolution of primates should be investigated in future studies.

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Review:

# Strategy for Prevention of HIV-1 Transmission

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**HIV-1 infection results in persistent viral replication progressing to AIDS. Recent advances in antiretroviral therapy have been expected to contribute to decrease the risk of viral transmission from HIV-1-infected people under therapy as well as their better prognosis. Precisely understanding of virological and pathological features of HIV-1 infection is important for preventing viral transmission via sexual intercourse or accidental exposure including iatrogenic infection and for avoiding unnecessary protective actions, leading to the world with HIV-1-infected and uninfected living comfortably together.**

**Keywords:** human immunodeficiency virus type-1 (HIV-1), acquired immunodeficiency syndrome (AIDS), route of HIV-1 transmission, HIV-1 testing, anti-HIV-1 agents

## 1. Introduction

In the 30 years since 1981, when the acquired immunodeficiency syndrome (AIDS) epidemic in the United States was first reported [1]. Despite great efforts to prevent human immunodeficiency virus type 1 (HIV-1) pandemic, 1.8 million people have died of AIDS-related disease in the world in 2009, estimated by UNAIDS, the Joint United Nations Program on HIV/AIDS) [2]. UNAIDS has set up a slogan "Getting to three Zeros; zero new HIV-1 infection, zero discrimination, and zero AIDS-related death" in 2011.

## 2. Virological and Immunological Aspects of HIV Infection

For preventing HIV-1 transmission, it is essential to know the HIV-1 infection route and the mechanism for disease progression. After exposure, HIV-1 replicates efficiently in CD4 positive cells, and several weeks later, plasma viral loads reach to the peak and then are reduced. Cytotoxic T lymphocyte (CTL) responses induced in the acute phase play a central role in this reduction of viral loads but fail to control viremia resulting in persistent HIV-1 replication. Anti-HIV-1 antibodies, in contrast, are not induced rapidly and remain undetectable during so-called "window period" in the very early phase of infection. Acquired immune response exerts suppressive pressure on HIV-1 replication, contributing to viral diversity by selecting viral genome mutations resulting in viral escape from the immune responses.

## 3. HIV-1/AIDS Epidemic

**Table 1** shows the global prevalence of the HIV-1 infected people in the world (estimated by UNAIDS). Today, 33 million persons are globally living with HIV-1; 2.6 million people were newly infected with HIV-1 and 1.8 million people died of AIDS in 2009. The main HIV-1 transmission route and the trends in HIV-1 infection differ by regions, so it is important to know the exact status of global HIV-1 epidemic even in domestic HIV-1 control.

### 3.1. HIV-1 Infection in Sub-Sahara

Twenty-two million out of 33 million of HIV-1-infected people (64%) in the world are living in the Sub-Saharan region. Despite great efforts for open access to antiretroviral medication, the number of the HIV-1 infected people increased and 1.4 million people died of AIDS in 2008. Regarding HIV-1 subtypes, HIV-1 clades A and C are prevalent, and the number of women suffers is significantly higher than that of men [2, 3].

### 3.2. HIV-1 Infection in Asia

In Asia, there are 4.3 million of HIV-1-positive people. The number of newly HIV-1-infected people was 3.5 hundred thousand in 2009; that peak was in 1990's. The improvement of access to highly-active antiretroviral therapy (HAART) has contributed to the reduction in the number of HIV-1 infected people in this region. Majority of HIV-1-positive people were infected with HIV-1 clade E [2, 3].

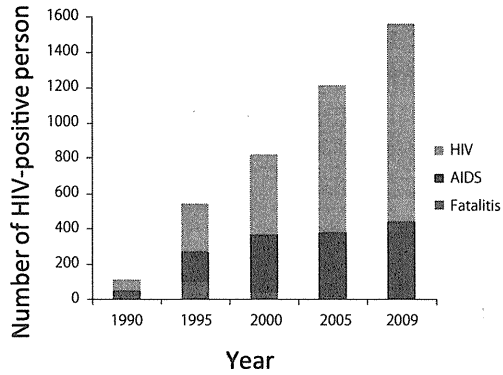
### 3.3. HIV-1 Infection in Japan

According to the committee on AIDS Trends in Japan, over 10,000 pwrsons are living with HIV-1, and the number of newly HIV-1-infected people was approximately one thousand in 2009. Most HIV-1 transmission was throguh homosexual contacts among men, but the heterosexual transmission incidence is currently increasing. Fewer 10 people died of AIDS in 2009 (**Fig. 1**).

**Table 1.** Epidemic in global in 2009.

| | Adults and children infected with HIV-1 | Newly infected people with HIV | Deaths due to AIDS |
|---|---|---|---|
| **Africa** | | | |
| Sub-Sahara | 22.5 million | 1.8 million | 1.3 million |
| Middle east and North | 460 000 | 75 000 | 24 000 |
| **Asia** | | | |
| South and South east Asia | 4.1 million | 270 000 | 260 000 |
| East Asia | 770 000 | 82 000 | 36 000 |
| **America** | | | |
| Central and South America | 1.4 million | 92 000 | 58 000 |
| North America | 1.5 million | 70 000 | 26 000 |
| **Other reagions** | | | |
| Europe | 82 000 | 31 000 | 26 000 |
| Oceania | 57 000 | 4500 | 1400 |
| Total | 33.3 million | 522.6 million | 1.8 milliom |

Data is from the by UNAIDS Report on the Global AIDS epidemic 2010. Refer to http://www.unaids.org/en/dataanalysis/epidemiology/, for details.




Data is provided in Annual reports by the committee on AIDS trend in Japan.
Refer to http://api-net.jfap.or.jp/status/2010.htm

**Fig. 1.** Trends in numbers of HIV-1 1infected people in Japan.

## 4. Diagnosis and Testing of HIV-1 Infection

There are two widely used methods to detect HIV-1 infection; one is based on anti-HIV-1 antibody detection and the other on the viral genome. Because the current screening test of HIV-1 infection is the former that is based on detection of anti-HIV-1 antibodies, we should be careful of the risk of HIV-1 transmission from those in the window period who are not recognized as HIV-1 positive in spite of their high viral loads [4, 5]. Thus, it is important to diminish the window period by improving the methods for detection of HIV-1 infection.

### 4.1. Detection of Anti-HIV-1 Antibodies

Generally, anti-HIV-1 antibodies are detected by Western blotting. This testing will be beneficial for diagnosis. If the false-negative is suspected, retest has been recommended 2 or 3 months later.

### 4.2. Detection of HIV-1 Genomes

The HIV-1 viral genome is detected using by PCR. PCR is highly sensitive and can potential to detect HIV-1 in blood from those in the window period. Thus, it has been widely used for HIV-1 screening of blood donated for transfusion. However, it is not easy to detect all the HIV-1 subtypes prevalent because of the HIV-1 genetic diversity [6–9]. Thus, primer sets for PCR have been modified for testing Blood Bank samples.

## 5. Routes for HIV-1 Transmission and Prophylaxis

It is important to understand the routes for HIV-1 transmission for effective prevention. Of note that the exposed viral load is a key risk factor for efficacy of HIV-1 transmission.

**Table 2.** Recommended prophylaxis for accidental and occupational HIV-1 exposure.

**a) For exposure by injured skin or needle-stick**

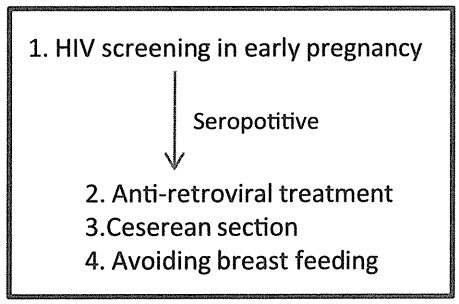
| | Infection status of source | | | |
|---|---|---|---|---|
| | HIV-negative | Source with HIV-risk factors | HIV-1 positive | |
| | | | VL<1500 | >1500 |
| Less severe (Example; superficial injure) | Not needed | Recommended 2-drugs | Recommended 2-drugs | 3-drugs |
| More sever (Example; deep puncture) | Not needed | 2-drugs | 3-drugs | 3-drugs |

Data is summarized from Guideline provided by MMWR[11].

**b) For exposure by mucosal or intact skin**

| | Infection status of source | | | |
|---|---|---|---|---|
| | HIV-negative | Source with HIV-risk factors | HIV-1 positive | |
| | | | VL<1500 | >1500 |
| Small volume (ex; a few drops) | No needed | Generally no warranted | Recommended 2-drugs | 2-drugs |
| Large volume | No needed | 2-drugs | 2-drugs | 3-drugs |

Data were summarized of Guideline provided by MMWR[11].




Refer to [10]; http://api-net.jfap.or.jp/library/guideLine/boshi/index.html, for details.

**Fig. 2.** Prevention strategy for maternal-infant transmission of HIV-1 infection.

### 5.1. Sexual Transmission

Medication is inappropriate to prevent HIV-1 infection in the form of sexual transmission. Promoting education concerning safer sex and screening/nationwide surveillance to detect potential HIV-1-infected person should be effective.

### 5.2. Mother-Infant Transmission

HIV-1-testing for pregnant women is considered the most effective prophylaxis to prevent maternal-infant HIV-1 transmission in Japan. A study group supported by the Ministry of Health, Labor and Welfare in Japan established a guideline for pregnant women in 2000, recommending HIV-1-testing in early pregnancy [10]. Additionally, in case of HIV-1 positive, mothers are also recommended to undergo antiviral therapy during pregnancy, to have cesarean section at delivery, and to avoid breast feeding. Recently, in Japan, some 97% of pregnant women in Japan undergo HIV-testing, and 9 in 100,000 pregnancies are founded to be in HIV-1-positive. The HIV-1 positive ratio is increasing slightly each year, but the risk of mother-to-child transmission is expected to be reduced to 0.5% if HIV-1-positive pregnant women receive antiretroviral therapy and elective cesarean section (**Fig. 2**).

### 5.3. Accidental and Occupational Exposure to HIV-1

Needle-stick injury at hospitals and laboratories is a representative example of accidental or occupational ex-

**Table 3.** Anti-HIV agents approved in Japan.

| Class | Agent |
|---|---|
| Nucleoside Reverse transcriptase inhibitor (NRTI) | Zidovudine;AZT, Lamivudine;ABC, Savudine; d4T, Didanosine, ddI Tenofovir;TDF, Emtricitabine;FTC |
| Non- Nucleoside Reverse transcriptase inhibitor (NNTI) | Nevirapine;NVP, Efavirenz;EFV Etravirine; ETR |
| Protease Inhibitor (PI) | Indinavir;IDV, Saqnavir; SQV Ritonaver;RTV Nelfinavir;NFV Fosamprenavir; FPV, Lopnavir;LPV Atazanavir; ATV, Darunavir; DRV |
| Integrase Inhibitor | Raltegravir;RAL |
| CCR5 Inhibitor | Maraviroc;MVC |

**Table 4.** Anti-HIV immunotherapy under development and clinical trials.

| **Candidate** | **Aim** | **Current status and Feasibility** |
|---|---|---|
| HAART initiation during acute HIV infection | Preserves HIV-1specific CTL | Needs further study |
| IL-7 therapy | Increases CD4 and CD8-positive cells | Impact clearly demonstrated in several studies |
| Therapeutic vaccination | Induces potential and long-lasting HIV-specific CTL | Not yet tested |

Refer to [13].

posure to HIV-1. Previous study reported that the average HIV-1 transmission risk after a precautious expose to HIV-1-infected blood has been estimated to be approximately 0.3%, which is lower than that of other viruses, such HBV and HCV. Mucosal membrane exposure runs an average risk of approximately 0.09% (95% CI = 0.006 – 0.5%). [11]. Moreover, the average risk of HIV-1 transmission after exposure through intact skin is considerably lowers than that though other routes. Viral input dose and infection routes thus strongly affect the infection risk. The immediate medication is the most effective to prevention HIV-1 infection in such accidental and occupational exposure to HIV-1 (**Table 2**). It was reported that the HIV-1 infection risk can be reduced by one-fifth if four-week HAART is started within the first 8 hours of viral exposure [12].

## 6. Current Anti-HIV-1 Therapy

Antiretrovirals are the most effective tool for decreasing viral loads in HIV-1-infected people. Inducting combination anti-retroviral therapy dramatically reduced the mortality due to AIDS in developed countries in the 1990s. The current standard treatment against HIV-1-infection is HAART, a combination regimen including three drugs at least. **Table 3** shows anti-retroviral drugs approved in Japan. The rise in drug-resistant HIV-1 variants and their adverse effects are the serious obstacles for continuous long-term therapy for AIDS prevention. Thus, many attempts toward development of a new anti-HIV-1 therapy such as immunotherapy have been performed (**Table 4**).

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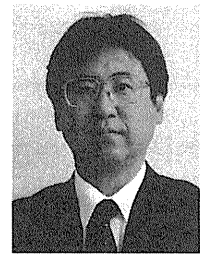
- The Japan association of Virology
- The Japan association for AIDS Research



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ORIGINAL ARTICLE

# Major histocompatibility complex class I-restricted cytotoxic T lymphocyte responses during primary simian immunodeficiency virus infection in Burmese rhesus macaques

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## ABSTRACT

**Major histocompatibility complex class I (MHC-I)-restricted CD8$^+$ cytotoxic T lymphocyte (CTL) responses are crucial for the control of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) replication. In particular, Gag-specific CTL responses have been shown to exert strong suppressive pressure on HIV/SIV replication. Additionally, association of Vif-specific CTL frequencies with *in vitro* anti-SIV efficacy has been suggested recently. Host MHC-I genotypes could affect the immunodominance patterns of these potent CTL responses. Here, Gag- and Vif-specific CTL responses during primary SIVmac239 infection were examined in three groups of Burmese rhesus macaques, each group having a different MHC-I haplotype. The first group of four macaques, which possessed the MHC-I haplotype *90-010-Ie*, did not show Gag- or Vif-specific CTL responses. However, Nef-specific CTL responses were elicited, suggesting that primary SIV infection does not induce predominant CTL responses specific for Gag/Vif epitopes restricted by *90-010-Ie*-derived MHC-I molecules. In contrast, Gag- and Vif-specific CTL responses were induced in the second group of two *89-075-Iw*-positive animals and the third group of two *91-010-Is*-positive animals. Considering the potential of prophylactic vaccination to affect CTL immunodominance post-viral exposure, these groups of macaques would be useful for evaluation of vaccine antigen-specific CTL efficacy against SIV infection.**

**Key words** cytotoxic T lymphocyte, human immunodeficiency virus, major histocompatibility complex, simian immunodeficiency virus.

Virus-specific CD8$^+$ CTL responses are crucial for the control of HIV and SIV replication (1–5). CTLs recognize specific epitopes which are presented on the target cell surface by binding to the MHC-I molecule. There have been many reports indicating association of MHC-I (HLA class I) genotypes with rapid or delayed AIDS progression in HIV-infected people (6–8). For instance, most of the HIV-infected individuals possessing *HLA-B*57* have a better prognosis and smaller viral loads, implicating HLA-B*57-restricted epitope-specific CTL responses in control

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**List of Abbreviations:** CTL, cytotoxic T lymphocyte; HIV, human immunodeficiency virus; HLA, human leukocyte antigen; IFN-$\gamma$, gamma interferon; MHC-1, major histocompatibility complex class I; PBMC, peripheral blood mononuclear cell; SIV, simian immunodeficiency virus.

of this virus (9, 10). Indian rhesus macaques possessing the MHC-I allele Mamu-B*17 tend to show smaller viral loads after SIVmac239 challenge (11). These findings imply possible HIV control by induction of particular effective CTL responses.

The potential of Gag-specific CTL responses to contribute to viral control was suggested by a cohort study indicating association of HIV control with the breadth of Gag-specific CTL responses (12). This was supported by an *in vitro* study indicating the ability of Gag-specific CTLs to respond rapidly to SIV infection (13). We previously developed a prophylactic AIDS vaccine using a Sendai virus vector expressing SIVmac239 Gag (14) and showed that Gag-specific CTL responses were responsible for vaccine-based SIV containment in a group of Burmese rhesus macaques possessing the MHC-I haplotype *90–120-Ia* (15, 16). Furthermore, our recent study analyzing the potential of $CD8^+$ cells to suppress SIV replication *in vitro* suggested association of *in vitro* anti-SIV efficacy with numbers of Vif-specific CTL frequencies (17). We also found weaker correlation between anti-SIV efficacy and numbers of Nef-specific CTL frequencies. These results imply the potency of Gag- and Vif-specific (and possibly Nef-specific) CTLs in suppressing HIV/SIV replication.

The immunodominance patterns of these potent CTL responses could be affected by host MHC-I genotypes (18, 19). Better understanding of these MHC-I-associated CTL immunodominance patterns during primary HIV/SIV infection would contribute to elucidation of the interaction between viral replication and host CTL responses. In the present study, we examined whether Gag- and Vif-specific CTL responses are efficiently induced during primary SIVmac239 infection in three groups of Burmese rhesus macaques possessing different MHC-I haplotypes. One group did not induce Gag- or Vif-specific CTL responses, whereas the other two groups elicited Gag- and Vif-specific CTL responses efficiently. These groups of macaques would be useful for analysis of the impact of Gag- and Vif-specific CTL responses on SIV replication *in vivo*.

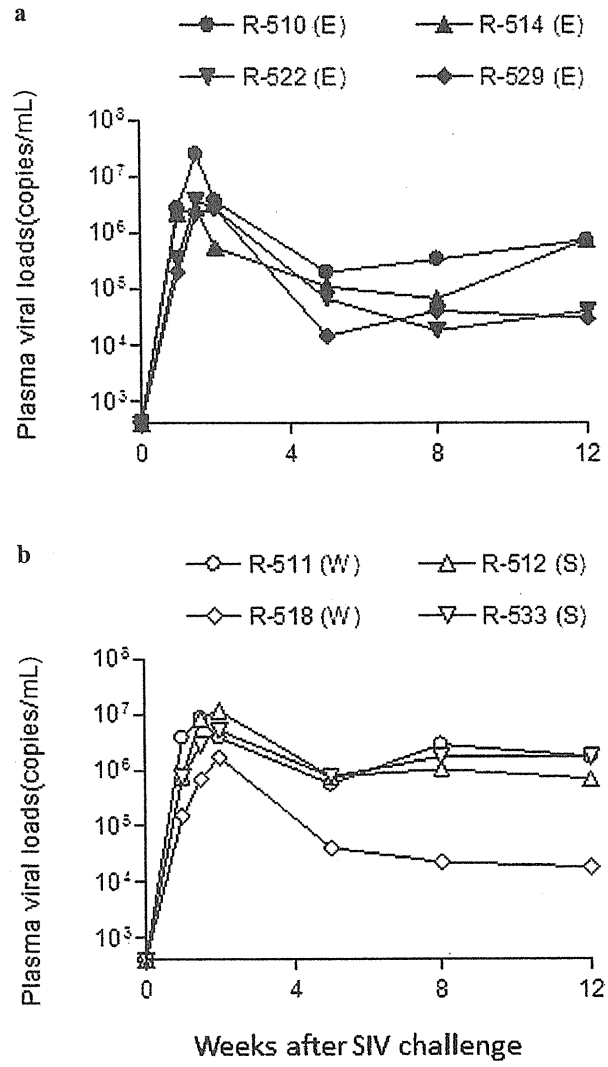


**Fig. 1. Plasma viral loads after SIV challenge.** (a) The first group of Burmese rhesus macaques, which possessed MHC-I haplotype *90-010-Ie* (R-510, R-514, R-522, and R-529) and (b) the second group, which possessed *89-075-Iw* (R-511 and R-518) and the third group, which possessed *91-010-Is* (R-512 and R-533) were challenged with SIVmac239. The viral loads (SIV gag RNA copies/mL) were determined as described previously (15).

## MATERIALS AND METHODS

### Animal experiments

Animal experiments using Burmese rhesus macaques (*Macaca mulatta*) possessing either the MHC-I haplotypes *90-010-Ie*, *89-075-Iw* or *91-010-Is* were performed in the Institute for Virus Research, Kyoto University, in accordance with the institutional regulations approved by the Committee for Experimental Use of Non-human Primates. The MHC-I haplotypes of macaques were determined as described previously (20, 21). These animals were challenged intravenously with 1000 50% tissue culture infective doses ($TCID_{50}$) of SIVmac239 (22).

### Analysis of virus-specific cytotoxic T lymphocyte responses

Virus-specific $CD8^+$ T-cell frequencies were measured by flow cytometric analysis of IFN-$\gamma$ induction after specific stimulation as described previously (17). PBMCs were cocultured with autologous herpesvirus papio-immortalized 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-$\gamma$ staining was performed with a Cytofix-Cytoperm kit (Becton Dickinson, Tokyo, Japan) and fluorescein isothiocyanate-conjugated anti-human CD4 (Becton Dickinson), peridinin chlorophyll protein-conjugated anti-human CD8 (Becton Dickinson), allophycocyanin-conjugated anti-human CD3 (Becton Dickinson), and phycoerythrin-conjugated anti-human IFN-$\gamma$ monoclonal antibodies (BioLegend, Tokyo, Japan). Specific CD8$^+$ T-cell frequencies were calculated by subtracting nonspecific IFN-$\gamma^+$ CD8$^+$ T-cell frequencies from those after peptide-specific stimulation. Specific CD8$^+$ T-cells counts of less than 100 per million PBMCs were considered negative.

## RESULTS

In the present study, we used eight Burmese rhesus macaques consisting of four animals possessing MHC-I haplotype *90-010-Ie*, two possessing *89-075-Iw*, and two possessing *91-010-Is*. After a SIVmac239 challenge, all these animals failed to control viral replication and had high set-point plasma viral loads (geometric mean: $3 \times 10^5$ copies/mL) (Fig. 1).

We examined SIV-specific CD8$^+$ T cell responses at week 2 and week 6 or 12 after SIV challenge in these animals by detection of specific IFN-$\gamma$ induction after stimulation using peptide mixtures (Figs. 2 and 3). At week 6 or 12, we examined CD8$^+$ T cell responses specific for the N-terminal half of Gag (Gag-N), the C-terminal half of Gag (Gag-C), Vif, Nef, the N-terminal half of Pol (Pol-N), the C-terminal half of Pol (Pol-C), Vpx, Vpr, the N-terminal half of Env (Env-N), the C-terminal half of Env (Env-C), Tat, and Rev. At week 2, however, we examined only Gag-N-, Gag-C-, Vif- and Nef-specific CD8$^+$ T cell responses because of limited availability of PBMCs.

In the first group of macaques, which possessed *90-010-Ie*, neither Gag- nor Vif-specific CD8$^+$ T cell responses were induced efficiently at week 2 (Fig. 2). Even at week 12, these responses were undetectable in most of the animals. In contrast, Nef-specific CD8$^+$ T cell responses were detected at week 2, 6, or 12 in all four animals. Env-specific CD8$^+$ T cell responses were detectable at week 12 in three of them. These results indicate that, during primary SIV infection in *90-010-Ie*-positive macaques, Gag- or Vif-specific CD8$^+$ T cell responses are not induced, however Nef-specific CD8$^+$ T cell responses are.

In the second group of macaques, which possessed *89-075-Iw*, Gag- and Vif-specific CD8$^+$ T cell responses were elicited efficiently (Fig. 3a). In the third group of macaques, which possessed *91-010-Is*, Gag-, Vif- and Nef-specific CD8$^+$ T cell responses were elicited efficiently (Fig. 3b). Other SIV antigen-specific CD8$^+$ T cell responses were not efficiently induced in these two groups except for Tat-specific CD8$^+$ T cell responses in macaque

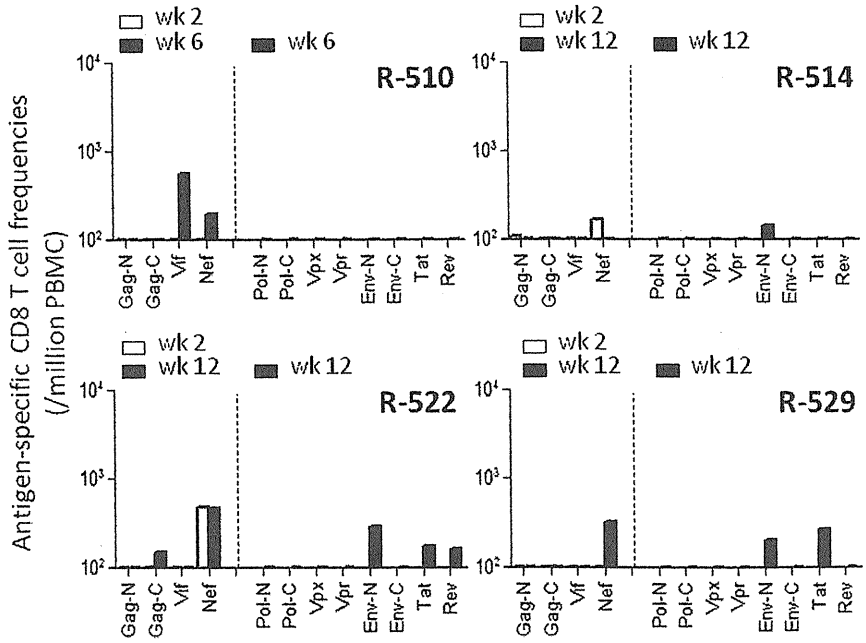


**Fig. 2. SIV antigen-specific CD8$^+$ T cell frequencies in the first group of macaques, which possessed *90-010-Ie*.** Gag-, Vif- and Nef-specific CD8$^+$ T cell frequencies at week 2 and Gag-, Vif-, Nef-, Pol-, Vpx-, Vpr-, Env-, Tat- and Rev-specific CD8$^+$ T cell frequencies at weeks 6 or 12 are shown.

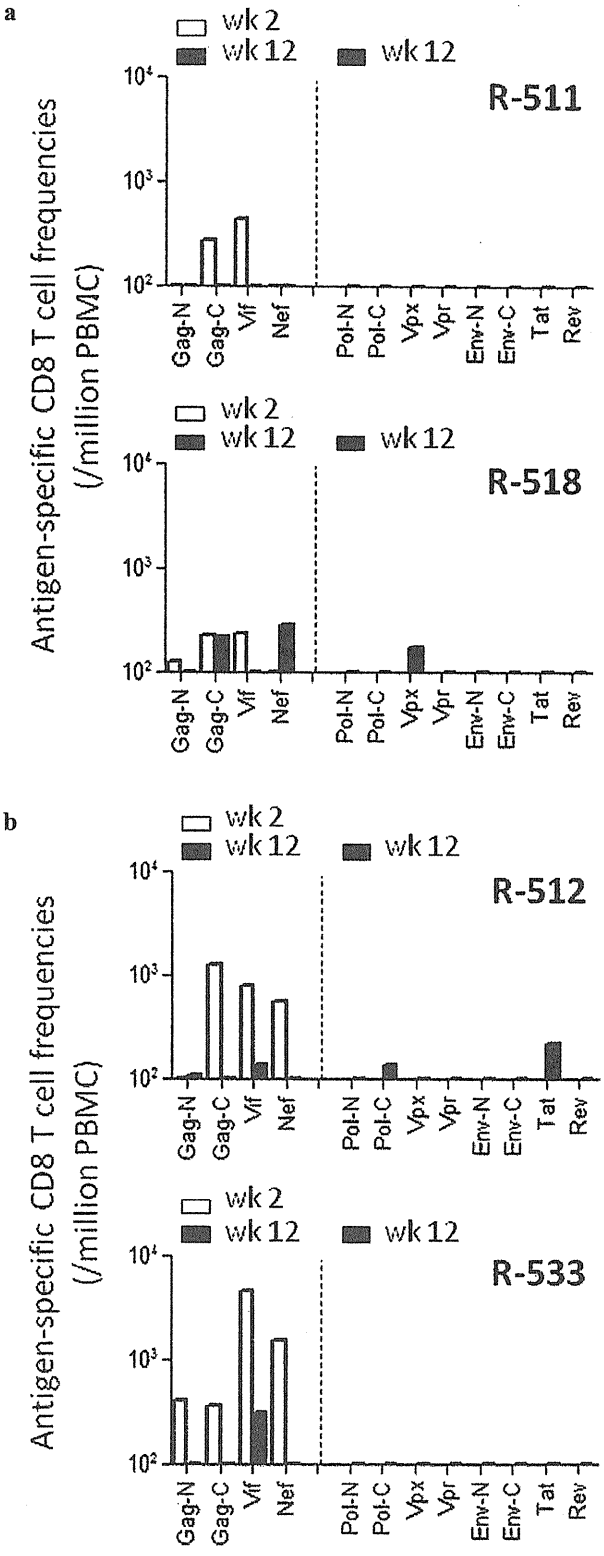


**Fig. 3.** SIV antigen-specific CD8$^+$ T cell frequencies in (a) the second group of macaques, which possessed *89-075-Iw* and (b) the third, which possessed *91-010-Is*.

R-512. Thus, in the four animals possessing *89-075-Iw* or *91-010-Is*, Gag- or Vif-specific CD8$^+$ T cell responses were induced more efficiently than Nef-specific ones at week 2. These responses in PBMCs were mostly diminished at week 12; possibly reflecting the considerable CTL consumption in the effector sites in animals with high viral loads.

## DISCUSSION

Previous studies have indicated the potential of Gag-specific CTL responses to suppress HIV/SIV replication *in vivo* (12, 13, 16). Further, our recent study suggested the potency of Vif-specific CTL responses (17). Then, in the present study, we examined Gag- and Vif-specific CTL responses during primary SIV infection in three groups of animals, each group having a different MHC-I haplotype. Although the numbers of CTL frequencies differed between groups, the CTL responses tended have similar patterns.

Our previous study showed vaccine efficacy in a group of macaques with the MHC-I haplotype *90-120-Ia* (15, 16). Unvaccinated *90-120-Ia*-positive macaques predominantly induce Gag-specific CTL responses but fail to control viremia, while vaccinated ones show enhanced Gag-specific CTL responses and control SIV replication. $Gag_{206–216}$ epitope-specific and $Gag_{241–249}$ epitope-specific CTL responses were shown to be responsible for this vaccine-based viral control (16). However, some Gag-specific CTLs may be effective while others are not. Further analysis of this type of vaccine efficacy would contribute to understanding the requisites for vaccine-based viral control. Possibly, the *89-075-Iw*-positive or *91-010-Is*-positive animals presented in this study may be a candidate model for such analysis.

In primary SIVmac239 infection, it is speculated that some MHC-I haplotypes (referred to as type 1) are associated with Gag/Vif-specific CTL responses while others (referred to as type 2) are not. The MHC-I haplotype *90-120-Ia* described above belongs to type 1. In the present study, the second group, which possess MHC-I haplotype *89-075-Iw*, and the third, which possess *91-010-Is*, both showed efficient Gag- and Vif-specific CTL responses in primary SIV infection, although it remains undetermined whether these MHC-I haplotypes belong to type 1. In contrast, the first group of macaques, which possess MHC-I haplotype *90-010-Ie* did not show efficient Gag- or Vif-specific CTL responses in primary SIV infection. Instead, Nef-specific CTL responses were induced in all four animals. This suggests that the MHC-I haplotype *90-010-Ie* belongs to type 2; that is, primary SIV infection induces no predominant CTL responses specific for Gag/Vif epitopes

restricted by *90-010-Ie*-derived MHC-I molecules. Our results imply that CTLs exerted selective pressure on SIV *gag* and *vif* in the second/third groups but not in the first group. Larger number of animals would enable us to compare those with type 1 and 2 MHC-I haplotypes, which would contribute to our understanding of the efficacy of Gag- and Vif-specific CTL responses against SIV infection.

In developing a prophylactic CTL-inducing AIDS vaccine, it would be important to induce CTL memory resulting in potent CTL responses post-HIV exposure, while prophylactic vaccination can affect the immunodominance patterns of CTL responses post-viral exposure (23, 24). Gag- and Vif-specific CTL memory induction may be a promising vaccine strategy, but the influence of prophylactic vaccination on the patterns of CTL responses post-viral exposure would be affected by MHC-I genotypes. In the hosts in which Gag- and Vif-specific CTL responses are induced during the natural course of SIV infection, Gag- and Vif-specific CTL memory induction by prophylactic vaccination would predominantly enhance these CTL responses. In contrast, in those in whom no Gag- or Vif-specific CTL responses occurred during the natural course of SIV infection, prophylactic vaccination inducing Gag- and Vif-specific CTL responses would result in broader CTL responses. Macaques in which both MHC-I haplotypes belong to type 2 may be ideal for evaluation of this type of vaccine efficacy, but it is very difficult to accumulate those animals. It would be reasonable to use groups of macaques possessing type 2 haplotypes such as the group 1 (*90-010-Ie*-positive macaques) presented in this study for such evaluation.

In summary, by focusing on Gag- and Vif-specific CTL responses, we found two types of rhesus macaques that showed different patterns of CTL responses during primary SIV infection; one elicited Gag- and Vif-specific CTL responses but the other did not. Accumulated analyses in both types of animals would contribute to understanding the impact of these potent CTL responses on primary SIV infection.

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# Intranasal Sendai viral vector vaccination is more immunogenic than intramuscular under pre-existing anti-vector antibodies

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ABSTRACT

Viral vectors are promising vaccine tools for eliciting potent cellular immune responses. Pre-existing anti-vector antibodies, however, can be an obstacle to their clinical use in humans. We previously developed a Sendai virus (SeV) vector vaccine and showed the potential of this vector for efficient CD8+ T-cell induction in macaques. Here, we investigated the immunogenicity of SeV vector vaccination in the presence of anti-SeV antibodies. We compared antigen-specific CD8+ T-cell responses after intranasal or intramuscular immunization with a lower dose (one-tenth of that in our previous studies) of SeV vector expressing simian immunodeficiency virus Gag antigen (SeV-Gag) between naive and pre-SeV-infected cynomolgus macaques. Intranasal SeV-Gag immunization efficiently elicited Gag-specific CD8+ T-cell responses not only in naive but also in pre-SeV-infected animals. In contrast, intramuscular SeV-Gag immunization induced Gag-specific CD8+ T-cell responses efficiently in naive but not in pre-SeV-infected animals. These results indicate that both intranasal and intramuscular SeV administrations are equivalently immunogenic in the absence of anti-SeV antibodies, whereas intranasal SeV vaccination is more immunogenic than intramuscular in the presence of anti-SeV antibodies. It is inferred from a recent report investigating the prevalence of anti-SeV antibodies in humans that SeV-specific neutralizing titers in more than 70% of people are no more than those at the SeV-Gag vaccination in pre-SeV-infected macaques in the present study. Taken together, this study implies the potential of intranasal SeV vector vaccination to induce CD8+ T-cell responses even in humans, suggesting a rationale for proceeding to a vaccine clinical trial using this vector.



## 1. Introduction

Virus-specific CD8+ T-cell responses are crucial for the control of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) replication [1–6]. Efficient induction of virus-specific CD8+ T-cell responses is an important strategy for AIDS vaccine development, and recombinant viral vectors are promising vaccine tools for CD8+ T-cell induction [7,8]. Recent studies have indicated the potential of prophylactic viral vector immunization to induce virus-specific CD8+ T-cell responses and reduce postchallenge viral loads in macaque AIDS models [9–13]. Most of the parental or related viruses of these vectors can induce natural infection in humans. Thus, pre-existing antibodies against the vector virus itself could be an obstacle to viral vector-based CD8+ T-cell induction in humans. Indeed, a clinical trial of a vaccine using adenovirus serotype 5 (AdV5) vectors has shown reduction in efficiency of vaccine-based CD8+ T-cell induction in people with pre-existing anti-AdV5 antibodies [14–17].

We previously developed an AIDS vaccine using a recombinant Sendai virus (SeV) vector and showed that intranasal SeV vector immunization results in efficient induction of antigen-specific CD8+ T-cell responses in macaques [9,18,19]. SeV, murine parainfluenza virus type 1 (PIV-1), is an enveloped virus with a negative-sense RNA genome. SeV replication is localized in the airway because it requires a protease localized in the airway epithelium for envelope protein processing [20]. Thus, replication-competent SeV vectors [21] have been administered intranasally, while replication-defective SeV vectors [22] may be administered intramuscularly as well as intranasally. However, we have not

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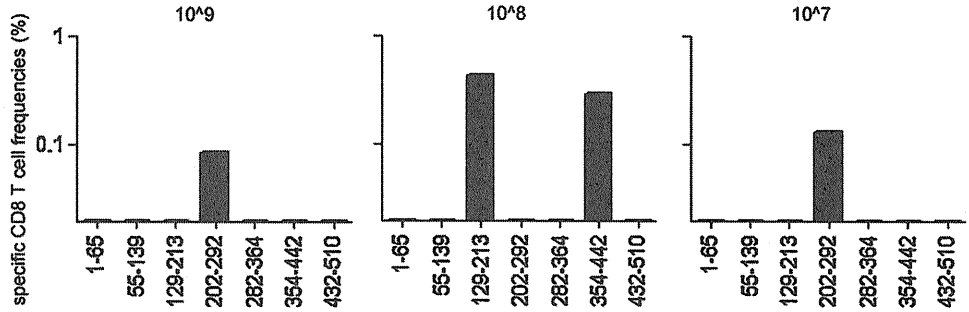


**Fig. 1.** Gag-specific CD8⁺ T-cell responses after intranasal boost with lower doses of F(−)SeV-Gag. Cynomolgus macaques received a DNA vaccination, and six weeks later, were intranasally boosted with $6 \times 10^9$ ($10\hat{9}$), $6 \times 10^8$ ($10\hat{8}$), or $6 \times 10^7$ ($10\hat{7}$) CIU of F(−)SeV-Gag, respectively. Gag peptide pool-specific CD8⁺ T-cell frequencies (percent in CD8⁺ T lymphocytes) two weeks after the boost are shown. A panel of overlapping peptides spanning the entire SIV Gag amino acid (aa) sequence was divided into 7 pools, 1–65 (corresponding to the 1st–65th aa in SIV Gag), 55–139 (55th–139th aa), 129–213 (129th–213th aa), 202–292 (202nd–292nd aa), 282–364 (282nd–364th aa), 354–442 (354th–442nd aa), and 432–510 (432nd–510th aa), and used for the stimulation to detect peptide pool-specific CD8⁺ T cells, respectively.

yet examined the immunogenicity of intramuscular SeV vector vaccination.

The natural host of SeV is mice and its natural infection has not been observed in primates including humans [20]. Antibodies against human PIV-1 (hPIV-1), whose natural infection frequently occurs in humans, are known to cross-react with SeV [23,24]. Our recent analyses in macaques showed efficient Gag-specific CD8⁺ T-cell induction by an intranasal immunization with $6 \times 10^9$ CIU of F(−)SeV-Gag more than one year after an initial SeV vector inoculation, suggesting a possibility of antigen-specific CD8⁺ T-cell induction by SeV vector administration in the presence of SeV-specific neutralizing antibody (NAb) responses [25,26]. However, it remains unclear to what extent SeV-specific NAbs could have adverse effect on CD8⁺ T-cell induction by SeV vector vaccination.

In the present study, we investigated antigen-specific CD8⁺ T-cell responses after intranasal or intramuscular immunization with a lower dose of SeV vector in macaques pre-infected with SeV to sensitively examine the effect of pre-SeV-infection on SeV-based CD8⁺ T-cell induction. Our results revealed that intranasal SeV administration is more immunogenic than intramuscular in the presence of anti-SeV NAbs and suggested the potential of this vector to induce antigen-specific CD8⁺ T-cell responses even in humans.

## 2. Materials and methods

### 2.1. Animal experiments

The animal experiments were conducted through the Cooperative Research Program in Tsukuba Primate Research Center (TPRC), National Institute of Biomedical Innovation with the help of the Corporation for Production and Research of Laboratory Primates. All animals were maintained in accordance with the guidelines for laboratory animals of the National Institute of Infectious Diseases. Blood collection and vaccination were performed under ketamine anesthesia. Cynomolgus macaques (*Macaca fascicularis*) of the TPRC breeding colonies derived from Indonesia, Malaysia, and the Philippines were used for this experiment. All animals received a DNA vaccine followed by a single boost with a replication-defective (non-transmissible) F-deleted SeV expressing SIVmac239 Gag, F(−)SeV-Gag, as described previously [9]. The DNA, CMV-SHIVdEN, used for the vaccination was constructed from an *env*- and *nef*-deleted $SHIV_{MD14YE}$ molecular clone DNA [27] and has the genes encoding SIVmac239 Gag, Pol, Vif, and Vpx, SIVmac239-HIV-1 chimeric Vpr, and HIV-1 Tat and Rev [19]. At the DNA vaccination, animals received 5 mg of CMV-SHIVdEN DNA intramuscularly. Six weeks after the DNA prime, animals intranasally or intramuscularly received a single boost with $6 \times 10^7$, $6 \times 10^8$, or $6 \times 10^9$ cell infectious units (CIU) of F(−)SeV-Gag [22,28]. Group II and IV animals were intranasally infected with $1 \times 10^8$ CIU of replication-competent (transmissible) V-knocked-out SeV [18,21] nine weeks before the DNA prime.

### 2.2. Measurement of Gag-specific CD8⁺ T-cell responses

We measured Gag-specific CD8⁺ T-cell levels by flow-cytometric analysis of gamma interferon (IFN-γ) induction after specific stimulation as described previously [9]. Peripheral blood mononuclear cells (PBMCs) were cocultured with autologous herpesvirus papio-immortalized B lymphoblastoid cell lines (B-LCLs) pulsed with peptide pools using panels of 117 overlapping peptides (mostly 15-mer) spanning the entire SIVmac239 Gag amino acid sequences [25] (Fig. 1) or a vaccinia virus vector expressing SIVmac239 Gag (Figs. 3 and 4) for Gag peptide pool-specific or Gag-specific stimulation. Intracellular IFN-γ staining was performed using CytofixCytoperm kit (BD, Tokyo, Japan) and the following monoclonal antibodies: fluorescein isothiocyanate (FITC)-conjugated anti-human CD4 (BD, #556615, M-T477), peridinin chlorophyll protein (PerCP)-conjugated anti-human CD8 (BD, #347314, SK1), allophycocyanin (APC)-conjugated anti-human CD3 (BD, #557597, SP34-2), and phycoerythrin (PE)-conjugated anti-human IFN-γ antibodies (BD, #557074, 4S.B3). Specific CD8⁺ T-cell levels were calculated by subtracting non-specific IFN-γ⁺ CD8⁺ T-cell frequencies from those after Gag peptide pool-specific or Gag-specific stimulation. Specific CD8⁺ T-cell levels less than 0.02% of CD8⁺ T lymphocytes were considered negative.

### 2.3. Measurement of anti-SeV IgG levels

The plasma anti-SeV immunoglobulin G (IgG) levels were measured by an enzyme-linked immunosorbent assay (ELISA) (Denka Seiken, Tokyo, Japan) using whole inactivated SeV (HVJ Z strain) particles and a peroxidase-conjugated anti-monkey IgG antibody [29].

### 2.4. Measurement of anti-SeV neutralizing titers

We measured plasma SeV-specific neutralizing titers on LLC-MK2 cells using a recombinant SeV expressing enhanced green