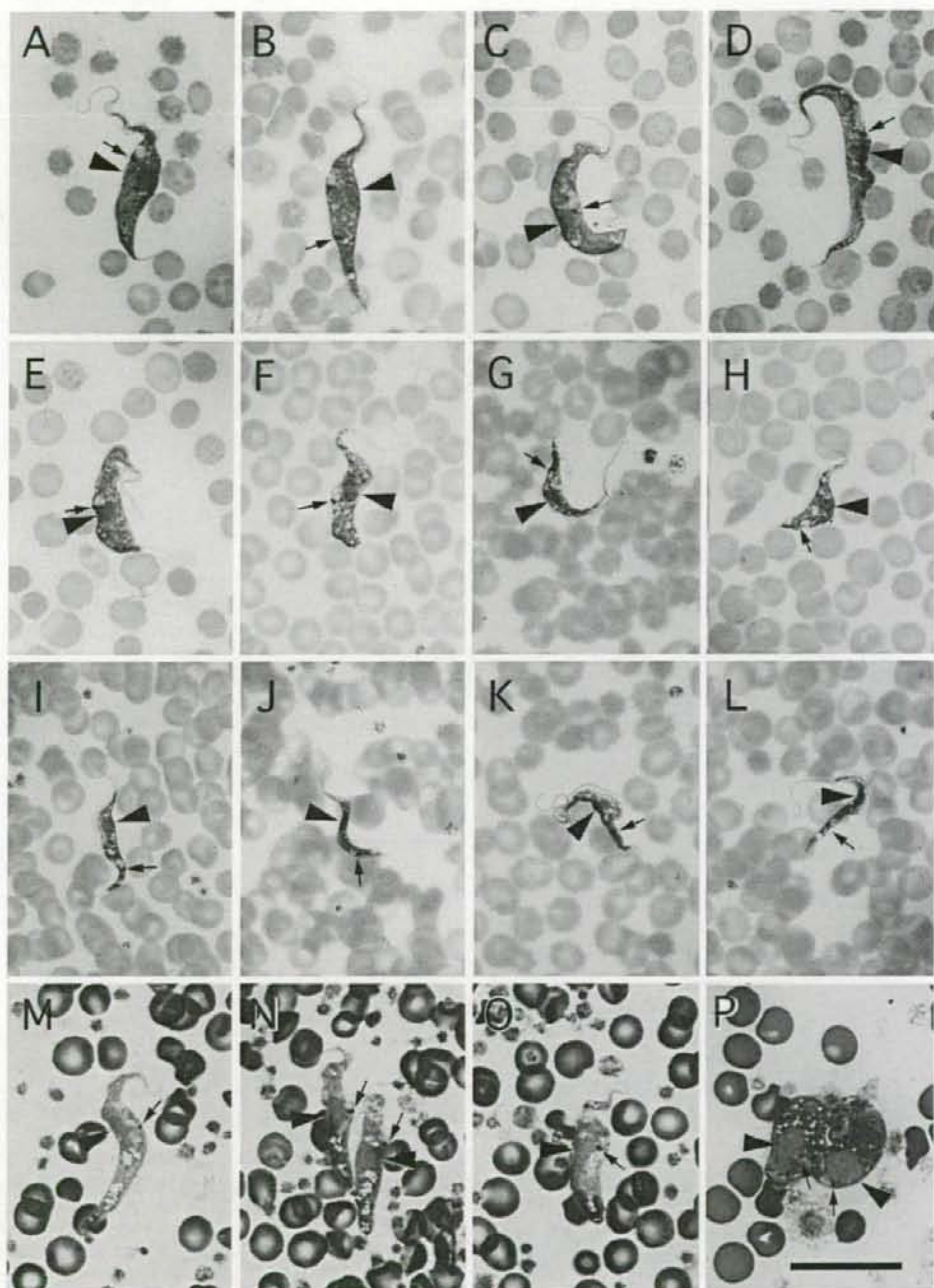

FIGURE 1. *Trypanosoma minasense* in the peripheral blood of squirrel monkeys (**A–L**) and red-handed tamarins (**M–P**). Arrow head indicates the nucleus and small arrow indicates the kinetoplast. The specimens have been stained with Giemsa's solution. The scale bar in (P) applies to all parts of figure and = 20 μm; all photographs are at the same magnification.

TABLE I. Morphometric comparison of *T. minasense* with previously recorded species of the subgenus *Megatrypanum* and *T. rangeli* in small neotropical monkeys.*

Parasite	Host	Number of samples	Reference	L
T. minasense	*Saimiri sciureus*	17	The present study (Natural infection)	29.7–46.7 (40.0 ± 4.8)
T. minasense	*Saimiri sciureus*	12	Ziccardi and Lourenço-de-Oliveira (1999) (Experimental infection)	26–40 (32.3 ± 1.33)
T. minasense	*Callithrix penicillata*	19	Ziccardi and Lourenço-de-Oliveira (1999) (Experimental infection)	30–40 (38.9 ± 0.8)
T. devei	*Midas midas* *Midas rufimanus* *Leontocebus tamarin*	? (multiple sources)	Hoare (1972)	38.7–45.5
T. lambrechti	*Cebus griseus* *Cebus albifrons*	?	Marinkelle (1968) cited by Hoare (1972)	30.1–43.2 (34.9)
T. rangeli	*Saimiri sciureus* *Saimiri ustus* *Callithrix penicillata*	17 (multiple sources)	Ziccardi and Lourenço-de-Oliveira (1998) (Experimental infection in marmosets and natural infection in squirrel monkeys)	29–36 (30.9 ± 0.49)

* Expressed as range, followed by mean or mean ± SD in parentheses.
† Calculated after Deane and Damasceno (1961), i.e., KI = PK/KN.
‡ Calculated after Hoare (1972), i.e., KI = (PK + KN)/KN.

rangeli; and the other 1 sequence from 1 tamarin (no. 15) situated near *T. rangeli*, *T. conorhini*, and *T. vespertilionis*, far from *T. theileri* and other trypanosome species with *T. theileri*-like morphology.

Microfilariae in the blood

Many microfilariae of *Mansonella* (*Tetrapetalonema*) *mariae* Petit, Bain and Roussihon, 1985, 319 ± 18 (range 295–357) μm in length and 2.7 ± 0.2 (range 2.2–2.8) μm in width (n = 10), were found in Giemsa-stained thin blood films from 22 squirrel monkeys (prevalence, 25.9%) (Fig. 6A). Several sheathed microfilariae of *Dipetalonema caudispina* (Molin, 1858) Diesing, 1861, 237 ± 17 (range 192–269) μm in length and 4.6 ± 0.3 (range 4.0–5.0) μm in width (n = 14), were found in Giemsa-stained thin blood films from 3 squirrel monkeys (prevalence, 3.5%) (Fig. 6B); 2 monkeys were infected with the former species. Microfilariae morphology of these 2 species were consistent with the description by Petit et al. (1985) and Eberhard et al. (1979), respectively. Partial sequences of ITS1 of *M. mariae*, newly obtained using the peripheral blood microfilariae from 1 infected squirrel monkey (DDBJ/EMBL/GenBank AB362562; 163bp 3′-end of 18S rDNA and 301bp 5′-end of ITS1), were closer to, but different from, available sequences of the corresponding region of *Mansonella ozzardi* (AF228559–AF228564), as well as from *Mansonella perstans* (DQ995498); these were the only species with currently available sequences among species of *Mansonella* in the database (Morales-Hojas et al., 2001). Tamarins had no microfilariae in their blood, probably due to injections of ivermectin at the time of transfer to a laboratory animal facility.

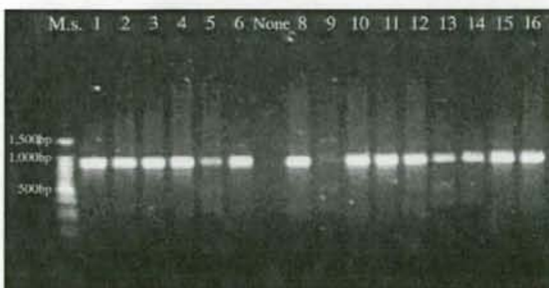

FIGURE 2. Ethidium bromide-stained SSU rDNA amplicons using a primer pair of TRY927F and TRY927R. Amplicons using respective blood DNA extracts of red-handed tamarins, nos. 1–16 (except for no. 7), and no template control (None) were separated by electrophoresis with molecular size markers (M.s.) on the left. Note that all amplicons (tamarin nos. 1–6, 8, and 10–16) had an identical molecular size less than 1,000 bp.

DISCUSSION

Due to the highly pleiomorphic nature of *T.* (*Megatrypanum*) *minasense* in the bloodstream, an accurate identification of the species based only upon morphology is often elusive (Ziccardi and Lourenço-de-Oliveira, 1997, 1998, 1999; Ziccardi et al., 2000, 2005). In an experimental infection using squirrel monkeys and marmosets (*Callithrix penicillata*), *T. minasense* sometimes looked like *T.* (*Tejeraia*) *rangeli* (Ziccardi and Lourenço-de-Oliveira, 1999). Our observations indicate that kinetoplasts of trypanosomes detected in the present study are located more anteriorly than *T. rangeli* (Table I). *Trypanosoma minasense*, and at least 2 other species of the subgenus *Megatrypanum*, i.e., *T.* (*M.*) *devei* and *T.* (*M.*) *lambretchi*, were recorded in Neotropical nonhuman primates (Deane and Damasceno, 1961; Lambrecht, 1965; Marinkelle, 1968; Hoare, 1972; Lanham et al., 1984; Ziccardi et al., 2000). *Trypanosoma devei* is a slender trypanosome, much narrower than *T. minasense*. *Trypanosoma lambretchi* has a nucleus in the posterior part of the body. Although the extreme scantiness of the infection in individual hosts, and polymorphic forms by individual hosts (Fig. 1), made the species identification difficult, the trypanosomes found in both squirrel monkeys and tamarins were closer to *T. minasense* than to *T. devei* or *T. lambretchi* (Hoare, 1972).

TABLE I. Extended.

BW	PK	KN	NA	F	N	K	KI†	KI‡	NI
1.8–5.0 (3.2 ± 1.0)	5.7–13.5 (7.7 ± 2.2)	2.8–10.6 (6.9 ± 1.9)	8.5–17.7 (11.9 ± 3.1)	7.8–19.8 (13.7 ± 3.4)	2.5–5.0 (3.3 ± 0.6)	0.7–1.4 (1.1 ± 0.2)	0.8–3.0 (1.3 ± 0.7)	1.8–4.0 (2.3 ± 0.7)	0.6–1.9 (1.3 ± 0.4)
1.8–2.3 (2 ± 0.04)	2.5–11 (5.8 ± 0.97)	5–8 (6.2 ± 0.34)	6–14 (9.4 ± 0.73)	6–10 (8.8 ± 0.37)	1.2–2 (1.6 ± 0.11)	0.8–1.1 (1 ± 0.03)	0.4–2.2 (1 ± 1.20)	— —	0.7–2.3 (1.4 ± 0.14)
2–4 (3 ± 0.16)	3–12 (10 ± 0.64)	3–11 (5.8 ± 0.44)	9–14 (11.3 ± 0.35)	6–10 (8.6 ± 0.26)	1–2.5 (1.8 ± 0.09)	0.8–1 (1 ± 0.02)	0.4–3.5 (1.9 ± 0.22)	— —	1–2 (1.4 ± 0.06)
2.0–3.0	8.7–16.5	4.0–9.5	—	6.5–7.2	—	—	—	ca. 3.0	1.2–1.5
ca. 2.5–4.0	—	3.0	—	ca. 11.6–13.2	—	—	—	—	—
1.2–3.0 (1.9 ± 0.12)	2–6 (3.8 ± 0.30)	7–9 (7.8 ± 0.18)	6–13 (8.6 ± 0.49)	5–11 (9.2 ± 0.41)	1.5–2 (2 ± 0.03)	0.5–1.1 (0.9 ± 0.03)	0.3–0.9 (0.5 ± 0.04)	— —	0.8–2 (1.4 ± 0.09)

FIGURE 3. Neighbor-joining phylogenetic tree based on alignment of SSU rDNA sequences. Bootstrap scores, expressed as percentages of 1,000 replicates, are given at nodes. The *T. minasense* examined in the present study is indicated by a closed arrow. The sources of the parasites were checked with reference to Votypka et al. (2004), Sato et al. (2005, 2007), Hamilton, Stevens, Gidley et al. (2005), and Hamilton, Stevens, Holz et al. (2005).

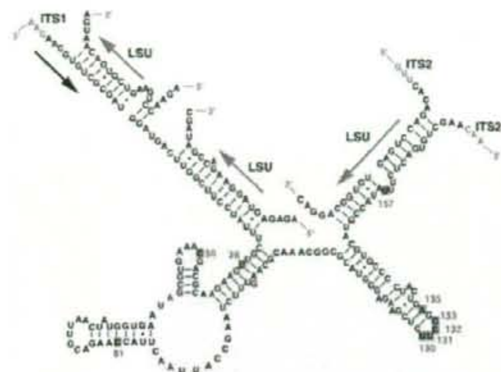

FIGURE 4. Putative secondary structure of the 5.8S rDNA of *T. minasense* with the associated 28S rDNA structure. Shaded nucleotides, with position numbers from the beginning of the 5.8S rDNA (DDBJ/EMBL/GenBank AB362411), are highly variable between trypanosomatid species or genus but absolutely conserved by species or genus, as shown in Table II.

Ziccardi et al. (2005) conducted sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on several strains of *T. rangeli*, *T. saimirii*-like trypanosomes isolated from squirrel monkeys, and *T. minasense*, and found that *T. saimirii*-like trypanosomes shared more than 80% of 20 polypeptides, ranging between 18 and 200 kDa, with *T. rangeli*, whereas *T. minasense* shared only 9 of 20 polypeptide bands with *T. rangeli* isolates. Based on the SDS-PAGE profiles mentioned above, as well as on no morphological or biological differences, the infectivity to triatomine bugs, successful axenic cultures, and successful PCR amplification of a randomly-amplified DNA fragment (Tra625) of *T. rangeli* and squirrel-monkey-driven trypanosomes (Ziccardi and Lourenço-de-Oliveira, 1998, 1999; Da Silva, Rodrigues et al., 2004), Ziccardi et al. (2005) concluded that *T. saimirii* is a junior synonym of *T. rangeli*. Moreover, *T. minasense* is an independent species from *T. rangeli*. The 2,184-bp sequence of SSU rDNA from the "LSTM" isolate of South American trypanosomes from a squirrel monkey (DDBJ/EMBL/GenBank AJ012413) was previously thought to be the sequence of *T. minasense*, but was recently concluded to be that of *T. rangeli* (Stevens et al., 1998, 1999; Da Silva, Noyes et al., 2004; Hamilton et al., 2007). The 2,176-bp SSU rDNA sequence of *T. minasense* in the present study was closer to those of *T. theileri* and other trypanosome species with *T. theileri*-like morphology and was distinct from *T. rangeli* (Fig. 3). Furthermore, the 5.8S rDNA sequence of *T. minasense* examined in the present study was again closer to *T. theileri* than to *T. cruzi* or *T. rangeli* (Table II). Although considerable variations were noted in 5.8S rDNA sequences of *T. rangeli* as single base insertion–deletion events or substitutions (Beltrame-Botelho et al., 2005), no nucleotide substitutions were seen at 9 highly variable base positions (shown in Table II), by species or higher taxa, in isolates of *T. rangeli*; however, 3 base substitutions existed between *T. rangeli* and *T. minasense*, as analyzed in the present study, indicating that these 2 were distinct species. Further analyses should be done on the present finding that the phylogenetic trees, based on a 18S rDNA alignment, found *T. bennetti* (kestrel trypanosome in North America) as the closest trypanosome species; these analyses should be performed after clarifying the 5.8S rDNA sequence of *T. bennetti* and after accumulation of more rDNA sequences of a variety of trypanosome spp. from mammals, birds, reptiles, and amphibians.

SSU rDNA sequence analyses give vastly different, and poorly supported, positions for species of *Trypanosoma* in kinetoplastid evolutionary trees, depending on the out-group taxon and the analysis method (Lukes et al., 1997; Stevens et al., 1998, 1999, 2001; Hughes and Piontkivska, 2003; Hamilton et al., 2004; Hamilton, Stevens, Gidley et al., 2005; Simpson et al., 2006). Recent use of cytoplasmic heat shock protein 90 (hsp90) genes (Simpson et al., 2002) or gGAPDH genes (Hamilton et al., 2004; Hamilton, Stevens, Gidley et al., 2005; Hamilton et al., 2007) for phylogenetic trees resolves the aforementioned problem with robust statistical support, resulting in rather different phylogenetic trees from those inferred from SSU rDNA sequences. According to this viewpoint, we have attempted to characterize the gGAPDH nucleotide sequence of *T. minasense*, but all 6 sequences fell into either *T. cruzi* or *T. rangeli* clades (Fig. 5). Of these, 5 gGAPDH sequences which had been amplified by the nested PCR are apparently parts of

TABLE II. Comparison of 5.8S rDNA sequences of some representative trypanosomatid species.

Trypanosomatid species	No. of isolates examined	Length (bp)	Position of base substitution*								
			38	50	81	130	131	132	133	135	157
T. minasense	1	172	U	C	C	U	U	C	G	A	U
T. rangeli	10	172–173	•	•	U	•	•	U	C	•	•
T. theileri	4	172	•	•	U	C	•	•	•	•	•
T. cruzi	5	172	•	•	U	C	•	•	A	•	•
T. grosi	3	172	A	•	U	•	•	U	U	•	A
T. otospermophili	2	172	A	•	U	•	•	U	U	•	A
T. kuseli	1	172	A	•	U	•	•	U	U	•	A
Leishmania spp. (9 species†)	12	169–171	•	U	U	•	A	U	U	U	–
Crithidia fasciculata	1	171	•	U	U	•	•	U	U	•	–

* Corresponding to the base position of *T. minasense* (DDBJ/EMBL/GenBank AB362411). Dot (•) indicates the identical base with *T. minasense*, dash (–) indicates a deletion of base.

† *Leishmania donovanii, L. ethiopica, L. infantum, L. major, L. tropica, L. amazonensis, L. braziliensis, L. chagasi, and L. mexicana.*

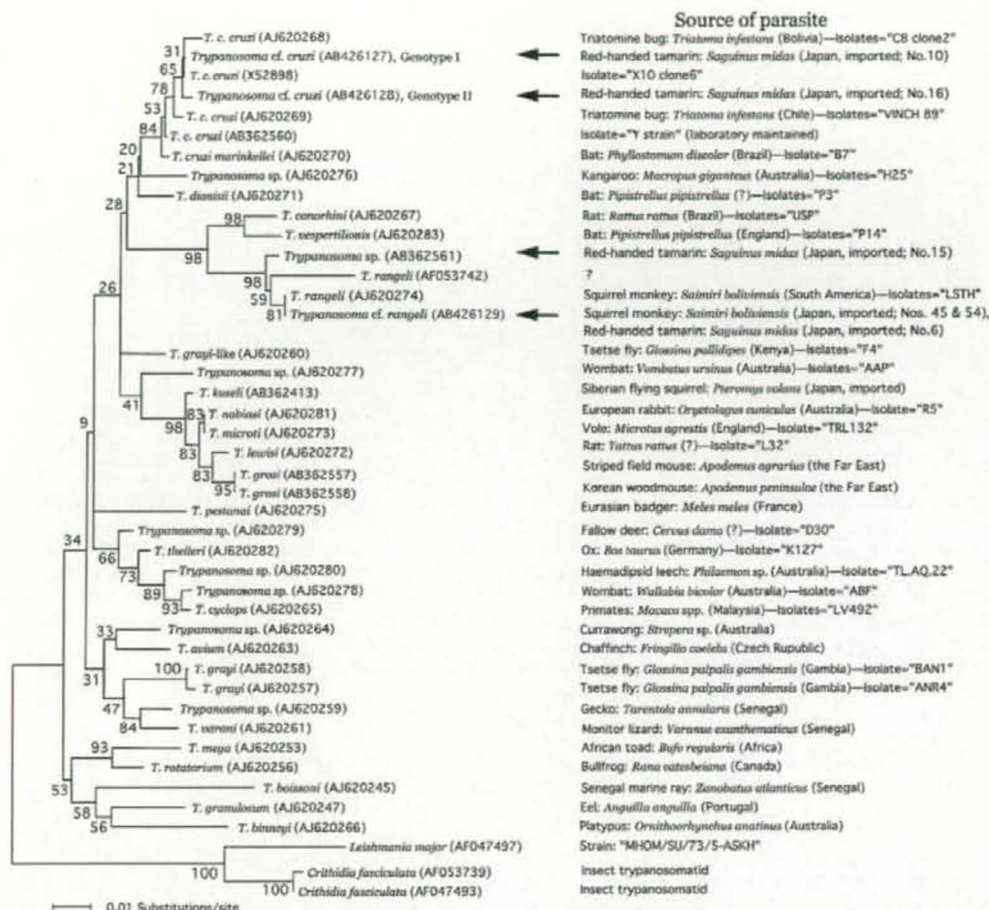

FIGURE 5. Neighbor-joining phylogenetic tree, based on alignment of gGAPDH gene sequences. Sequences obtained in the present study are indicated by arrows. Bootstrap scores, expressed as percentages of 1,000 replicates, are given at nodes. The sources of the parasites were checked as described in the legend for Figure 2.

the genes of *T. cruzi* or *T. rangeli*, whereas the 971-bp long sequence (DDBJ/EMBL/GenBank AB362561) from the blood of a tamarin (no. 15) situates near a group of *T. rangeli* isolates, but branches off from it (Fig. 5). At present, we could not determine whether or not this gGAPDH sequence is that of *T. minasense*, thus failing to identify its proper phylogenetic position based on the gGAPDH gene.

As shown in Figure 2, specific bands amplified by the primer pair of TRY927F and TRY927R, universal primers for the highly variable region of trypanosome 18S rDNA, seem to be single for all 14 positive tamarins, and by this primer pair are clearly less than 1,000 bp in size and the calculated sizes of *T. cruzi* and *T. rangeli* 18S rDNA amplicons. To the contrary, the g-GAPDH primers used in the present study preferentially amplified the *T. rangeli* and *T. cruzi* gGAPDH genes. To clarify the gGAPDH gene sequence of *T. minasense*, and to construct the proper phylogenetic tree based on it, for the special amplification we should select primers using an adequate trypanosome sample collected in the endemic regions and specifically diagnosed by multiple criteria (Ziccardi et al., 2005).

Stevens et al. (1999) proposed that use of the names *Herpetosoma* and *Megatrypanum* should be discontinued until their status is clarified because these subgenera are clearly polyphyletic and lack evolutionary and taxonomic relevance. As shown in Figure 3, clade I, which we refer to as "*Megatrypanum*-type trypanosome clade" and which contains *T. avium* and *T. theileri*, was poorly supported by bootstraps. In addition, several species, e.g., *T. conorhinii* and *T. talpae*, classically defined in the subgenus *Megatrypanum*, are positioned in different clades. Hoare (1972) stated that the subgenus *Megatrypanum* is a rather heterogeneous group of large mammalian trypanosomes, and may be regarded, phylogenetically, as the most primitive representative of the genus *Trypanosoma* in mammals because it shows affinities with some corresponding parasites of amphib-

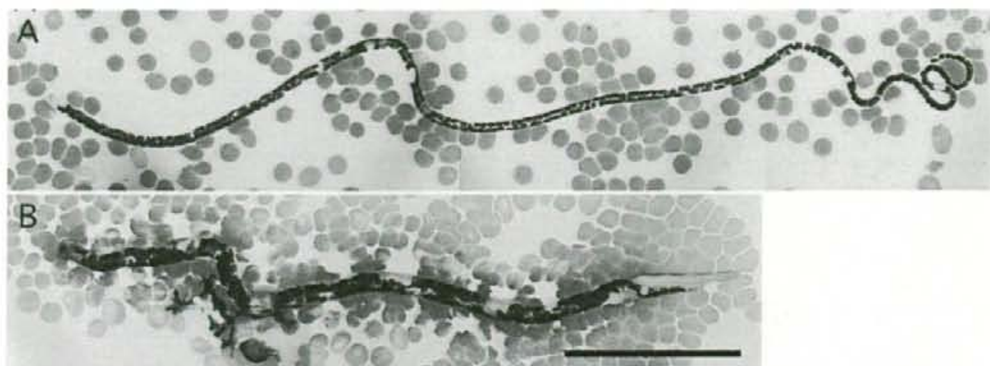

FIGURE 6. Microfilariae of *Mansonella (Tetrapetalonema) mariae* (**A**) and *Dipetalonema caudispina* (**B**) in the peripheral blood of squirrel monkeys. The specimens have been stained with Giemsa's solution. The scale bar in (B) applies to both A and B and = 50 μm; the 2 photographs are at the same magnification.

ians and reptiles. Accumulation of more molecular data is currently needed about trypanosomes having morphological characters of the subgenus *Megatrypanum*; such studies on trypanosomes of indigenous Australian vertebrates (Noyes et al., 1999; Hamilton, Stevens, Gidley et al., 2005) provided fascinating insights on the issue. Similarly, as seen in Figure 2, *T. talpae* from moles, along with 2 unnamed trypanosomes from shrews of the genera *Anourosores* and *Crocidura*, formed a distinct clade of insectivore trypanosomes exhibiting *Megatrypanum* morphology.

Trypomastigotes of *T. minasense* have been recorded in 32 species or subspecies of Neotropical nonhuman primates, primarily in monkeys of the Cebidae and Callitrichidae (Dunn et al., 1963; Deane et al., 1974; De Resende et al., 1994; Ziccardi et al., 1996). This species is the only trypanosome for which a circadian rhythm of parasitemia was noted (Deane et al., 1974). Furthermore, scanty parasitemia by *T. minasense* was sustained for at least 17 or 18 mo in marmosets (*Callithrix penicillata*) during captivity (De Resende et al., 1994; Ziccardi et al., 1996). Ziccardi and Lourenço-de-Oliveira (1997) examined 165 squirrel monkeys (70 *Saimiri sciureus* and 95 *S. ustus*) caught in the Brazilian Amazon basin, and found *T. rangeli* in 58 (35.2%), *T. minasense* in 55 (33.3%), and *T. cruzi* in 58 (35.2%) monkeys. Another study by the same group reported parasitemia by *T. minasense* in 4.3% (2 tamarins) of 46 nonhuman primates at a different locality in Brazil (Ziccardi et al., 2000). Due to apparent effects caused by the source of primates, the timing of blood aspiration for examination, and the applied techniques for examination, the reported prevalence (23.5%) of *T. minasense* in 85 squirrel monkeys, examined only by Giemsa-stained blood smears, might be an underestimation. More sensitive methods such as hematocrit and PCR-based detection techniques, applied in this study for tamarins only, as well as hemoculture, would most likely increase the prevalence. As mentioned above, we should note that the use of universal primers for a broad range of *Trypanosoma* spp. often detect only a single species, even though the animal has a mixed infection with multiple species.

Along with trypanosomes, microfilariae of *Dipetalonema* (*D. obtusa*, *D. gracile*, *D. caudispina*, *D. graciliforme*, and *D. robini*) and *Mansonella* (*M.* [*Tetrapetalonema*] *marmosetae*, *M.* [*T.*] *tamarinae*, and *M.* [*T.*] *mariae*) are the most frequently encountered helminth infections in New World monkeys (Dunn and Lambrecht, 1963; Esslinger and Gardiner, 1974; Petit et al., 1985). Prevalence often exceeds 70%, and multiple infections with 2–4 species are common in endemic areas for Neotropical nonhuman primates. The prepatent periods for species of *Mansonella* were estimated to be of moderate length, on the order of 5–6 mo (Orihel et al., 1981; Eberhard and Lowrie, 1987), and those for species of *Dipetalonema* were longer, 9–10 mo (Travie et al., 1985). Again, the prevalences of microfilariae reported in this study (25.9% for *M. mariae* and 3.5% for *D. caudispina*) may be underestimations. The squirrel monkey was refractory to experimental infection with the human filarial worm, *Mansonella ozzardi* (Orihel et al., 1981). Therefore, it is not unusual to refer to a newly obtained nucleotide sequence of the ITS region of microfilariae from our squirrel monkeys (DDBJ/EMBL/GenBank AB362562) as *M. mariae*, although ITS nucleotide sequences of these 2 species closely resembled each other.

Sullivan et al. (1993) discussed, in detail, the possibility that the natural trypanosome or filarial infections may modify the experimental infection of these monkeys, confounding the variables that these infections can induce; they also discussed the potential risk for animal handlers and laboratory staff to infection by *T. cruzi*. Detection of scanty *T. minasense*, *T. rangeli*, and *T. cruzi* parasitemia is difficult, particularly by Giemsa-stained blood films, and sustained infection must have been commonly overlooked to date. We do not know the effects of this background infection to the aimed experimental analyses using Neotropical nonhuman primates. Furthermore, the vector transmitting *T. minasense* is still unknown at present (Dunn et al., 1963; Hoare, 1972). During transportation and laboratory maintenance, possible transmission of the trypanosomes from infected Neotropical nonhuman primates to uninfected individuals remains a possibility. The partial molecular characterization of *T. minasense* in the present study enables us to identify the vector(s) for this species, to survey the accurate prevalence of this trypanosome infection in natural habitats, or to diagnose the infection in captivity. Accumulation of more data on the

biology of *T. minasense*, as well as on trypanosome species of the subgenus *Megatrypanum* in Neotropical nonhuman primates, is required.

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Gag-Specific Cytotoxic T-Lymphocyte-Based Control of Primary Simian Immunodeficiency Virus Replication in a Vaccine Trial[▽]

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Gag-specific cytotoxic T lymphocytes (CTLs) exert strong suppressive pressure on human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) replication. However, it has remained unclear whether they can actually contain primary viral replication. Recent trials of prophylactic vaccines inducing virus-specific T-cell responses have indicated their potential to confer resistance against primary SIV replication in rhesus macaques, while the immunological determinant for this vaccine-based viral control has not been elucidated thus far. Here we present evidence implicating Gag-specific CTLs as responsible for the vaccine-based primary SIV control. Prophylactic vaccination using a Gag-expressing Sendai virus vector resulted in containment of SIVmac239 challenge in all rhesus macaques possessing the major histocompatibility complex (MHC) haplotype *90-120-Ia*. In contrast, *90-120-Ia*-positive vaccinees failed to contain SIVs carrying multiple *gag* CTL escape mutations that had been selected, at the cost of viral fitness, in SIVmac239-infected *90-120-Ia*-positive macaques. These results show that Gag-specific CTL responses do play a crucial role in the control of wild-type SIVmac239 replication in vaccinees. This study implies the possibility of Gag-specific CTL-based primary HIV containment by prophylactic vaccination, although it also suggests that CTL-based AIDS vaccine efficacy may be abrogated in viral transmission between MHC-matched individuals.

Despite tremendous efforts to develop AIDS vaccines eliciting virus-specific T-cell responses, whether this approach actually does result in controlling human immunodeficiency virus (HIV) replication remains unknown. Recent trials have shown reductions in postchallenge viral loads by prophylactic vaccination eliciting virus-specific T-cell responses in macaque AIDS models (19, 22, 34), but the first advanced human trial of a T-cell-based vaccine was halted because of a lack of efficacy (5). Hence, it is quite important to determine which T-cell responses are responsible for primary HIV control.

Cytotoxic T-lymphocyte (CTL) responses have been indicated to play an important role in the control of HIV and simian immunodeficiency virus (SIV) infections (2, 9, 10, 17, 23, 29). Above all, the potential of Gag-specific CTL responses to contribute to viral control has been suggested by a cohort study indicating an association of HIV control with the breadth of Gag-specific CTL responses (15). In support of this, a recent in vitro study revealed their ability to rapidly respond to SIV infection (28). However, it has remained unclear whether Gag-specific CTL-based viral containment can be achieved by prophylactic vaccination.

We previously developed a prophylactic AIDS vaccine regimen consisting of a DNA prime followed by a boost with a Sendai virus (SeV) vector expressing SIVmac239 Gag (SeV-Gag) (22, 32). Our trial showed potential for efficiently inducing Gag-specific T-cell responses and containment of SIVmac239 challenge in a group of Burmese rhesus macaques sharing the major histocompatibility complex class I (MHC-I) haplotype *90-120-Ia* (22). A follow-up study revealed the reappearance of plasma viremia at >1 year postchallenge in some of these *90-120-Ia*-positive SIV controllers. In these transient controllers, multiple CTL escape mutations were accumulated in the viral *gag* gene, resulting in viremia reappearance and thus suggesting the involvement of $Gag_{206-216}$ (IINEEAADWDL) epitope-specific, $Gag_{241-249}$ (SSVDEQIQW) epitope-specific, and $Gag_{373-380}$ (APVPIPFA) epitope-specific CTLs in sustained viral control (12). Nonetheless, it has remained undetermined whether such Gag-specific CTL responses were responsible for the vaccine-based primary SIV control in *90-120-Ia*-positive vaccinees. In the present study, we challenged the *90-120-Ia*-positive vaccinees with SIVs carrying the *gag* CTL escape mutations to determine the role of Gag-specific CTLs in primary SIVmac239 control.

MATERIALS AND METHODS

Viral competition assay. SIV molecular clone DNAs with *gag* mutations were constructed by site-directed mutagenesis from the wild-type SIVmac239 (14) molecular clone DNA. Virus stocks were obtained by transfection of COS-1 cells with wild-type or mutant SIV molecular clone DNAs, and their titers were

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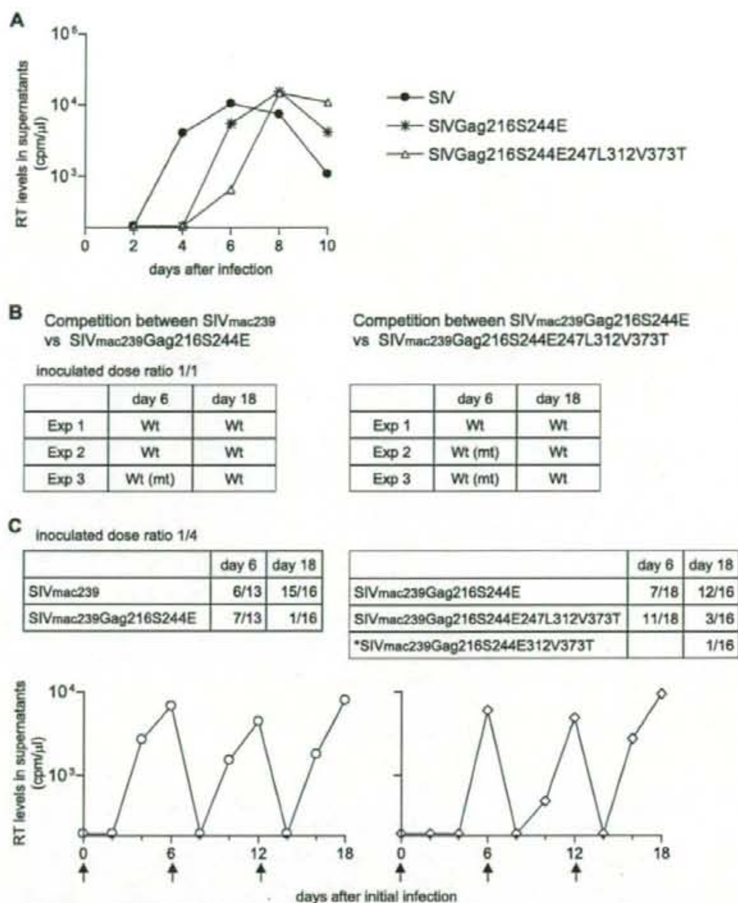

	day 6	day 18
Exp 1	Wt	Wt
Exp 2	Wt	Wt
Exp 3	Wt (mt)	Wt

	day 6	day 18
Exp 1	Wt	Wt
Exp 2	Wt (mt)	Wt
Exp 3	Wt (mt)	Wt

	day 6	day 18
SIVmac239	6/13	15/16
SIVmac239Gag216S244E	7/13	1/16

	day 6	day 18
SIVmac239Gag216S244E	7/18	12/16
SIVmac239Gag216S244E247L312V373T	11/18	3/16
*SIVmac239Gag216S244E312V373T		1/16

FIG. 1. Replication of mutant SIVs in vitro. (A) Wild-type and mutant SIV replication kinetics in HSC-F cells. HSC-F cells were infected with SIVmac239 (closed circles), SIVmac239Gag216S244E (asterisks), or SIVmac239Gag216S244E247L312V373T (open triangles). Virus production was monitored by measuring RT activity in the culture supernatants. Representative results from three sets of experiments are shown. (B) Viral competition assay. HSC-F cells were coinfected with SIVmac239 and SIVmac239Gag216S244E (left) or with SIVmac239Gag216S244E and SIV mac239Gag216S244E247L312V373T (right) at a ratio of 1:1. Viral *gag* fragments were amplified by RT-PCR from viral RNAs from the culture supernatants at days 6 and 18 postinfection and then sequenced. Dominant amino acid sequences at the 216th and 244th aa (left) or the 247th, 312th, and 373rd aa (right) in Gag in three sets of experiments are shown. Wt, only the wild-type sequence was detected; Wt (mt), the wild type was dominant, but the mutant was detectable (the mutant/wild-type ratio was $<1/2$). (C) Viral competition assay. HSC-F cells were coinfected with SIVmac239 and SIVmac239Gag216S244E (left) or with SIVmac239Gag216S244E and SIVmac239Gag216S244E247L312V373T (right) at a ratio of 1:4. The amplified *gag* fragments were subcloned into plasmids and sequenced. Frequencies of the indicated SIV clones (number of indicated clone per total number of clones) are shown. Changes in RT levels in the culture supernatants are shown in the bottom panels. The arrows indicate the time points of coinfection (at day 0) and viral passage for the second (at day 6) and the third (at day 12) cultures.

measured by reverse transcription (RT) assay as described previously (25, 33). For analysis of viral replication, HSC-F cells (herpesvirus saimiri-immortalized macaque T-cell line) (1) were infected with wild-type or mutant SIVs (normalized by RT activity), and virus production was monitored by measuring RT activity in the culture supernatants. For competition, HSC-F cells were coinfected with two SIVs at a ratio of 1:1 or 1:4, and the culture supernatants were harvested every other day and used for RT assays. On day 6, the supernatant was added to fresh HSC-F cells to start the second culture. Similarly, on day 12 after the initial coinfection, the second culture supernatant was added to fresh HSC-F cells to start the third culture. RNAs were extracted from the initial culture supernatant on day 6 and from the third culture supernatant on day 18 postcoinfection. The fragment (nucleotides 1231 to 2958 in SIVmac239 [GenBank accession number M33262]) containing the entire *gag* region was amplified from the RNA by RT-PCR and sequenced. Alternatively, it was subcloned into plasmids to determine dominant sequences.

Animal experiments. Burmese rhesus macaques (*Macaca mulatta*) were maintained in accordance with the guidelines for animal experiments performed at the National Institute of Infectious Diseases (26). Three animals, R01-007, R02-003, and R02-012, that received a prophylactic DNA prime/SeV-Gag boost vaccine and contained SIVmac239 challenge have been reported previously (22). In the present study, macaques R06-015, R06-035, R06-041, R05-004, R05-027, and R07-005 also received the DNA prime/SeV-Gag boost vaccine. The DNA used for the vaccination, CMV-SHIVdEN, was constructed from *env*- and *nef*-deleted simian-human immunodeficiency virus $SHIV_{MD14YE}$ molecular clone

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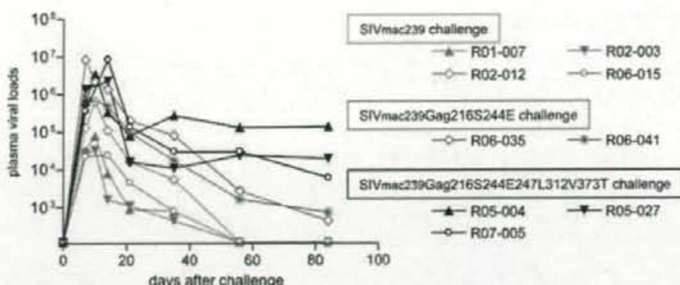

FIG. 2. Plasma viral loads after wild-type or mutant SIV challenge. The *90-120-Ia*-positive vaccinees were challenged with SIVmac239 (red lines), SIVmac239Gag216S244E (blue lines), or SIVmac239Gag216S244E247L312V373T (black lines). Plasma viral loads (SIV *gag* RNA copies/ml plasma) were determined as described before (22). The lower limit of detection is approximately 4×10^2 copies/ml.

DNA (SIVGP1) (31, 32) and has the genes encoding SIVmac239 Gag, Pol, Vif, and Vpx, SIVmac239-HIV chimeric Vpr, and HIV Tat and Rev. At the DNA vaccination, animals received 5 mg of CMV-SHIVdEN DNA intramuscularly. Six weeks after the DNA prime step, animals received a single boost intranasally with 6×10^9 cell infectious units of F-deleted replication-defective SeV-Gag (21, 32). Approximately 3 months after the boost, animals were challenged intravenously with 1,000 50% tissue culture infective doses of SIVmac239, SIVmac239 Gag216S244E, or SIVmac239Gag216S244E247L312V373T. The challenge virus stocks were prepared by virus propagation on rhesus macaque peripheral blood mononuclear cells (PBMCs). Sequence analysis confirmed the absence of *gag* mutations except for the two or five mutations in the challenge viruses.

Immunostaining of $CD4^+$ T-cell memory subsets. PBMCs were subjected to immunofluorescence staining by using fluorescein isothiocyanate-conjugated anti-human CD28, phycoerythrin-conjugated anti-human CD95, peridinin chlorophyll protein-conjugated anti-human CD4, and allophycocyanin-conjugated anti-human CD3 monoclonal antibodies (Becton Dickinson, Tokyo, Japan). The central memory subset of $CD4^+$ T cells was defined by possession of a $CD28^+$ $CD95^+$ phenotype, as described previously (13, 27).

Measurement of virus-specific $CD8^+$ T-cell responses. We measured virus-specific $CD8^+$ T-cell levels by flow cytometric analysis of gamma interferon (IFN-γ) induction after specific stimulation, as described previously (13, 22). In brief, PBMCs were cocultured with autologous herpesvirus papio-immortalized B-lymphoblastoid cell lines infected with a vaccinia virus vector expressing SIVmac239 Gag for Gag-specific stimulation or a vesicular stomatitis virus G protein-pseudotyped SIVGP1 for SIV-specific stimulation. The pseudotyped virus was obtained by cotransfection of COS-1 cells with a vesicular stomatitis virus G protein expression plasmid and the SIVGP1 DNA. Alternatively, B-lymphoblastoid cell lines were pulsed with 1 to 10 μM peptides for peptide-specific stimulation (11, 12). The 15-mer $Gag_{367-381}$ peptide was used to detect $Gag_{367-381}$-specific CTLs, including $Gag_{373-380}$-specific CTLs. Intracellular IFN-γ staining was performed using a Cytofix Cytoperm kit (Becton Dickinson). Peridinin chlorophyll protein-conjugated anti-human CD8, allophycocyanin-conjugated anti-human CD3, and phycoerythrin-conjugated anti-human IFN-γ antibodies (Becton Dickinson) were used. Specific T-cell levels were calculated by subtracting nonspecific IFN-γ^+ T-cell frequencies from those after Gag-specific, SIV-specific, or peptide-specific stimulation. Specific T-cell levels of <100 cells per million PBMCs were considered negative.

Statistical analysis. Statistical analysis was performed with Prism software, version 4.03, with significance set at P values of <0.05 (GraphPad Software, Inc., San Diego, CA). Central memory $CD4^+$ T-cell counts before challenge were not significantly different between the wild-type SIV-challenged ($n = 4$) and the mutant SIV-challenged ($n = 5$) macaques ($P = 0.70$ by unpaired two-tailed t test with Welch's correction and $P = 0.73$ by nonparametric Mann-Whitney U test). Ratios of the central memory $CD4^+$ T-cell counts from a few months postchallenge to those prechallenge were log transformed and compared between the two groups by an unpaired two-tailed t test and the Mann-Whitney U test. Gag-specific $CD8^+$ T-cell frequencies postvaccination (prechallenge) or postchallenge were also log transformed and compared between the two groups in the same statistical manner.

RESULTS

Comparison of viral fitness in wild-type and mutant SIVs. We used two mutant SIVs for challenge of the *90-120-Ia*-positive vaccinees. The first, designated SIVmac239Gag216S244E, carries two *gag* mutations, GagL216S and GagD244E, leading to a leucine (L)-to-serine (S) substitution at the 216th amino acid (aa) and an aspartic acid (D)-to-glutamic acid (E) substitution at the 244th aa in Gag. The second, designated SIVmac239Gag216S244E247L312V373T, carries five *gag* mutations, GagL216S, GagD244E, GagI247L (isoleucine [I] to L at the 247th aa), GagA312V (alanine [A] to valine [V] at the 312th aa), and GagA373T (A to threonine [T] at the 373rd aa). In our previous study (12), the former became dominant in the early phase (at approximately 4 months postchallenge) during the period of viral control, and the latter was dominant at viremia reappearance in a transient controller. GagL216S, GagD244E and GagI247L, and GagA373T mutations result in viral escape from recognition by $Gag_{206-216}$-specific, $Gag_{241-249}$-specific, and $Gag_{373-380}$-specific CTLs, respectively, while it remains unclear whether GagA312V was selected for by CTLs.

We first compared viral fitness in wild-type and mutant SIVs. In HSC-F cells (a macaque T-cell line), not only the wild type but also the mutant SIVs were able to replicate, but SIVmac239Gag216S244E replication was less efficient than that of wild-type SIVmac239, and SIVmac239Gag216S244E247L312V373T replication was even less efficient (Fig. 1A). In competitions between two SIVs, HSC-F cells were coinfected with both viruses, and viral genome sequences in the culture supernatants were assessed to establish which SIV became predominant. In culture supernatants of HSC-F cells after coinfection with SIVmac239 and SIVmac239Gag216S244E inoculated at a ratio of 1:1, the wild type rapidly became dominant (at day 6) (Fig. 1B). Coinfection at a ratio of 1:4 resulted in equivalence at day 6, but the wild type again dominated by day 18 (Fig. 1C). These results indicate a lower replicative ability of SIVmac239 Gag216S244E than of wild-type SIVmac239. In addition, competition between SIVmac239Gag216S244E and SIVmac239 Gag216S244E247L312V373T showed the lower replicative ability of the latter (Fig. 1B and C).

Challenge of *90-120-Ia*-positive vaccinees with wild-type or mutant SIVs. Next, we challenged *90-120-Ia*-positive macaques

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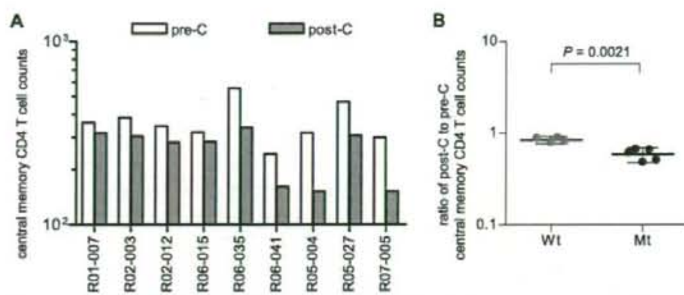

FIG. 3. Changes in central memory CD4$^+$ T-cell counts after wild-type or mutant SIV challenge. (A) Peripheral central memory CD4$^+$ (CD4$^+$ CD95$^+$ CD28$^+$) T-cell counts (/μl) prechallenge (pre-C) and a few months postchallenge (post-C). (B) Statistical comparison of central memory CD4$^+$ T-cell loss between the wild-type SIV-challenged (Wt) and the mutant SIV-challenged (Mt) macaques. The ratios of central memory CD4$^+$ T-cell counts postchallenge to those prechallenge are plotted. The longer bars indicate geometric mean values, and the regions between the shorter bars indicate the 95% confidence intervals. The ratios in the mutant group (n = 5) were significantly lower than those in the wild-type group (n = 4) (P = 0.0021 by unpaired t test and P = 0.0159 by Mann-Whitney U test).

with the mutant SIVs after DNA prime/SeV-Gag vaccination. Remarkably, all three vaccinees (R05-004, R05-027, and R07-005) challenged with SIVmac239Gag216S244E247L312V373T failed to control viral replication and showed high set point plasma viral loads, while all four vaccinees (R01-007, R02-003, R02-012, and R06-015) challenged with wild-type SIVmac239 contained viral replication, with undetectable set point plasma viral loads (Fig. 2). Even the two vaccinees (R06-035 and R06-041) challenged with SIVmac239Gag216S244E failed to contain viral replication, although with lower plasma viral loads, at approximately 10^3 RNA copies/ml at 3 months postchallenge. Central memory CD4$^+$ T-cell counts before challenge were not significantly different between the wild-type SIV-challenged (n = 4) and mutant SIV-challenged (n = 5) macaques, but ratios of the counts at a few months postchallenge to prechallenge for the latter group were significantly lower than those for the former (P = 0.0021 by unpaired t test and P = 0.0159 by Mann-Whitney U test) (Fig. 3). Thus, *90-120-Ia*-positive vaccinees can contain wild-type SIVmac239 but not SIVmac239Gag216S244E or SIVmac239Gag216S244E247L312V373T challenge.

Viral *gag* sequence analysis confirmed the rapid selection for the GagL216S mutation in all wild-type SIVmac239-challenged macaques, as described previously (22). All of the *gag* mutations in the challenge mutant viruses were maintained during the observation period (Table 1). SIVmac239Gag216S244E247L312V373T-challenged macaques showed no additional dominant *gag* mutations, whereas animals challenged with SIVmac239Gag216S244E rapidly selected viruses with a GagV145A (V to A at the 145th aa) mutation. Recovery of viral fitness by this mutation was not observed, and whether it was selected for by CTLs was unclear in our previous study (12).

Gag-specific CTL responses were induced after SeV-Gag boost in all vaccinees, and there was no significant difference in the levels between the wild-type and mutant challenges (P = 0.1198 by unpaired t test and P = 0.1111 by Mann-Whitney U test). However, secondary Gag-specific CTL responses were

TABLE 1. Dominant sequences in SIV Gag in macaques after challenge

Macaque	Time (wk) of plasma sample	Amino acid change in Gag at position[a]:								
		140	145	206	216	244	247	312	341	373
R01-007	5				L216S					
R02-003	5				L216S					
R02-012	5				L216S					
R06-015	5			(I206M)	L216S					
R06-035	5				L216S*	D244E*				
	12		V145A		L216S*	D244E*			(N341Y)	
R06-041	5		(V145A)		L216S*	D244E*				
	12		V145A		L216S*	D244E*				
R05-004	5				L216S*	D244E*	I247L*	A312V*		A373T*
	12	(I140V)			L216S*	D244E*	I247L*	A312V*		A373T*
R05-027	5				L216S*	D244E*	I247L*	A312V*		A373T*
	12				L216S*	D244E*	I247L*	A312V*		A373T*
R07-005	5				L216S*	D244E*	I247L*	A312V*		A373T*
	12				L216S*	D244E*	I247L*	A312V*		A373T*

[a] A fragment containing the entire *gag* region was amplified from plasma RNA by nested RT-PCR and then sequenced. We were unable to amplify the fragment from plasmas obtained at week 12 from the wild-type SIVmac239-challenged macaques with undetectable viremia. Dominant *gag* mutations resulting in amino acid changes are shown. Asterisks indicate the mutations included in the challenge inoculums. Parentheses indicate that both the wild-type and mutant sequences were detected equivalently at that position.

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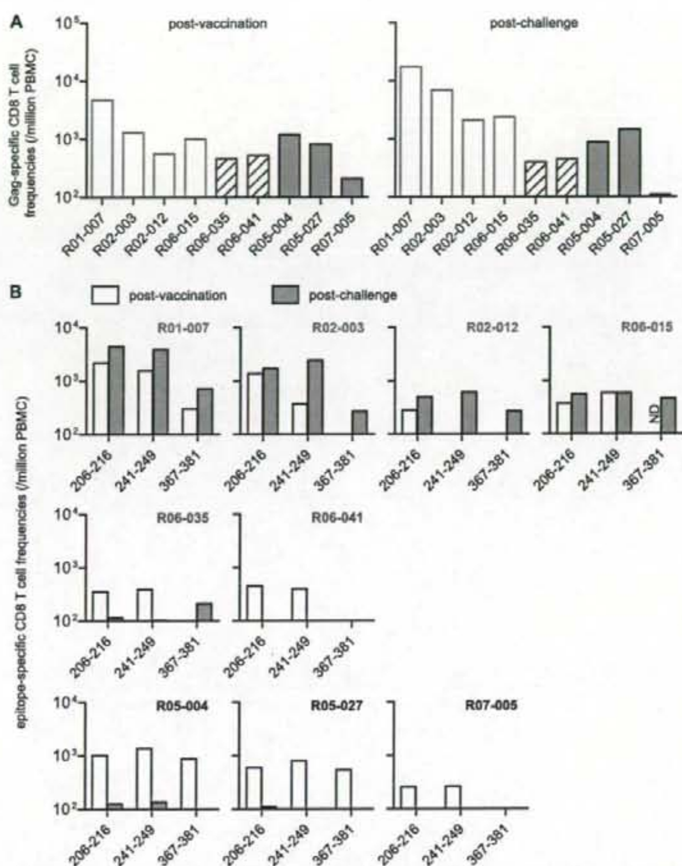

FIG. 4. Gag-specific CD8$^+$ T-cell responses before and after wild-type or mutant SIV challenge. Macaques R01-007, R02-003, R02-012, and R06-015 were challenged with SIVmac239; macaques R06-035 and R06-041 were challenged with SIVmac239Gag216S244E; and macaques R05-004, R05-027, and R07-005 were challenged with SIVmac239Gag216S244E247L312V373T. (A) Gag-specific CD8$^+$ T-cell frequencies at 2 weeks postboost (postvaccination) (left) and 2 weeks postchallenge (right). (B) $Gag_{206-216}$-specific, $Gag_{241-249}$-specific, and $Gag_{367-381}$-specific CD8$^+$ T-cell frequencies at 2 weeks (all except for R02-012) or 4 weeks (in R02-012) postboost (postvaccination) and 5 weeks (in R01-007, R02-003, R02-012, R06-035, R06-041, and R05-004) or 6 weeks (in R06-015, R05-027, and R07-005) postchallenge. ND, not determined.

less efficient after challenge with mutant SIV than after challenge with wild-type SIV ($P = 0.0095$ by unpaired t test and $P = 0.0159$ by Mann-Whitney U test) (Fig. 4A).

SeV-Gag boost induced efficient $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses in all vaccinees and $Gag_{367-381}$-specific CTL responses in some of them (Fig. 4B). Challenge with wild-type SIVmac239 resulted in efficient secondary responses of these three epitope-specific CTLs, whereas SIVmac239Gag216S244E247L312V373T challenge evoked none of them (Fig. 4B). SIVmac239Gag216S244E challenge did not result in secondary responses of $Gag_{206-216}$-specific or $Gag_{241-249}$-specific CTLs but did induce $Gag_{367-381}$-specific CTL responses in one case (Fig. 4B). These results indicate that SIVmac239Gag216S244E evades recognition by $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTLs and that SIVmac239Gag216S244E247L312V373T evades recognition by $Gag_{206-216}$-specific, $Gag_{241-249}$-specific, and $Gag_{367-381}$-specific CTLs.

We next examined Gag-specific and SIV-specific CTL responses after mutant SIV challenge (Fig. 5A). We used an *env*- and *nef*-deleted SHIV molecular clone DNA, SIVGP1, that has the genes encoding SIVmac239 Gag, Pol, Vif, Vpx, and a part of Vpr and measured the frequencies of CTLs responding to SIVGP1-transduced cells (referred to as SIV-specific CTLs) as described previously (13, 32). SIV-specific CTL frequencies at week 12 were much higher than those at week 2 for all five macaques challenged with mutant SIVs. In contrast, Gag-specific CTL frequencies at week 12 were lower than those at week 2 for four of five animals; the remaining macaque, R06-035, mounted $Gag_{367-381}$-specific CTL responses. Importantly, in all animals challenged with mutant SIVs, SIV-specific CTL

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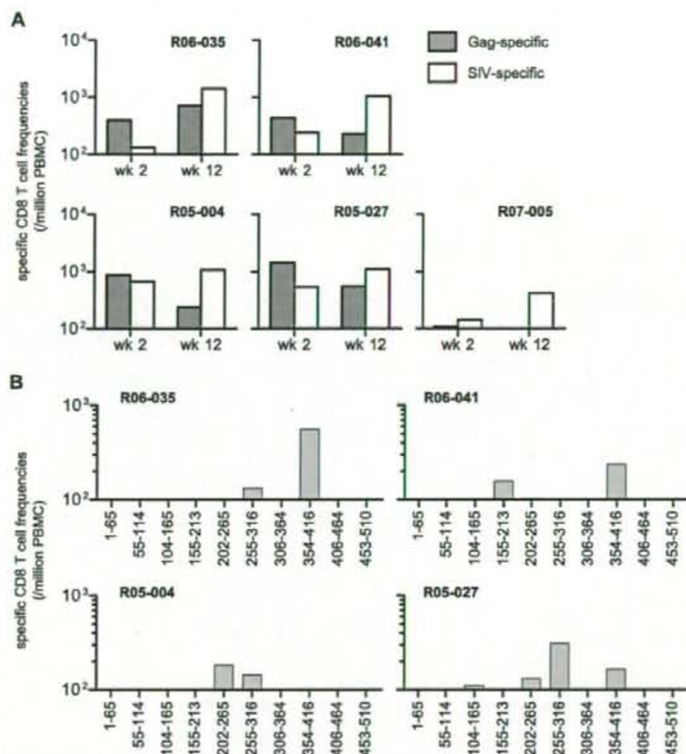

FIG. 5. SIV-specific CD8$^+$ T-cell responses after mutant SIV challenge. (A) Gag-specific (closed boxes) and SIV-specific (open boxes) CD8$^+$ T-cell frequencies at 2 weeks or 12 weeks postchallenge. (B) Frequencies of CD8$^+$ T cells specific for pools of SIV Gag peptides. A panel of 117 overlapping peptides (15 to 17 aa in length and overlapping by 10 to 12 aa) spanning the entire SIV Gag amino acid sequence were divided into the following 10 pools (each consisting of 11 or 12 peptides): pool 1, 1st to 65th aa in SIV Gag; pool 2, 55th to 114th aa; pool 3, 104th to 165th aa; pool 4, 155th to 213th aa; pool 5, 202nd to 265th aa; pool 6, 255th to 316th aa; pool 7, 306th to 364th aa; pool 8, 354th to 416th aa; pool 9, 406th to 464th aa; and pool 10, 453rd to 510th aa. The pools were used for stimulation to detect peptide pool-specific CD8$^+$ T cells.

frequencies were at marginal levels or lower than Gag-specific CTL frequencies at week 2, but the former became higher than the latter at week 12. These results indicate an induction of CTL responses specific for SIV antigens other than Gag in all five macaques after mutant SIV challenge.

At week 12 after mutant SIV challenge, Gag-specific CTL responses were undetectable in macaque R07-005 but were still detected in the other four macaques. We then analyzed Gag-specific CTL responses in these four macaques by using a panel of overlapping peptides spanning the entire SIV Gag amino acid sequence (Fig. 5B). In both SIVmac239Gag216S244E-challenged animals, R06-035 and R06-041, exhibiting detectable $Gag_{367-381}$-specific CTL responses (data not shown), CTL responses specific for the peptide mixture corresponding to the 354th to 416th aa in SIV Gag were detected at week 12. In addition, we found $Gag_{255-316}$-specific CTL responses in macaque R06-035 and $Gag_{155-213}$-specific CTL responses in macaque R06-041. SIVmac239Gag216S244E247L312V373T-challenged macaques R05-004 and R05-027 showed responses specific for several Gag peptide mixtures, including $Gag_{202-265}$-specific and $Gag_{255-316}$-specific CTL responses. These results indicate an induction of CTL responses specific for Gag epitopes other than the $Gag_{206-216}$, $Gag_{241-249}$, and $Gag_{373-380}$ epitopes after mutant SIV challenge.

DISCUSSION

In the present study, SIVs carrying multiple *gag* CTL escape mutations showed lower replicative abilities than that of the wild type; nonetheless, the *90-120-Ia*-positive vaccinees were able to contain only the latter. This demonstrates that Gag-specific CTL responses did play a central role in the vaccine-based primary containment of wild-type SIVmac239 replication in *90-120-Ia*-positive macaques.

Elicitation of virus-specific T-cell responses by prophylactic vaccination is believed to be a promising strategy for HIV control (3, 24); whether this approach can actually result in HIV control remains unknown. Recent studies have indicated the possibility of reductions in set point viral loads after SIV challenge by prophylactic vaccination inducing T-cell responses in rhesus macaques (19, 22, 34), yet the immune component crucial for the vaccine-based viral control has not been

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determined. No clear evidence for a contribution of vaccine-induced CTLs to this viral control has been forthcoming to date, although virus-specific CTL responses have been implicated in exerting strong suppressive pressure on HIV/SIV infection (9, 22). Indeed, viral replication persists even in the presence of CTL responses in the natural course of infection; it has thus remained unclear whether HIV/SIV replication can be controlled by vaccine-induced CTLs. The evidence from the present study now strongly implicates Gag-specific CTL responses as responsible for vaccine-based primary SIV control. This offers the possibility of Gag-specific CTL-based HIV containment by prophylactic vaccination and provides insight into the development of CTL-based AIDS vaccines.

The containment of SIVmac239 but failure to contain SIVmac239Gag216S244E in the vaccinees documents a crucial role for $Gag_{206-216}$-specific and/or $Gag_{241-249}$-specific CTL responses in vaccine-based SIVmac239 containment. Furthermore, challenge with SIVmac239Gag216S244E247L312V373T, possessing diminished viral fitness compared to SIVmac239Gag216S244E, tended to result in higher viral loads, indicating the involvement of $Gag_{373-380}$-specific CTL responses in viral control, while more complete viral evasion of $Gag_{241-249}$-specific CTL recognition by addition of the GagI247L mutation may also contribute to the difference between SIVmac239Gag216S244E and SIVmac239Gag216S244E247L312V373T challenge. Taken together, we conclude that these two or three epitope-specific CTL responses are crucial for primary SIVmac239 control in *90-120-Ia*-positive vaccinees. Conversely, this study implies that viral evasion of recognition by two dominant epitope-specific CTLs can result in failure of primary viral containment but may not be sufficient for abrogation of vaccine efficacy. Thus, analysis of CTL-based vaccine efficacy against SIVs carrying single or multiple CTL escape mutations could contribute to an evaluation of its potential for controlling the replication of highly diversified HIVs.

Our results suggest that SIV- but non-Gag-specific CTLs became predominant after mutant SIV challenge. Additionally, CTLs recognizing Gag regions other than the $Gag_{206-216}$, $Gag_{241-249}$, and $Gag_{373-380}$ epitopes were detected in most cases. These CTL responses may exert suppressive pressure on viral replication but are considered insufficient for controlling replication of the mutant SIVs with lower viral fitness.

Finally, this study also provides evidence indicating a possible abrogation of CTL-based AIDS vaccine efficacy in viral transmission between MHC-I-matched individuals. Indeed, even the mutant SIVs carrying multiple CTL escape mutations were able to replicate persistently in vivo, despite their diminished replicative ability. Transmission of these viruses can result in persistent viral infection and AIDS progression (30). CTL escape mutations resulting in a loss of viral fitness may revert to the wild-type sequence after transmission into MHC-I-mismatched hosts (4, 8, 9, 16, 18, 20), but such reversion does not occur rapidly; alternatively, some may be retained with additional compensatory mutations (6, 7, 30). Thus, there may be a risk of transmission and accumulation of HIV CTL escape variants even among MHC-I-mismatched individuals, resulting in abrogation of CTL-based AIDS vaccine efficacy in a population.

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ORIGINAL PAPER

Natural selection in the *TLR*-related genes in the course of primate evolution

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Abstract The innate immune system constitutes the front line of host defense against pathogens. Toll-like receptors (TLRs) recognize molecules derived from pathogens and play crucial roles in the innate immune system. Here, we provide evidence that the TLR-related genes have come under natural selection pressure in the course of primate evolution. We compared the nucleotide sequences of 16 TLR-related genes, including *TLR*s (*TLR1–10*), *MYD88*, *TILAP*, *TICAM1*, *TICAM2*, *MD2*, and *CD14*, among seven primate species. Analysis of the non-synonymous/synonymous substitution ratio revealed the presence of both strictly conserved and rapidly evolving regions in the TLR-related genes. The genomic segments encoding the intracellular Toll/interleukin 1 receptor domains, which exhibited lower rates of non-synonymous substitution, have undergone purifying selection. In contrast, *TLR4*, which carried a high proportion of non-synonymous substitutions in the part of extracellular domain spanning 200 amino acids, was found to have been the suggestive target of positive Darwinian selection in primate evolution. However, sequence analyses from 25 primate species, including eight hominoids, six Old World monkeys, eight New World monkeys, and three prosimians, showed no evidence that the pressure of positive Darwinian selection has shaped the pattern of sequence variations in *TLR4* among New World monkeys and prosimians.

Keywords Toll-like receptor · Natural selection · Primate evolution

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Introduction

Toll-like receptors (TLRs) recognize molecules derived from pathogens and play crucial roles in the innate immune system. TLRs are type I integral membrane glycoproteins characterized by the extracellular domains with varying numbers of leucine-rich-repeat motifs (LRR) and a cytoplasmic signaling domain termed the Toll/interleukin 1 receptor (TIR) domain (Akira et al. 2006; Bowie and O'Neill 2000). Different TLRs recognize a variety of

pathogen-associated molecules, including lipids and nucleic acids, and all TLRs transduce signals through TIR domains to activate immune cells (Akira et al. 2006; Bowie and O'Neill 2000). Stimulation with TLR ligands recruits adaptor proteins such as Myeloid differentiation factor 88 (MYD88), TIR domain-containing adaptor protein (TIRAP), TIR domain-containing adaptor molecule 1 (TICAM1), and TICAM2, all of which also have a TIR domain, to the cytoplasmic portion of the TLRs and activate signaling cascades to produce proinflammatory cytokines and chemokines (Akira et al. 2006; Bowie and O'Neill 2000).

Viral, bacterial, and parasitic infections have been postulated to be among the strongest selective pressures on primate evolution. It is also widely accepted that the susceptibility to infectious pathogens, such as *Mycobacterium tuberculosis* bacilli and HIV-1, are different among primate species (Isaza 2003; Stremlau et al. 2004). Given that TLRs play crucial roles in the innate immune system, the intriguing hypothesis that TLRs have emerged under the intense pressure of natural selection in the course of primate evolution is rising. Actually, it is suggested that *TLR1*, *TLR6*, and *TLR10* have come under particular natural selection pressures in the human population, because the sequence variations of these three genes display considerable geographical diversity in the British population (Wellcome Trust Case Control Consortium 2007). Moreover, it has been reported that natural selection has acted on *TLR4* in humans, since excess of rare non-synonymous polymorphisms in *TLR4* are observed in humans (Smirnova et al. 2001).

To investigate the natural selection hypothesis, we analyzed the nucleotide sequences of 16 TLR-related genes, including ten *TLRs* (TLR1–10), four genes linked to signal transduction (*MYD88*, *TILAP*, *TICAM1*, and *TICAM2*), and two genes linked to *TLR4* (*MD2* and *CD14*) in primates. MD2 and CD14 are key molecules of the LPS signaling through TLR4 (Poltorak et al. 1998; Shimazu et al. 1999; Nagai et al. 2002). Our study shows that the genomic segments encoding the intracellular TIR domains have undergone purifying selection and that the extracellular domain of *TLR4* has been the suggestive target of positive Darwinian selection in the course of primate evolution. We concluded that natural selection has indeed shaped the sequence patterns of TLR-related genes in primate evolution.

Materials and methods

DNA sequences

DNA samples from 25 primates, including human (*Homo sapiens*), chimpanzee (*Pan troglodytes*), bonobo (*Pan paniscus*), gorilla (*Gorilla gorilla*), orangutan (*Pongo pygmaeus*), black gibbon (*Hylobates concolor*), white-handed gibbon (*Hylobates lar*), siamang (*Hylobates syndactylus*), crab-eating macaque (*Macaca fascicularis*), rhesus macaque (*Macaca mulatta*), hamadryas baboon (*Papio hamadryas*), black and white colobus (*Colobus guereza*), silvered lutong (*Trachypithecus cristatus*), dusky lutong (*Trachypithecus obscurus*), common marmoset (*Callithrix jacchus*), cotton-top tamarin (*Saguinus oedipus*), red-handed tamarin (*Saguinus midas*), lion tamarin (*Leontopithecus rosalia*), common squirrel monkey (*Saimiri sciureus*), tufted capuchin (*Cebus apella*), long-haired spider monkey (*Ateles belzebuth*), and Central American spider monkey (*Ateles geoffroyi*), tarsiers (*Tarsius* spp.), lesser galago (*Galago senegalensis*), and ring-tailed lemur (*Lemur catta*) were analyzed. Overlapping primer sets covering all coding exons of 16 genes including the *TLRs* (*TLR1–10*), *MYD88*, *TILAP*, *TICAM1*, *TICAM2*, *MD2*, and *CD14* were designed on the basis of size and overlap of PCR amplicons. Genomic DNA was subjected to PCR amplification followed by sequencing using the BigDye Terminator cycling system. Sequencing analysis was performed in an ABI3130x automated DNA sequencer (Applied Biosystems).

Statistical analysis

Sequence alignments were performed by the Clustal X program (Thompson et al. 1997). All values for Ka, Ks, Ka/Ks, %GC, and Codon Bias Index (CBI) were evaluated by DnaSP (Rozas et al. 2003). The Bn–Bs program (Zhang et al. 1998) was applied to evaluate the Ka/Ks ratio in individual branches of the primate phylogenetic tree. We studied positive Darwinian selection for the target region of TLR4 by using the MEGA version 4.0 program (Tamura et al. 2007). Ancestral amino acid sequence was estimated by a parsimony method using PROTPARS program in PHYLIP (Felsenstein 1989).

Results

The nucleotide sequences of tn *TLRs* (TLR1–10), *MYD88*, *TILAP*, *TICAM1*, *TICAM2*, *MD2*, and *CD14* were determined among seven primates, including human, chimpanzee, bonobo, gorilla, orangutan, crab-eating macaque, and rhesus macaque. All sequences were newly determined in the study, and all accession numbers were shown in Table S1. The lengths of the deduced coding sequences for each gene differed among the seven primates, as summarized in Table 1.

To evaluate the non-synonymous/synonymous substitution ratio, we applied the Bn–Bs program (Zhang et al. 1998). This program uses a modified Nei–Gojobori method (Nei and Gojobori 1986) to estimate pairwise synonymous

Table 1 The non-synonymous and synonymous substitution ratio for 16 TLR-related genes among seven primates

Gene	Chromosome (Human)	Size of coding region (bp)	Entire coding sequence			TIR domain			Non-TIR region		
			Σbn	Σbs	Σbn/ Σbs	Σbn	Σbs	Σbn/ Σbs	Σbn	Σbs	Σbn/ Σbs
TLR1	4p14	2,358	0.041	0.095	0.429	0.019	0.103	0.164	0.046	0.093	0.491
TLR2	4q32	2,352, 2,349 Orangutan	0.025	0.086	0.290	0.009	0.058	0.157	0.028	0.091	0.311
TLR3	4q35	2,712	0.032	0.121	0.267	0.018	0.070	0.259	0.035	0,130	0.270
TLR4	9q32–33	2,517, 2,490 Orangutan, 2,478 Crab-eating, Rhesus	0.038	0.085	0.447	0.006	0.118	0.053	0.045	0.079	0.566
TLR5	1q41–42	2,574	0.030	0.108	0.282	0.018	0.173	0.105	0.032	0.097	0.339
TLR6	4p14	2,388, 2,385 Bonobo	0.030	0.120	0.240	0.021	0.199	0.105	0.031	0.105	0.293
TLR7	Xp22.3–p22.2	3,147	0.014	0.069	0.202	0.003	0.087	0.034	0.016	0.066	0.236
TLR8	Xp22.3–p22.2	3,123, 3,120 Crab-eating, Rhesus	0.020	0.095	0.209	0.003	0.056	0.054	0.023	0.101	0.224
TLR9	3p21.3	3,096	0.029	0.153	0.187	0.003	0.202	0.016	0.032	0.145	0.224
TLR10	4p14	2,433	0.024	0.106	0.228	0.018	0.045	0.402	0.026	0.118	0.216
MYD88	3p22–p21.3	888	0.009	0.096	0.094	0.000	0.087	0.000	0.017	0.102	0.165
TIRAP	11q23–q24	663, 660 Crab-eating, Rhesus	0.035	0.164	0.216	0.032	0.254	0.126	0.037	0.110	0.341
TICAM1	19p13.3	2,139[a]	0.039	0.171	0.227	0.007	0.163	0.041	0.046	0.172	0.269
TICAM2	5q23.1	705	0.020	0.119	0.167	0.000	0.132	0.000	0.033	0.110	0.300
MD2	8q21.11	480	0.015	0.054	0.269	–	–	–	–	–	–
CD14	5q31.1	1,125	0.013	0.040	0.332	–	–	–	–	–	–

[a] *TICAM1* has a CCT(Pro)-repeat variation

and non-synonymous distances among the sequences and then estimates the branch lengths in terms of synonymous (bs) and non-synonymous substitutions (bn) per site by using the ordinary least-squares method, while the tree topology is given. Σbn and Σbs indicate the value summing up bn and bs, respectively, in the lineages. When the value of Σbn and Σbs and the ratio of Σbn/Σbs were evaluated for the entire coding sequences from each gene, there was no evidence to support that these genes have come under the pressure of positive natural selection. All of the values of the Σbn/Σbs ratio from the analyzed genes were much lower than 1.0, which suggested that these genes have been under the pressure of negative selection (Table 1).

To identify the genomic segments, which have undergone natural selection, a sliding window plot analysis (600-bp window with 30-bp steps) was performed throughout these genes. Analysis of the Σbn/Σbs ratio revealed the presence of both strictly conserved and rapidly evolving regions in the TLR-related genes. Three candidate segments, where the pressure of negative or positive natural selection might have operated, were identified in *TLR7*, *MYD88*, and *TLR4* (Fig. 1a).

Two target segments showed little non-synonymous nucleotide difference among the seven primates (Fig. 1a and Supplementary material, Fig. S1). One was located at the coding segment encoding the C-terminal of TLR7 and the other at the segment encoding the C-terminal of MYD88, both of which encode the TIR domain (Fig. 1a). Phylogenetic comparisons from 14 human sequences encoding TIR domains reveal no obvious similarity between *TLR7* and *MYD88* (Fig. 2a). We then evaluated the Σbn/Σbs ratios for the TIR domains for 14 genes with TIR domains. The sizes of the genomic segments encoding TIR domains [average 393 bp (249–426 bp)] were smaller than the window size (600 bp) used in our analysis so that our window analysis would underestimate the Σbn/Σbs ratios for TIR domains. The values of Σbn and Σbn/Σbs ratio for TIR domains displayed lower values when compared with those of the non-TIR coding sequences except for Σbn/Σbs ratio from *TLR10* (Table 1 and Fig. 2b). In particular, *TLR7*, *TLR8*, *TLR9*, *MYD88*, and *TICAM2* have much lower values for Σbn at the TIR domains. Taken together, it is suggested that the TIR domains have been under the control of negative/purifying selection.

On the other hand, sequence comparisons among the seven primates support the positive Darwinian selection at the extracellular domain of TLR4, for which the Σbn/Σbs ratios were much higher than 1.0 (the highest value in the 600-bp window is 2.37, with a statistical significance in *Z* test; Zhang et al. 1998, Tamura et al. 2007; *Z* score 2.16; p value <0.01; Fig. 1). Among analyzed windows fro TLR-related genes, *TLR4* and *TICAM1* have extreme high values of Σbn. However, the windows of *TICAM1* harboring high values of Σbn also have the high value of Σbs. The high values of %GC seem to be associated with the high-nucleotide substitution rate in *TICAM1* (Fig. 1a and b). A lower value of CBI was also correlated with a lower synonymous nucleotide substitution rate (data not shown).

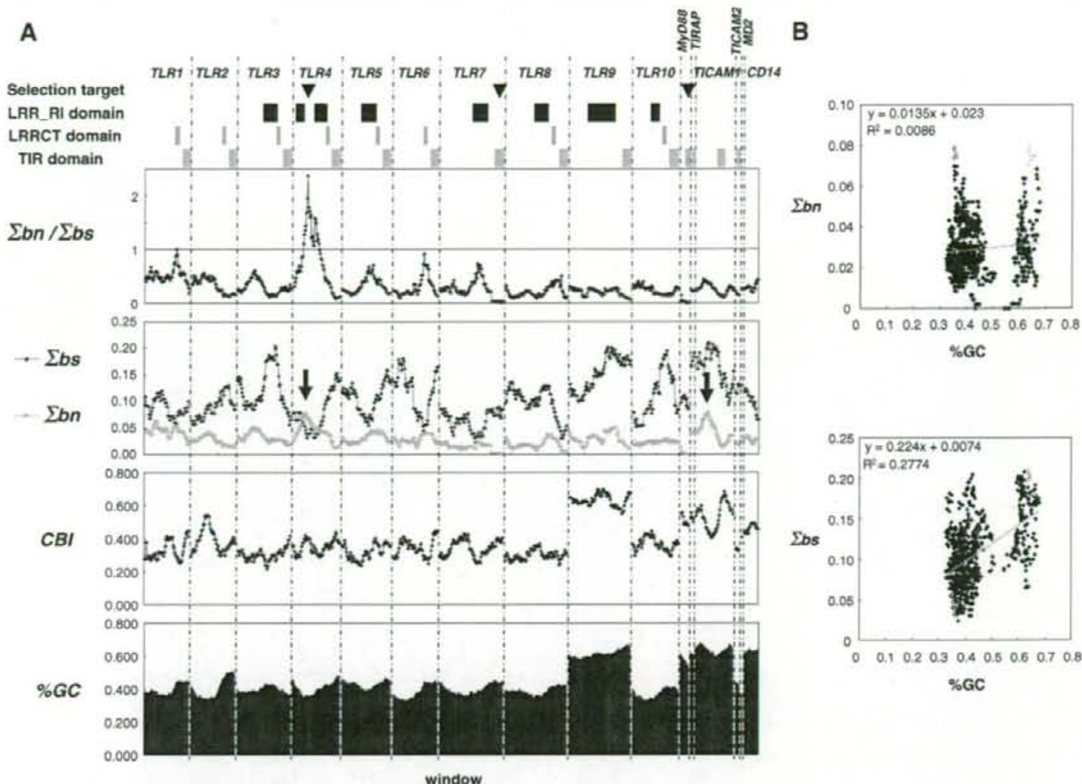

Fig. 1 **a** The values of Σbn/Σbs, Σbn, Σbs, CBI, and %GC based on the sliding window plot analysis for the TLR-related gene (600-bp window with 30-bp steps). The *arrow heads* indicate the candidate segments for the pressure of positive or negative natural selection. The *arrows* indicate that *TLR4* and *TICAM1* have extreme high values of Σbn among analyzed windows. CBI is a measure of the deviation from the equal use of synonymous codons, which indicates the extent to which a gene uses a subset of optimal codons (Bennetzen and Hall 1982). Three conserved domain structures, *LRR_RI* (leucine-rich repeats, ribonuclease inhibitor-like subfamily), *LRRCT* (leucine-rich repeat C-terminal domain), and *TIR* (Toll/interleukin-1 receptor homology domain), are referred from CD-search (Marchler-Bauer and Bryant 2004). **b** Pairwise comparisons between Σbn and %GC and between Σbs and %GC. All values of Σbn, Σbs, and %GC were based on the sliding window plot analysis for the TLR-related gene. *TLR4* (*white lozenge*) and *TICAM1* (*gray lozenge*) have several windows with extreme high values of Σbn

In the window of *TLR4* harboring the highest value of Σbn, its level of CBI (0.398) was almost equivalent to the average level among analyzed windows (0.387±0.114).

The estimated values of bn and bs of each lineage at *TLR4* target region were shown in Fig. 3. The values of bn in three lineages since the emergence of great apes were significantly higher than those of bs. These lineages have a relatively low value of bs; however, the values of bn were much larger than the estimated value of bs for entire TLR4 coding sequences in each lineage except for orangutan lineage (Supplementary material, Fig. S1). These lines of evidence suggested that the extracellular domain of TLR4 has been the possible target of positive Darwinian selection in the course of primate evolution.

To evaluate this finding further, we determined the sequences of a ~600 bp *TLR4* target region from additional 18 primates, including three gibbons (black gibbon, white-handed gibbon, and siamang), four Old World monkeys (hamadryas baboon, black and white colobus, silvered lutong, and dusky lutong), eight New World monkeys (common marmoset, cotton-top tamarin, red-handed tamarin, lion tamarin, common squirrel monkey, tufted capuchin, long-haired spider monkey, and Central American spider monkey), and three prosimians (tarsiers, lesser galago, ring-tailed lemur). Each of target sequences from three gibbons, two lutongs, and two tamarins is identical so that a total of 21 sequences were advanced to further analyses.