Fig. 1. The Δ5G was derived by site-directed mutagenesis of an SIVmac239 infectious DNA clone so that the asparagine residues for the N-glycosylation sites at aa 79, 146, 171, 460 and 479 in gp120 were converted to glutamine residues [2,3]. The Δ5G-ver1, Δ5G-ver2 and Δ3G were also constructed by site-directed mutagenesis from the series of deglycosylated mutants reported previously [2]. The stocks of deglycosylated mutants were prepared by DNA transfection of respective proviral DNAs into 293T cells. The stock of SIVsmE543-3 was prepared as previously described [4]. These virus stocks were propagated in phytohemagglutinin-stimulated peripheral PBMC from rhesus macaques as previously described [3,16].

Animals

Juvenile rhesus macaques originating from Burma were used following negative results of screening for SIV, simian T-cell lymphotropic virus, B virus, and type D retrovirus infection prior to study inception. All animals were housed in individual cages and maintained according to the rules and guidelines for experimental animal welfare as outlined by National Institute of Infectious Diseases and National Institute of Biomedical Innovation, Japan. Full details of the study were approved (Approval number: 507006) by National Institute of Infectious Diseases Institutional Animal Care and Use Committee in accordance with the recommendations of the Weatherall report. Early endpoints are adopted including frequent monitoring of viral loads and immunological parameters, and humane euthanasia is conducted once any manifestation of clinical AIDS or signs of fatal disease is noted.

Vaccination and challenge infection

Three animals per group were intravenously inoculated with 100 $TCID_{50}$ of either of 4 deglycosylation mutants (Δ5G, Δ5G-ver1, Δ5G-ver2 and Δ3G) as shown in Fig. 1. At 40 weeks post infection, 4 SIV-infected animals: Mm0301 (Δ5G), Mm0513 (Δ5G-ver1), Mm0307 (Δ5G-ver2), Mm0304 (Δ3G) and three naive animals (Mm0608, Mm0521, and Mm0522) were intravenously inoculated with 1000 $TCID_{50}$ of SIVmac239 for purposes of homologous virus challenge studies.

To examine the efficacy of the live attenuated vaccine against heterologous virus, 11 vaccinees were intravenously inoculated with 1000 $TCID_{50}$ of SIVsmE543-3.3 as follows: Mm0517 (Δ5G), Mm0511 (Δ5G-ver1), and Mm0512 (Δ5G-ver2) were challenged at 50 weeks post vaccination with the deglycosylation mutant; Mm0409 (Δ5G), Mm0303 (Δ5G-ver1), and Mm0518 (Δ5G-ver2) were challenged at 61 weeks post vaccination; Mm0515 (Δ3G) and Mm0516 (Δ3G) were challenged at 117 weeks post vaccination. 3 naïve animals (Mm0309, Mm0626, Mm0627) were infected with SIVsmE543-3 as vaccine-naïve controls. Furthermore, three of SIVmac239-challenged animals, Mm0301, Mm0513 and Mm0304 (Mm0307 died with a SIV-infection-unrelated cause) were re-challenged with SIVsmE543-3 at 117 weeks post vaccination and 77 weeks post SIVmac239 challenge.

Plasma viral load measurements

SIV infection was monitored by measuring the plasma viral RNA load using a highly sensitive quantitative real-time RT-PCR. Viral RNA was isolated from plasma samples from infected animals using MagNA PureCompact Nucleic Acid Isolation Kit (Roche Diagnostics). Real-time RT-PCR was performed by using QuantiTec Probe RT-PCR kit (Qiagen) and Sequence detection system SDS7000 (Applied Biosystems). To detect SIVmac239 gag and SIVsmE543-3 gag separately, primers and probe sets were synthesized as follow; SIVsmE543-3 gag specific primers: 5′- FAM-GCAGAGGAGGAAATTACCCAGTGC-3′, 5′-CAATTTTACCCAAGCATTTAATGTT- TAMRA- 3′ and probe 5′-TGTCCACCTACCCTTAAGTCCAA-3′, SIVmac239 specific gag primers: 5′-GCAGAGGAGGAAATTACCCAGTAC-3′, 5′-CAATTTTACCCAGGCATTTAATGTT-3′ and probe 5′-FAM-TGTCCACCTGCCATTAAGTCCCGA-TAMRA-3′. These primers and probes do not cross-react with SIVmac239 RNA and SIVsmE543-3 RNA. The detection sensitivity of plasma viral RNA by this method was calculated to be 100 viral RNA copies per ml of plasma.

Sequencing of SIV RNA and proviral DNA

Viral RNA was isolated using MagNA PureCompact Nucleic Acid Isolation Kit (Roche Diagnostics) and cDNA was synthesized with two-step qRT-PCR kit (Invitrogen). PBMC from vaccine recipients were suspended with lysis buffer (10mM Tris 0.5% NP-40 and 0.5% Tween20) with Proteinase K (200 mg/ml), and incubated at 55°C for 1 hour, then heat-inactivated at 95°C for 5 min. Serial 10-fold diluted cDNA or cell lysate was subjected to nested PCR with the Ex-Taq PCR kit (Takara, Tokyo, Japan) with the following condition: 1 cycle of 97°C for 1 min. and then 25 cycles of amplification (94°C for 30 s, 55°C for 30 s, 72°C for 2.5 min) and 72°C for 10 min. and then 4°C for 5 min. Primers were designed to target the several overlapping sequences spanning the open reading frames of SIVmac239 or SIVsmE543-3 as shown in File S1. Positive PCR products were sequenced by using BigDye terminator cycle sequencing kits (Applied Biosystems) and analyzed by using ABI3100 or ABI 3130xl Genetic Analyzer (Applied Biosystems). Sequences were assembled using ATGC version 4.2 (Genetyx Corporation).

SIV specific T cell responses

The T cells in the animals were examined for virus specific cellular response against the vaccine virus and the challenge virus by using pooled peptides covering overlapping sequences of all viral proteins of SIVmac239 and SIVsmE543-3 respectively. Briefly, cryopreserved PBMC were thawed, resuspended at 2×10^6 cells/ml in R10 (RPMI1640 supplemented with 10% heat-inactivated FCS, 55 μM 2-mercaptoethanol, 50 U/ml penicillin and 50 μg/ml streptomycin), and rested for 2 h at 37°C. The cells were washed and aliquots of 10^5 cells were stimulated with each pool of peptides at a final concentration of 2 mg/ml in an anti-IFN-g Ab-coated plate overnight. ELISPOT assay for the detection of IFN-g secreting cells were performed using a commercial ELISPOT kit (U-CyTech Bioscience). Peptides based on sequences of SIVmac239 viral proteins were synthesized by the Microchemical Facility, Emory University School of Medicine, Atlanta, GA, USA. Peptides based on the sequences of SIVsmE543-3 viral proteins were synthesized by Sigma-Aldrich Japan.

Neutralization assay

Virus neutralizing antibodies were tested according to a protocol using CEMx174/SIVLTR-SEAP cells [31] as described previously [3]. Serially diluted heat-inactivated plasma was tested for inhibition of the corresponding vaccine virus or the challenge virus (SIVsmE543-3) in CEMx174/SIVLTR-SEAP cells. SEAP activity in the culture supernatant was assayed using a commercial SEAP reporter gene assay chemiluminescent kit (Roche Diagnostics).

Statistical analysis

Correlation analysis was performed using Spearman's non-parametric rank test and Mann-Whiney 'U' test by using Graph

pad Prism 4.0 software. Correlations were considered statistically significant when P values were <0.05.

DNA sequence data deposition

The SIV sequences reported in this paper have been deposited in the DNA Data Bank of Japan (accession nos. AB553915 to AB554013).

Supporting Information

File S1

Found at: doi:10.1371/journal.pone.0011678.s001 (0.28 MB PDF)

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Author Contributions

Conceived and designed the experiments: CS AK MM YS NY KM. Performed the experiments: CS SW TN EK NU KU HS KM. Analyzed the data: CS SW TN EK TS NU KU HS KM. Contributed reagents/materials/analysis tools: SO VH. Wrote the paper: CS FV AAA YN KM.

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ORIGINAL PAPER

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

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

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

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Introduction

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

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

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

To evaluate the efficacy of SIV vaccination, it is necessary to characterize the MHC alleles because the presentation of antigenic peptides by MHC molecules to T cells, more specifically the binding of antigenic peptide to the MHC molecule, depends on the structure of the MHC allele. We have previously developed a reference strand conformation analysis-based typing system for *Mamu* class I genes and reported that the number of expressing genes varies among macaques of Burmese or Laotian origin; we could identify at least 16 different *Mamu* class I locus haplotypes that were composed of different numbers of *Mamu* class I genes (Tanaka-Takahashi et al. 2007). In addition, we reported that a haplotype of *Mamu* class I genes, *90-120-Ia*, exerted a protective vaccination against SIVmac239 challenge (Matano et al. 2004). Furthermore, it was revealed that one of highly expressed *Mamu-A* allele of the *90-120-Ia* haplotype, *Mamu-A1*065:01* (previously designated as *Mamu-A*90120-5*), encoded a Mamu-A molecule that could efficiently present a SIV-derived $Gag_{241\text{-}249}$ peptide to cytotoxic T cells from the vaccinated macaques (Tsukamoto et al. 2008).

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

Materials and methods

Animals

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

Sequencing analysis of cDNAs from *Mamu* class I genes

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

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

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

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

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

Table 2 (continued)

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

[a] New alleles are indicated as novel

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

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

[d] Identical sequences found in *Mafa* or *Mane* alleles

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

Data analyses and nomenclature for *Mamu* class I alleles

Nucleotide sequences of cDNAs were analyzed and aligned using Genetyx Ver. 8 software package (Genetyx Corp., Japan). When at least three clones from independent PCR or from different individuals showed identical sequences, we submitted the sequences to DNA Data Bank of Japan database and to the Immuno Polymorphism Database for nonhuman primate MHC (http://www.ebi.ac.uk/ipd/mhc/submit.html; Robinson et al. 2003) to obtain official nomenclature for novel alleles of *Mamu-A* and *-B* genes. Phylogenetic analysis of *Mamu-A* sequences corresponding to exon 2, 3 and a part of exon 4 obtained in this study was done by using Gentetyx Ver. 8 software package. *Mamu-A1* 001:01* was included in the analysis as a reference. Neighbor-joining trees were constructed with the Kimura 2 parameter method. Bootstrap values were based on 5,000 replications.

Results

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

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

clones with identical sequences were obtained from two independent PCR for an individual or from at least two individuals, the nucleotide sequences were considered to be real and not artifacts. We identified 73 different *Mamu-A* sequences in 100 individuals. Among them, 44 (60.2%) were novel, whereas the other 29 (39.8%) were identical to those reported mainly from Chinese- or Indian-origin macaques (Table 2). In addition, 50 sequences were from *Mamu-A1*, while 14, 1, 3, 3, and 2 sequences were from *Mamu-A2*, *-A3*, *-A4*, *-A5*, and *-A6*, respectively (Table 2). A neighbor-joining analysis showed that the sequences from the same minor *Mamu-A* genes were clustered with each other (Fig. 1).

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

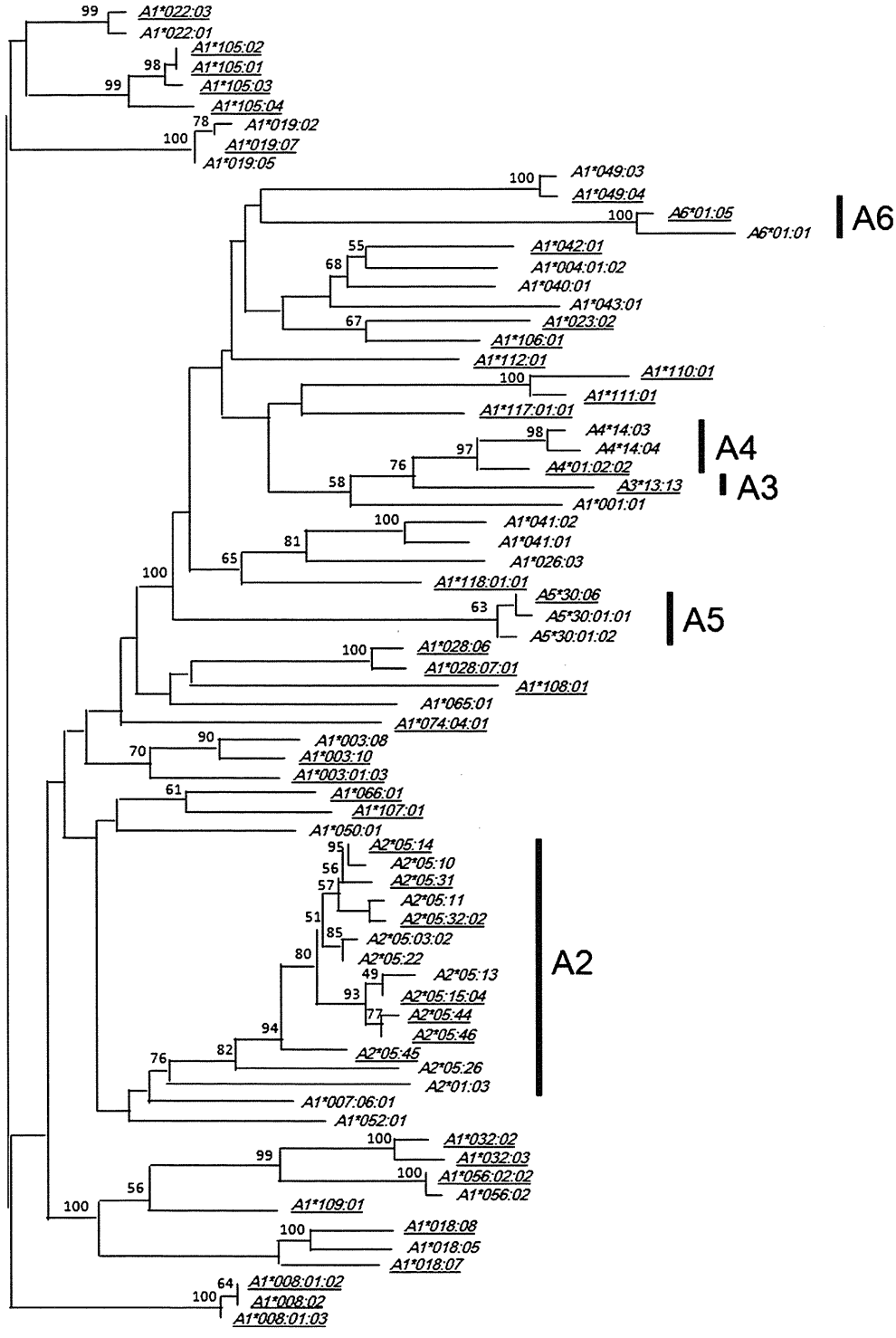

Fig. 1 Phylogenetic tree of *Mamu-A* alleles detected in this study. The tree was constructed using neighbor-joining method with bootstrap values of 5,000 replications. The values are indicated as percentages and those values less than 50% are not shown. *Mamu-A1* 001:01* was included in the analysis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

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

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

Table 3 (continued)

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

Table 3 (continued)

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

[a] New alleles are indicated as novel

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

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

[d] Identical sequences found in *Mafa* or *Mane* alleles

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

Mamu class I haplotypes observed in Burmese-origin macaques

From the cDNA analyses of genetically related macaques, we could identify the *Mamu-A* and *Mamu-B* sequences comprising 13 different haplotypes from seven paternal lineages (haplotype 'w' was shared by 89-075 and its offspring 91-010F1) and eight other haplotypes in the colonies; the *Mamu* class I haplotype consisted of one to three expressing *Mamu-A* genes and one to five expressing *Mamu-B* (including Mamu-I) genes, confirming that the number of expressed *Mamu* class I genes varied with the haplotype (Table 4). Examples of family pedigrees are shown in Fig. 2. Although usually only one *Mamu-A1* allele could be identified in the haplotypes, the *90-120-a* haplotype carried two different *Mamu-A1* alleles, which was confirmed by the analysis of family pedigree (Fig. 2a). In addition, *Mamu-B*001* alleles were tightly linked to a *Mamu-B*007* allele (Table 4).

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

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

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

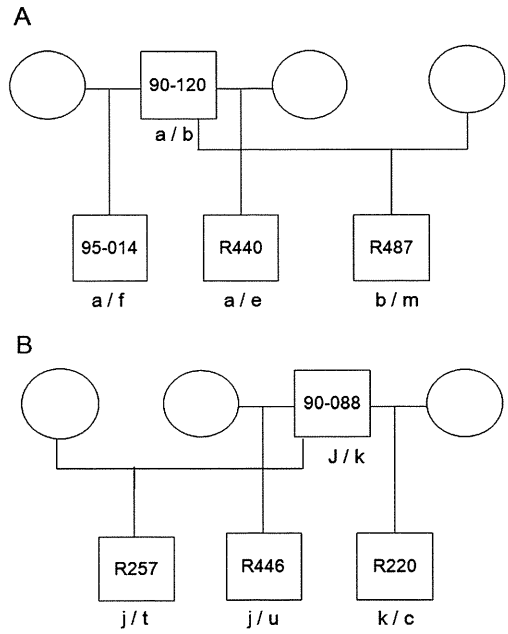

Fig. 2 Segregation of *Mamu* class I haplotypes in the pedigrees of macaques. Pedigree information and haplotype information are indicated along with ID of macaques. A. *Mamu* class I haplotypes of *a* and *b* in the parent (90-120) were segregated to its offspring 95-014, R440, and R487. B. *Mamu* class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

Discussion

The rhesus macaque is widely used in the experimental design for developing a vaccine against HIV. Indian-origin macaques are well characterized as a model system and it has been reported that there are several "elite controller" alleles such as *Mamu-A*001* and *Mamu-B*017*, with which most macaques showed lower viral loads after SIVmac239 challenge (Friedrich et al. 2004). In this study, we did not observe *Mamu-A1*001* in Burmese-origin macaques, while we previously reported that a group of animals carrying the MHC class I haplotype 90120a ('a' haplotype designated in this study, Table 4) showed vaccine-based control of SIVmac239 replication (Matano et al. 2004). This haplotype contains *Mamu-A*065:01* (previously noted as *Mamu-A*90120-5*) allele, and cytotoxic T lymphocyte (CTL) responses specific for an SIVmac239 $Gag_{241\text{-}249}$ (SSVDEQIQW) epitope restricted by this *Mamu-A1* allele are responsible for the SIV control in the vaccinated macaques carrying the 90120a haplotype (Kawada et al. 2008). Interestingly, the SIV $Gag_{241\text{-}249}$ epitope is overlapped with a HLA-B*5701-restricted HIV-1 $Gag_{240\text{-}249}$ epitope, TW10 (TSTLQEQIAW), and TW10-specific CTL responses have also been indicated to exert strong suppression on HIV-1 replication resulting in lower viral loads (Tsukamoto et al. 2008; Goulder and Watkins 2008).

Among 73 *Mamu-A* sequences detected in this study, only four sequences were reported to be found in the Indian-origin macaques. In clear contrast, 25 *Mamu-A* sequences were also found in the Chinese-origin macaques, implying that the genetic background of Burmese-origin macaques might be closer to Chinese-origin macaques than to Indian-origin macaques. However, 27 and 25 *Mamu-B* sequences were identical to those reported in Chinese- and Indian-origin macaques, respectively, demonstrating that Burmese-origin macaques represent a mixture of geographically distinct Chinese- and Indian-origin macaque populations. In addition, more than half of *Mamu* class I alleles found in this study were novel, indicating that the regional difference in MHC allelic distribution exists similar to that in human HLA. Because the habitat of Burmese-origin rhesus macaques is overlapped in part with the habitat of crab-eating macaques (cynomolgus rhesus, *Macaca fascicularis*) and Southern pig-tailed macaques (*Macaca nemestrina*), it is interesting to investigate whether the identical sequences to *Mamu* class I alleles would be frequently found in *Mafa* or *Mane* class I alleles. As shown in Tables 2 and 3, about 10% of *Mamu* class I alleles had identical sequences to equivalent *Mafa* or *Mane* class I alleles, as has been observed in the other macaque populations (Campbell et al. 2009; Otting et al. 2009), demonstrating that the frequency of shared MHC class I alleles was relatively constant in different populations of macaques.

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

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

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

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

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

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

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Original Article

Expression of proinflammatory cytokines and its relationship with virus infection in the brain of macaques inoculated with macrophage-tropic simian immunodeficiency virus

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The pathogenesis of acquired immunodeficiency syndrome dementia complex (ADC) is still poorly understood. Many studies suggest that proinflammatory cytokines such as IL-1β and TNF-α released by microglia/macrophages or astrocytes play a role in CNS injury. A microscopic finding of a microglial nodule with multinucleated giant cells (MNGCs) is a histopathologic hallmark of ADC and named HIV encephalitis. However, in vivo expression of these cytokines in this microenvironment of HIV encephalitis is not yet clarified. One of the main reasons is complexities of brain pathology in patients who have died from terminal AIDS. In this study, we infected two macaques with macrophage-tropic Simian immunodeficiency virus SIV239env/MERT and examined expression of TNF-α and IL-1β in inflammatory lesions with MNGCs and its relation to virus-infected cells using immunohistochemistry. One macaque showed typical inflammatory lesions with MNGCs in the frontal white matter. Small microglial nodules were also detected in the basal ganglia and the spinal cord. SIVenv positive cells were detected mainly in inflammatory lesions, and seemed to be microglia/macrophages and MNGCs based on their morphology. Expression of IL-1β and TNF-α were detected in the inflammatory lesions with MNGCs, and these positive cells were found to be negative for SIVenv by double-labeling immunohistochemistry or immunohistochemistry of serial sections. There were a few TNF-α positive cells and almost no IL-1β positive cells in the area other than inflammatory lesions. Another macaque showed scattered CD3+ cells and CD68+ cells in the perivascular regions of the white matter. SIVenv and TNF-α was demonstrated in a few perivascular macrophages. These findings indicate that virus-infected microglia/macrophages do not always express IL-1β and TNF-α, which suggests an indirect role of HIV-1-infected cells in cytokine-mediated pathogenesis of ADC. Our macaque model for human ADC may be useful for better understanding of its pathogenesis.

Key words: cytokines, HIV encephalitis, macaque model, macrophage-tropic, simian immunodeficiency virus.

INTRODUCTION

Human immunodeficiency virus-1 (HIV-1) can induce a clinical triad of progressive cognitive decline, motor dysfunction, and behavioral abnormalities named acquired immunodeficiency syndrome dementia complex (ADC). Although the introduction of highly active anti-retroviral therapy (HAART) has been successful to reduce progression of acquired immunodeficiency syndrome (AIDS), controversial results have been reported that the prevalence of dementia may eventually increase, corresponding to a longer life span of people with HIV-1 infection.[1-3]

Acquired immunodeficiency syndrome dementia complex is histopathologically identified by diffuse and

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nodular microgliosis with formation of multinucleated giant cells (MNGCs) in the white mater of the brain and is termed HIV encephalitis.[4,5] Myelin pallor[6] and axonal damage[7–9] with abundant HIV-infected macrophages and microglial cells have been demonstrated in the white mater.[5,10] Many studies suggest proinflammatory cytokines such as IL-1β and TNF-α released by microglia and macrophages which play a role in CNS injury.[11,12] On the other hand, infection of astrocytes may also occur with limited virus replication,[13] and astrocytes could cause CNS injury by secreting cytokines.[14–17] These observations suggest a role of proinflammatory cytokines in ADC. However, in vivo expression of these cytokines in the microenvironment of HIV encephalitis, microglial nodules with MNGCs, the histopathologic hallmark of ADC, is not yet clarified. One of the main reasons is presumed to be the complexities of brain pathology in patients who have died from terminal AIDS.

Simian immunodeficiency virus (SIV) infection of macaques has been shown to recapitulate key features of HIV infection of the human CNS, including the development of encephalitis with characteristic histopathological changes and psychomotor impairment.[18,19] Our previous study demonstrated that a macrophage-tropic SIV, SIV239env/MERT, caused typical microglial nodules with MNGCs without development of AIDS. Using this animal model of HIV-1 encephalitis, we explored which cell types expressed TNF-α and IL-1β and whether it is related to viral infection in the microenvironment of SIV encephalitis.

MATERIALS AND METHODS

Virus and animal

SIV239env/MERT is a macrophage-tropic virus the pathogenic properties of which have been previously described. This virus comprises four amino acid substitutions in the env of SIVmac239 backbone, and replicates as efficiently as the highly macrophage-tropic virus SIVmac316 in the alveolar macrophages.[20]

The rhesus macaques were screened and found to be seronegative for SIV, STLV, B virus, and type D retroviruses. Two macaques, #531 and #626, were infected intravenously with 100 TCID50 of SIV239env/MERT. Three uninfected macaques were used as controls. The animals were housed in individual cages and maintained according to the rules and guidelines of the National Institute for Infectious Diseases (NIID) for experimental animal welfare.

CD4+ cell count and viral RNA load

CD4+ cell counts were performed in peripheral blood specimens at the time of autopsy. To measure the level of virus replication in the periphery, viral RNA was quantified in plasma at autopsy. Viral RNA in the plasma of inoculated macaques was measured by real-time RT_PCR.

Histopathological examination

Routine histopathological methods applied are described elsewhere.[21] In brief, coronal sections of the brain and spinal cord were embedded in paraffin. For routine light microscopy, the paraffin sections were stained with HE and KB.

We used EnVision system (Dako, Carpinteria, CA, US) for immunohistochemistry. The following antibodies were used as the first antibodies: a mouse monoclonal antibody (mAb) anti-human TNF-α (1:400, abcam K. K., Tokyo, Japan), a rabbit polyclonal antibody IL-1β (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, US), a mAb anti-GFAP (1:100; Chemicon International, Temecula, CA, US), a rabbit anti-human CD3 (1:50; Dako, Glostrup, Denmark), a mAb anti-human macrophage CD68 (KP1; 1:50; Dako), a rabbit anti-Iba1 antibody (1:500, Wako Pure Chemical Industries, Osaka, Japan), and a mAb anti–SIV envelope gp160/gp32, KK41 (1:50; National Institute for Biological Standards and Control, Herts, UK). Immunoreactivity was visualized using either diaminobenzidine/peroxidase or 3-amino-9-ethylcarbazole (Dako, Carpinteria, CA, US) substrate–chromogen system (Dako). Light counterstaining was done with hematoxylin. For the SIV envelope gp160/gp32 immunostaning, a lymph node section from an SIV-infected rhesus macaque was used as a positive control. In the same way, for the TNF-α and IL-1β immunostaining, a tonsil section was used as a positive control.

Double-label immunohistochemistry

We performed double-label immunohistochemistry for IL-1β and SIVKK41 using the same section to examine the expressions of IL-1β correlating with the SIVenvgp160/gp32- positive cells. This entailed performing immunohistochemistry for IL-1β, followed by immunohistochemistry for SIVKK41. Double labeling was performed using AEC/peroxidase followed by Vector blue/alkaline phosphatase.

RESULTS

Clinical manifestation

Macaque #531 showed very slow progression of clinical course. A CD4+ cell count remained moderately decreased, 270/μL, even long after infection, and was sacrificed for autopsy 154 weeks after infection. Plasma viral load was relatively high, 277 800 copies/mL, at autopsy. Macaque #626 also showed also progression of clinical

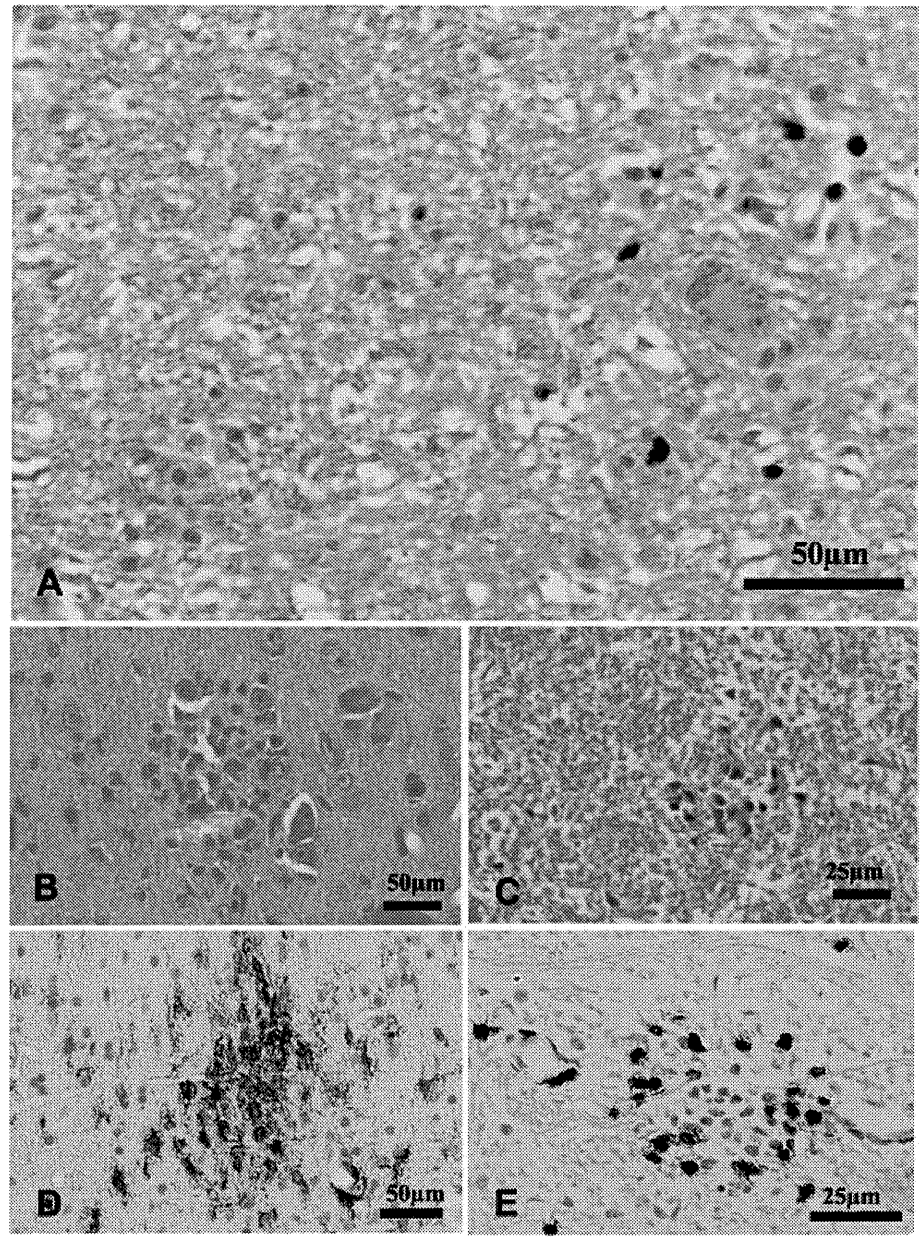

Fig. 1 Histopathological findings of the brain in macaque #531. A typical inflammatory lesion with microglial nodule with multinucleated giant cells (MNGCs) in the frontal white matter (A). Small microglial nodules seen in the basal ganglia (B), and the spinal cord (C). Microglial nodules were mainly composed of microglia/macrophages (D), and CD3+ T-cells (E). A, B, and C: HE; D: CD68; E: CD3. A: frontal white matter, B and D: basal ganglia, C and E: spinal cord.

course. CD4+ cell count remained moderately decreased, 220/μL, even long after infection. It was sacrificed for autopsy 218 weeks after infection. Plasma viral load remained low, 1000 copies/mL, at autopsy. These two macaques did not show obvious neurological symptoms or behavior abnormality.

Histopathological findings of the brain and lymph nodes

Macaque #531 showed a pathological hallmark of AIDS encephalopathy such as typical inflammatory lesions with MNGCs in the frontal white matter, (Fig. 1A). To a lesser extent, microglial nodules were also detected in the basal ganglia and spinal cord (Fig. 1B–C). Microglial nodules were mainly composed of microglia/macrophages (Fig. 1D), and CD3+ T-cells were scattered in the surrounding areas (Fig. 1E). Astrocytic gliosis was not accentuated in the areas of microglia nodules. On the other hand, no abnormality was observed in the cerebral cortex and cerebellum. Another macaque, #626, showed scattered CD3+ cells and CD68+ cells in the perivascular regions of the white matter and the meninges. However, microglial nodules with MNGCs could not be found. No other pathologic abnormality was found in the brain.

Virus-infected cells were detected by the immunostaining of the SIVenvgp160/gp32. In macaque #531, positive cells were detected mainly in inflammatory lesions

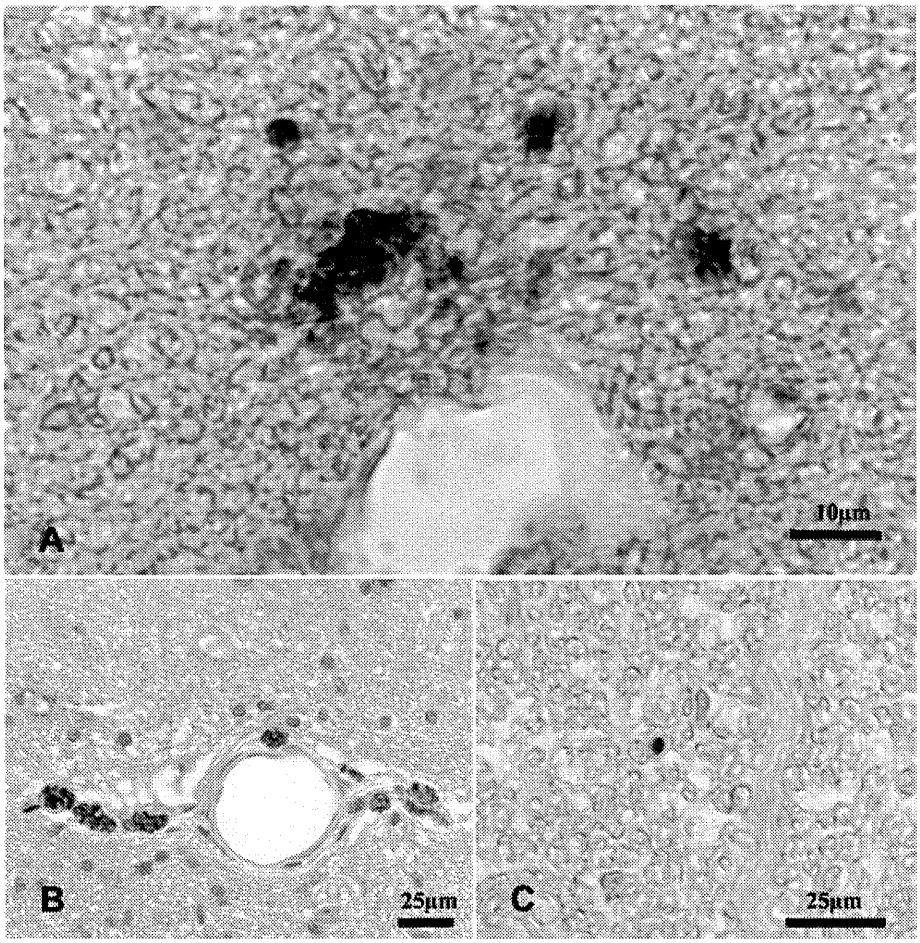

Fig. 2 Expression of a simian immunodeficiency virus (SIV) envelope protein by the immunostaining of SIVenvgp160/gp32 in macaque #531. SIV envelope protein is demonstrated in an inflammatory lesion in the frontal white matter (A), some perivascular macrophages in the basal ganglia (B), and a few in the spinal cord (C).

(Figs 2A,4B), and seemed to be microglia/macrophages and MNGCs based on their morphology. Some perivascular macrophages were also positive in basal ganglia (Fig. 2B). We also detected a few positive cells in the cerebellum and the spinal cord (Fig. 2C) as well as meningeal mononuclear cells. In another macaque, #626, SIVenvgp160/gp32 positive cells were limited to a few perivascular and meningeal mononuclear cells.

The lymph nodes of two virus-infected animals showed hyperplasia of follicles and their germinal centers showed irregular shapes. Decrease of CD3+ T-cells in the paracortical region was not evident.

All control macaques showed no abnormality in both brains and lymph nodes.

The expressions of TNF-α and IL-1β in inflammatory lesions

Since macaque #531 showed typical inflammatory lesions with MNGCs, we further examined expression of proinflammatory cytokines by immunohistochemistry. IL-1β-positive cells showed intracytoplasmic labeling. Positive cells were detected only in inflammatory lesions with MNGCs of the frontal white matter, that is to say, we could not detect IL-1β-positive cells in the parenchyma of basal ganglia as well as in the spinal cord. In order to investigate the relation between expression of IL-1β and virus infection, we performed double-label immunohistochemistry for IL-1β and SIVenvgp160/gp32. Interestingly, the IL-1β positive cells were found around the SIVenvgp160/gp32-positive cells, but not SIVenvgp160/gp32-positive cells (Fig. 3). The brain parenchyma of macaque #626 did not showed any IL-1β-positive cells.

TNF-α was also labeled as cytoplasmic staining. Positive cells were detected in some mononuclear cells of inflammatory lesions in the frontal white matter and basal ganglia, as well as a few perivascular macrophages. We could not detect TNF-α-positive cells in the spinal cord. TNF-α-positive cells seemed to be SIVenvgp160/gp32-negative cells in comparison with distribution of SIVenvgp160/gp32-positive cells stained using a serial section (Fig. 4). In macaque #626, a few TNF-α-positive cells were detected in perivascular and meningeal mononuclear cells.

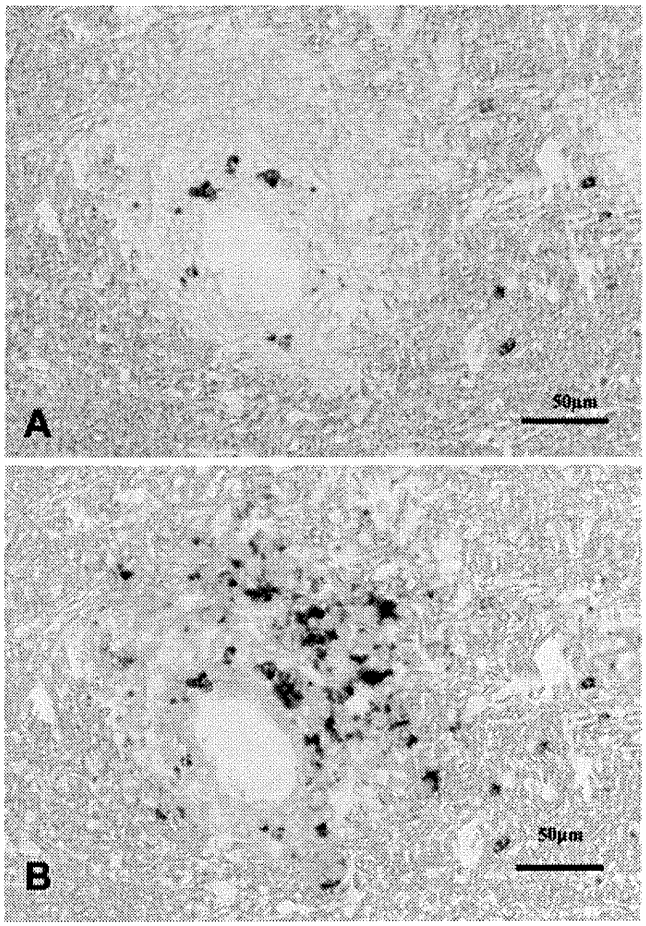

Fig. 3 Expression of IL-1β and SIVenvgp160/gp32 in an inflammatory lesion with microglial nodule with multinucleated giant cells (MNGCs). (A) IL-1β-positive cells are detected only in an inflammatory lesion with MNGCs seen in the frontal white matter of macaque #531. (B) IL-1β positive cells were found around the SIVenvgp160/gp32-positive cells but not SIVenvgp160/gp32-positive cells demonstrated by double-labeling immunohistochemistry performed using the same section of (A). A: anti-IL-1β; B: double-label immunohistochemistry for IL-1β (red) and SIVenvgp160/ gp32 (dark blue).

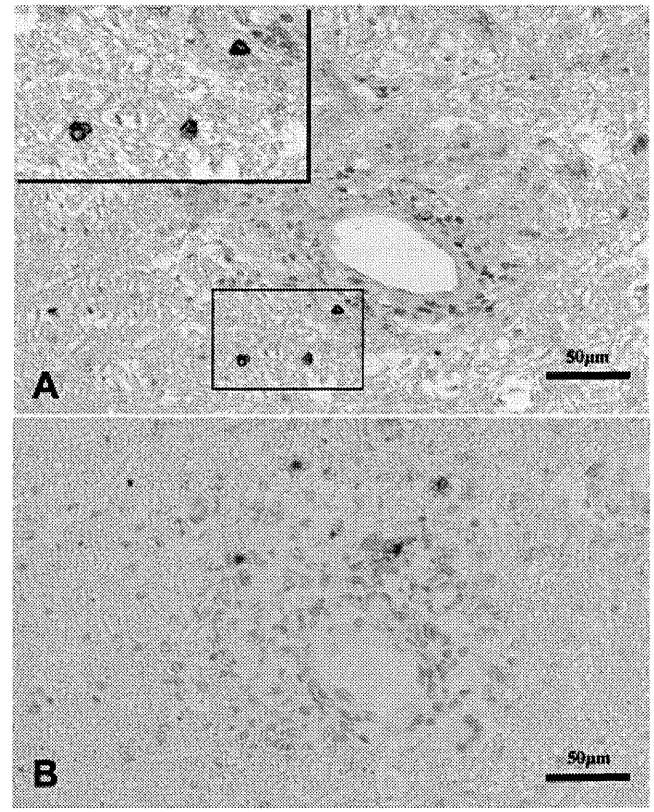

Fig. 4 Expression of TNF-α and SIVenvgp160/gp32 in an inflammatory lesion with microglial nodule with multinucleated giant cells (MNGCs). (A): TNF-α-positive cells are detected in mononuclear cells of an inflammatory lesion with MNGCs seen in the frontal white matter of macaque #531. (B): Distribution of SIVenvgp160/gp32-positive cells differs from that of TNF-α-positive cells demonstrated by SIVenvgp160/gp32 immunohistochemistry of serial section. A: anti-TNF-α, B: anti-SIVenvgp160/gp32.

DISCUSSION

Cytokines such as TNF-α and IL-1β may have toxic effects on CNS cells and have been postulated to contribute to the pathogenesis of the neurological complications of human immunodeficiency virus (HIV) infection.[22] However many of such studies were done by in vitro experiments; exposure of macrophages and microglia to either gp120 or Tat resulted in up-regulation of TNF-α expression,[23,24] and exposure of microglia to gp120 resulted in the production of IL-1β.[25,26] In contrast, there are only a few reports which demonstrated proinflammatory cytokines in the AIDS brain tissues directly in vivo. Tyor *et al.*[11] reported that there were significant increases in IL-1β and TNF-α in HIV-positive patients compared with HIV-negative brains, but no correlation was found between levels of cytokines and the presence or absence of CNS disease among HIV-positive individuals. In addition, in vivo expression of these cytokines in the microenvironment of HIV encephalitis, microglial nodules with MNGCs, was not demonstrated in their study. Zhao *et al.*[27] reported that IL-1β was expressed at high levels in areas of microglial nodules in HIV encephalitis. Because some MNGCs were positive for IL-1β in their report, they suggested that IL-1β was induced by HIV-1 infection.

In our present study, expression of IL-1β and TNF-α were detected in the inflammatory lesions with MNGCs, and these positive cells were found to be negative for SIVenvgp160/gp32. There were a few TNF-α positive cells and almost no IL-1β positive cells in the area other than inflammatory lesions including microglial nodules. Our findings indicate that virus-infected microglia/ macrophages do not always express IL-1β and TNF-α. The

findings seen in macaque #531 might indicate a limited role of IL-1β and TNF-α in the very early stage of ADC. In order to understand a precise role of proinflammatory cytokines in ADC, further studies are required focusing on origin or nature of the cells expressing proinflammatory cytokines.

The differences between previous reports[22,27] and our present data about in vivo expression of cytokines might be explained by complexities of brain pathology in patients who have died from terminal AIDS. Human autopsies were usually performed in the advanced stages of AIDS. In such conditions, the brains may contain a variety of pathologic conditions other than HIV encephalitis such as diffuse poliodystrophy, another pathologic event of ADC, many kinds of opportunistic infections and tumors, and/or effects of anti-viral agents. Our macaque #531 with typical pathologic findings of SIV encephalitis was not in the stage of AIDS, and opportunistic diseases or diffuse poliodystrophy were not observed. We can also exclude the effects of chemotherapy.

In the present study, macaque #531 with typical SIV encephalitis did not show obvious neurological symptoms or behavior abnormality. This reminded us of a previous report in which the brains of asymptomatic HIV-1-positive individuals who died accidentally revealed HIV-1 infection and inflammatory response in the cerebral white matter.[28] These observations indicate that histopathologic findings of HIV encephalitis might be subclinical in many individuals infected with HIV-1. Another macaque (#626) did not show microglial nodules with MNGCs. The plasma viral load of this animal was much lower than that of macaque #531. This suggested that presence or absence of HIV encephalitis might simply depend on the value of plasma viral load.

Our macaque infected with SIV239env/MERT induced typical microglial nodules with MNGCs as a model of HIV encephalitis, and this macaque model may be useful for the better understanding of HIV encephalitis pathogenesis.

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