(R5 virus). Two single nucleotide polymorphisms (SNP) in *RANTES* promoter; *-403G/A* and *-28C/G*, were identified [2]. The *RANTES-28G* variant was shown to elevate promoter activity *in vitro* [2,3] and is associated with a slower rate of CD4 cell depletion in HIV-1 infected Japanese patients [2]. Although this result was not confirmed in subsequent studies in other ethnic groups in which the *RANTES-28G* frequency was lower than in the Japanese [3–5], McDermott *et al.* reported that patients with *RANTES-28G* showed a clear trend towards slower rate of CD4 cell depletion in HIV-1 infected European Americans [6].

With respect to *RANTES-403A* variants, HIV-1 infected European Americans with *RANTES-403A* were initially reported to progress more slowly to AIDS [6]. However, subsequent studies with a larger sample size showed that *RANTES-403A* or *RANTES In1.1C*, a *RANTES* intron polymorphism which is in a strong linkage disequilibrium with *RANTES-403A* and which negatively affects *RANTES* transcription *in vitro*, was reported to be associated with an increased rate of disease progression [3,4].

A variant of the interleukin-4 (*IL4*) promoter, *-589T* was found to be associated with elevated levels of serum IgE and to accelerate phenotypic switches of HIV-1 from non-syncytium inducing (NSI) to syncytium-inducing (SI) type in HIV-infected Japanese patients probably due to the suppressive effect of IL4 on CCR5 expression together with its enhancing effect on CXCR4 expression [7]. This allele was subsequently found to be associated with a lower viral load and a slower rate of disease progression in HIV-infected French patients [8,9]. However, studies in other ethnic groups did not show consistent results [10,11].

There is a considerable variation in allele frequencies at these loci among different ethnic groups. Furthermore, diverse strains of HIV-1 circulate in different geographical areas and effects of host genetic polymorphisms may differ in individuals infected with different HIV-1 subtypes. Therefore, it is important to study influences of host genetic polymorphisms in different populations; however, such data in Asian populations remain sparse. In this paper, we report that polymorphisms in *IL4* and *RANTES* influence HIV load, CD4 cell count and survival time in Thai patients infected with HIV-1 subtype E.

Materials and methods

Patients and samples

We conducted a prospective cohort study at the HIV clinic in the Day Care Center of Lampang Hospital in northern Thailand. The Lampang Hospital is a government referral hospital with approximately 800 beds. It is located in the center of Lampang province, a province 600 km to the north of Bangkok. We approached all HIV-1 infected individuals who attended the clinic from 6 July 2000 to 12 July 2001 and asked them to participate in this cohort study. After giving written informed consent, all participants were interviewed by trained study coordinators, and clinically examined by two designated doctors. EDTA-treated blood was taken from each individual. Plasma and buffy coat fractions were separated and stored at −80°C until use. For a control group we collected blood samples from 119 female blood donors at the blood bank of the Lampang Hospital.

Plasma HIV-1 RNA copy number was measured using a commercial kit (Amplicor HIV-1 Monitor Test, Roche Molecular System, Inc. Branchburg, New Jersey, USA), which has a lower limit of detection of 400 copies/ml. CD4 cell count was measured by FACScan (BD Biosciences, California, USA) using fresh EDTA-treated whole blood samples at the time of recruitment. The survival status of participants until 1 October 2003 was ascertained from the cohort database, mailing letters, and death certificates at the Lampang Provincial Health Office. Data were double entered and validated using the access program. This study was approved by the Ethical Review Committee for Research in Human Subjects, Ministry of Public Health, Thailand in January 2000.

Polymorphism genotyping

Genomic DNA was extracted and purified from the buffy coat fraction using QIAamp mini blood kit (QIAGEN GmbH, Hiden, Germany). Each specimen was analyzed for *IL4-589C/T*, *RANTES-403G/A*, *RANTES-28C/G*, *RANTES In1.1T/C*, and *RANTES 3'222T/C* by PCR–RFLP as described elsewhere [2,3,7].

Sample analysis

We conducted this study strictly in blinded manner so that field investigators were masked from any host genetic polymorphism data and laboratory investigators were masked from any clinical data. The data set was analyzed only after deleting sample and patient's identification number. Continuous variables of two groups with different genetic background were compared by a non-parametric Kruskal–Wallis test. Qualitative variables of two groups were compared by the Chi-square test. Significance in Kaplan–Meier analysis was determined by the log-rank test. Statistical analyses were carried out using Epi Info version 3.01 (US-CDC). *RANTES* haplotypes were constructed and calculated for their frequencies by an Expectation-Maximization algorithm using software (Arlequin version 2.01, Genetica and Biometry Laboratory, Geneva, Switzerland). D′ measure for linkage disequilibrium was also calculated by using Arlequin. Incidence rate ratios were calculated by STATA (Stata Corp., Texas, USA). Crude and adjusted relative risks (RR) and their 95% confidence intervals (CI) were calculated by Cox proportional hazard models using StatView (SAS Institute Inc. North Carolina, USA).

Results

Baseline characteristics of study subjects

During the study period, 487 HIV-infected individuals participated in this study. There were 187 males and 300 females; baseline CD4 cell counts and antiretroviral (ARV) drug use information was available in 185 males and 297 females. We found that HIV-disease was significantly more advanced in men than in women. Median [interquartile range (IQR)] CD4 cell count at the time of recruitment in men [66 (15–258) cells/μl) was significantly lower ($P < 0.0001$) than that in women [262 (90–418)]. The proportion of patients diagnosed as AIDS was also higher ($P < 0.0001$) in men (77/185; 41.6%) than in women (44/297; 14.8%). Because the aim of this study was to investigate the influence of host genetic polymorphisms on disease progression and the majority of male individuals were already in the advanced stage of HIV disease, we analyzed the data of men and women separately and in this paper we present our analysis on the 297 females.

Among these females, 186 were ARV drug naive and 111 were ARV drug experienced: 51 were receiving ARV drugs and 60 reported prior ARV treatment but were not on ARV at the time of recruitment. The latter group included 18 females who were given zidovudine (ZDV) as prophylaxis for HIV mother-to-child transmission. The remaining 42 subjects stopped ARV drugs because of financial constraints.

All females reported that they had contracted HIV-1 through heterosexual contact. Baseline characteristics of ARV drug naive and ARV drug interrupted groups were similar (Table 1). In this analysis, we excluded the 51 individuals who were on ARV drug treatment, because on-going ARV drug treatment considerably affects CD4 cell count and viral load so that an association between these parameters and genetic polymorphisms may be obscured.

Allele frequencies of *IL4-589T* and *RANTES-403A, RANTES-28G, RANTES In1.1C*, and *RANTES 3'222C*

We genotyped 246 ARV drug-free females and 119 blood donors for *IL4-589C/T, RANTES-403G/A, RANTES-28C/G, RANTES In1.1T/C*, and *RANTES 3'222T/C*. Allele frequency of *IL4-589T* was 0.774 in 246 ARV drug-free females and 0.798 in 119 blood donors. Frequencies of four *RANTES* SNP in ARV drug-free females and blood donors are shown at the bottom of Table 2. There was no difference in frequency of those five SNP among subgroups, including the ARV-treated group (data not shown). All of the observed genotypes were in Hardy–Weinberg equilibrium (data not shown).

Table 2 shows estimated *RANTES* haplotype distribution in 246 ARV drug-free females and 119 blood donors. As reported in European- and African-Americans, the haplotype R1 was the most common. In European- and African-Americans, approximately 75% and 55% of *RANTES-403A* was associated with *RANTES In1.1C*, respectively. *RANTES-403A* was more closely associated with *RANTES In1.1C* in Thai patients than in European- and African-Americans, since 93.4% of *RANTES-403A* were associated with *RANTES In1.1C* in 246 ARV drug-free female patients ($D'=1.0$, Table 2). Therefore, we focused on *RANTES-28G* and *RANTES In1.1C* in the subsequent analysis among four *RANTES* polymorphisms, as *RANTES-28G* and *RANTES In1.1C* were shown to be functional and actually affected RANTES promoter activity [2,3], while roles of *RANTES-403A* and *RANTES 3'222T/C* as functional alleles were not yet confirmed.

Genotype variation and HIV disease status (clinical, CD4 cell count, viral load)

We analyzed viral load, CD4 cell count, and clinical status among ARV drug-free females at recruitment by stratifying individuals according to their host genetic background (Table 3). We found that patients with the homozygous *IL4-589T* allele showed a significantly lower HIV-1 viral load ($P = 0.005$) and higher CD4 cell count ($P = 0.003$) than patients with heterozygous *IL4-589C/T* or homozygous *IL4-589C* alleles. Furthermore the proportion of symptomatic individuals was lower among females with homozygous *IL4-589T* allele than among others, although this trend did not reach statistical significance ($P = 0.183$).

Patients with *RANTES-28G* allele (*C/G* or *G/G*) showed a weak trend toward a higher CD4 cell count

Table 1. Baseline data of ARV drug-free HIV-1 infected females.

Characteristics	ARV drug naive (n = 186)	ARV treatment interrupted (n = 60)	Combined (ARV drug free) (n = 246)
Age at recruitment: median years (IQR)	31 (28–36)	31 (27.5–38)	31 (28–36)
CD4 cell count [median cells/μl (IQR)]	269 (71–422)	259.5 (63–400.5)	262 (67–421)
HIV-1 load [median $\log_{10}$ HIV RNA copies/ml (IQR)]	5.084 (4.424–5.549)	5.0194 (3.897–5.592)	5.078 (4.331–5.571)
AIDS- related symptoms [% (n)]	37.1(69)	45(27)	39 (96)
95% CI	30.1–44.5	32.1–58.4	32.9–45.4
Diagnose of AIDS [%(n)]	15.6 (29)	20 (12)	16.7 (41)
95% CI	10.7–21.6	10.8–32.3	12.3–21.9

ARV, Antiretroviral; IQR, interquartile range; CI, confidence interval.

Table 2. *RANTES* haplotype frequencies in ARV drug free HIV-1 infected females and female blood donors.

RANTES Haplotype	*RANTES* polymorphism site				ARV drug free[a] (n = 246)	Blood donor[b] (n = 119)
	−403	−28	In1.1	3′222		
R1	*G*	*C*	*T*	*T*	0.697	0.731
R2	*A*	*C*	*T*	*T*	0.020	0.034
R3	*A*	*C*	*C*	*T*	0.002	0.000
R4	*A*	*C*	*C*	*C*	0.191	0.160
R5	*A*	*G*	*C*	*T*	0.089	0.076

[a]Allele frequencies of *RANTES-403A* = 0.303, *RANTES-28G* = 0.089, *RANTES In1.1C* = 0.283, and *RANTES 3′222C* = 0.191.
[b]Allele frequencies of *RANTES-403A* = 0.269, *RANTES-28G* = 0.076, *RANTES In1.1C* = 0.235, and *RANTES 3′222C* = 0.160. ARV, Antiretroviral.

($P = 0.165$), although the differences were not statistically significant. There was no significant difference in HIV-1 viral load or proportion of symptomatic individuals between patients with and without the *RANTES-28G* allele. Patients with *RANTES In.1C* showed no difference from those without this allele in HIV-1 viral load, CD4 cell count, or proportion of symptomatic cases.

Genotype variation and survival status

Of 246 ARV drug-free females, we obtained follow-up information from 238 (96.7%): the median (IQR) of follow-up was 964 (495–1072) days. During the follow-up period, 55 patients had started ARV drug treatment. Among 524.05 persons-years of follow up, 54.16 persons-years were ARV drug treatment positive. Therefore, the majority (89.7%) of our study subjects was ARV drug-free. There was no difference in proportion of patients who started ARV drugs during the observation period among each genotype group (Table 3). During the follow up period, 65 deaths occurred (including one suicide). Death was then used as the marker for HIV-1 disease progression. We found that the mortality rate among homozygous *IL4-589T* individuals was slightly lower than for the other groups, but this difference did not reach statistical significance [incidence rate ratio (IRR), 0.68; 95% CI, 0.40–1.14; $P = 0.121$) (Table 3). For *RANTES* promoter, there was significantly lower mortality rate among individuals with *RANTES-28G* allele when compared to those without this allele (IRR, 0.41; 95%CI, 0.15–0.95; $P = 0.024$) (Table 3). In contrast, there was no difference in the mortality rate between individuals with *RANTES In1.1C* and those without this allele (IRR. 1.11; 95%CI, 0.66–1.84; $P = 0.67$).

Table 3. Median HIV-1 load and CD4 cell count, HIV-1 related symptoms, diagnosis of AIDS and number of deaths/person years of observation (PYO) stratified by *IL4-589C/T, RANTES-28C/G,* and *RANTES In1.1T/C* genotype group.

	IL4-589		*RANTES-28*		RANTES In1.1	
	C/C and C/T (n = 99)	T/T (n = 147)	C/C (n = 205)	C/G and G/G (n = 41)	T/T (n = 133)	T/C and C/C (n = 113)
Median viral load [log_{10} copies/ml (IQR)]	5.381 (4.519–5.650)	4.908 (4.214–5.441)	5.107 (4.406–5.578)	4.843 (4.177–5.491)	5.098 (4.287–5.615)	5.053 (4.433–5.507)
P (Kruskal–Wallis test)		0.005		0.275		0.929
Median CD4 count [(cells/μl (IQR)]	171 (46–354)	303 (144–433)	261 (58–412)	297 (144–451)	285 (66–414)	244 (71–421)
P (Kruskal–Wallis test)		0.003		0.165		0.684
HIV-1 related symptoms [%(n)]	44.4(44)	35.4(52)	40.5(83)	31.7(13)	37.6(50)	40.7(46)
(95% CI)	(34.5–54.8)	(27.7–43.7)	(33.7–47.5)	(18.1–48.1)	(29.3–46.4)	(31.6–50.4)
P (Chi^2 test)		0.153		0.293		0.618
Diagnosis of AIDS [% (n)]	19.2(19)	15(22)	17.1 (35)	14.6 (6)	16.5(22)	16.8(19)
(95% CI)	(12.0–28.3)	(9.6–21.8)	(12.2–22.09)	(5.6–29.2)	(10.7–24.0)	(10.4–25.0)
P (Chi^2 test)		0.383		0.702		0.954
Dealths (n)	30	35	59	6	34	31
PYO	192.59	331.90	420.84	103.66	288.17	236.33
Mortility rate (%)	15.58	10.54	14.02	5.79	11.8	13.12
(95% CI)	(10.01–21.15)	(7.05–14.03)	(10.44–17.60)	(1.16–10.42)	(7.83–15.77)	(8.5–17.74)
Incidence rate ratio of death (95% CI)		0.68 (0.40–1.14)		0.41 (0.15–0.95)		1.11 (0.66–1.84)
P (Chi^2 test)		0.121		0.024		0.67
ARV treatment during follow-up	22	33	44	11	32	23
Observed (n)	94	144	197	41	129	109
P (Chi^2 test)		0.930		0.534		0.499

IQR, Interquartile range; CI, confidence interval.

Consistent with the results on mortality rate, the Kaplan–Meier analysis demonstrated an insignificant better survival in homozygous *IL-589T* ($P = 0.125$) (Fig. 1a), a significant better survival in individuals carrying *RANTES-28G* ($P = 0.037$) (Fig. 1b), and no difference between individuals carrying *RANTES In1.1C* and those without this allele ($P = 0.668$) (Fig. 1c). Since *RANTES-28G* was associated with *RANTES In1.1C* (Table 3), we then grouped the patients as: (i) individuals who posses sed neither *RANTES-28G* nor *RANTES In1.1C*; (ii) individuals who possessed *RANTES In1.1C* but not *RANTES-28G*; and (iii) individuals who possessed

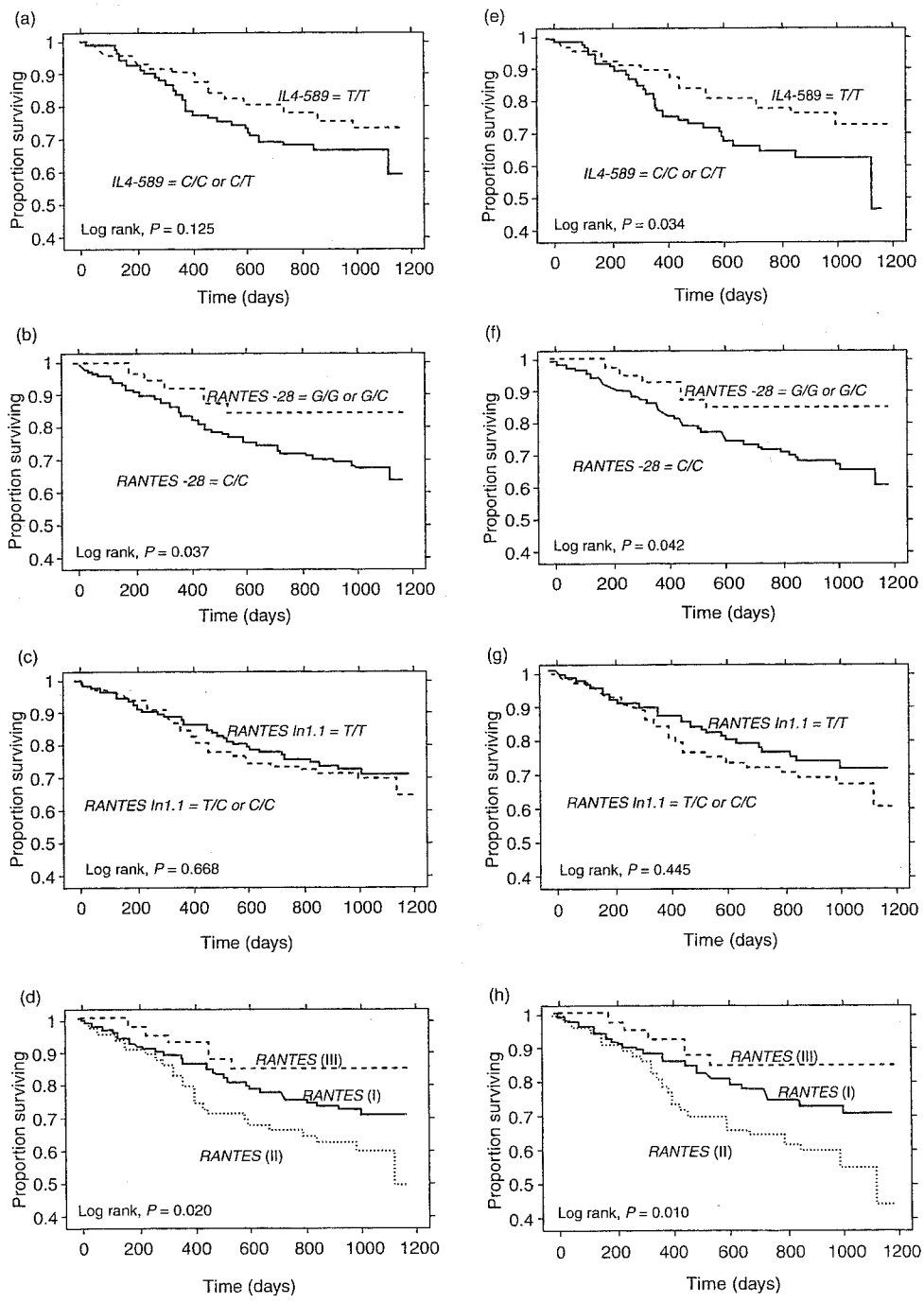

Fig. 1. Kaplan–Meier analysis of the effect of promoter polymorphism of *IL4* and *RANTES* on survival in ARV drug-free HIV-1 infected Thai females during the total observation period (a–d) or untreated period (e–h). (a and e) *IL4-589T*; (b and f) *RANTES-28*; (c and g) *RANTES-403*; and (d and h): grouped *RANTES* as (I) individuals who possessed neither *RANTES In1.1C* nor *RANTES-28G*, (II) individuals who possessed *RANTES in1.1C* but not *RANTES-28G*, and (III) individuals who possessed *RANTES-28G*.

RANTES-28G. The analysis clearly showed a poorer survival in the group (ii) and a better survival in group (iii) when compared with group (i) ($P = 0.020$) (Fig. 1d). These results suggested that *RANTES-28G* had a protective effect against HIV-1 disease progression in Thai patients, and that *RANTES In1.1C* without *RANTES-28G* had an accelerating effect. We obtained virtually the same results as above when we used *RANTES-403A* instead of *RANTES In1.1C* (data not shown).

We then subtracted the numbers of days after patients started to receive ARV drugs from the observation period in order to adjust possible effects of ARV treatment, although the majority of our study subjects was ARV drug-free during the observation period. As shown in Fig. 1e–h, we obtained almost identical results to those shown in Figure 1a–d, except that difference between homozygous *IL4-589T* individuals and the others became statistically significant ($P = 0.034$, Fig. 1e). Thus, the risk of death for homozygous *IL4-589T* patients during the untreated period was lower than the other patients with heterozygous *IL4-589T* or homozygous *IL4-589C* (RR, 0.59 Cox model; 95% CI, 0.36–0.97; Fig. 1e). The RR of death for patients with *RANTES-28G* during the untreated period was also lower than those without *RANTES-28G* (RR, 0.43; 95% CI, 0.19–0.99; Fig. 1f). The RR of death for patients with *RANTES In1.1C* during the untreated period was not significantly higher than those without this allele (RR, 1.21; 95% CI, 0.74–1.98; Fig. 1g).

However, after adjustment for *RANTES-28G*, the RR of death for patients with *RANTES In1.1C* became significantly higher (adjusted RR, 1.75; 95% CI, 1.03–2.95). Similarly, the RR of death for patients with *RANTES-28G* became lower after adjustment for *RANTES In1.1C* (adjusted RR, 0.30; 95% CI, 0.12–0.74). These results were most likely caused by the fact that protective *RANTES-28G* was in strong linkage disequilibrium with deleterious *RANTES In1.1C* as shown in Table 2. Further adjustment for *IL4-589T* did not affect RR of death for patients with *RANTES-28G* (adjusted RR, 0.30; 95% CI, 0.12–0.74) or patients with *RANTES In1.1C* (adjusted RR, 1.79; 95% CI, 1.06–3.02). Similarly, adjustment for *RANTES-28G* and *RANTES Int1.1C* did not influence the RR of death for homozygous *IL4-589T* patients (adjusted RR, 0.59; 95% CI, 0.35–0.96). These results suggested that *IL4* and *RANTES* were independent factors involved in protection against HIV-1 disease progression.

Further adjustment for age at recruitment and previous ARV drug history did not affect the RR of death during the untreated period for homozygous *IL4-589T* patients (adjusted RR, 0.54; 95% CI, 0.32-0.89), patients with *RANTES-28G* (adjusted RR, 0.27; 95% CI, 0.11–0.68) or patients with *RANTES In1.1C* (adjusted RR, 1.94; 95% CI, 1.14–3.30). Adjustment for diagnosis of AIDS at the time of recruitment only slightly affected the RR of death for homozygous *IL4-589T* patients (adjusted RR, 0.61; 95% CI, 0.37–1.00; $P = 0.051$). However, the protective effect of homozygous *IL4-589T* became no longer significant after adjustment for serum virus load (adjusted RR, 0.76; 95% CI, 0.45–1.27; $P = 0.293$) or CD4 cell count (adjusted RR, 0.86; 95% CI, 0.51–1.44; $P = 0.563$). Adjustment for virus load, CD4 cell count, and diagnosis of AIDS at the time of recruitment almost completely diminished the protective effect of homozygous *IL4-589T* (adjusted RR, 0.95; 95% CI, 0.56–1.62; $P = 0.854$). These results suggested that the protective effect of *IL4-589T* was mediated through a lower virus load and higher CD4 cell count. In a sharp contrast, the protective effect of *RANTES-28G* and the deleterious effect of *RANTES In1.1C* were not influenced by adjustment for virus load, CD4 cell count, and diagnosis of AIDS at the time of recruitment (adjusted RR of *RANTES-28G*, 0.27; 95% CI, 0.11–0.68; adjusted RR of *RANTES In1.1C*, 1.98; 95% CI, 1.15–3.41).

Discussion

This is the first host-genetic polymorphism study with a substantial sample size in the Southeast Asia, addressing an effect on HIV-1 disease progression. The *IL4-589T* allele was very common (0.78) in Thai patients as in other Asians – 0.69 in Japanese, 0.77 in Chinese – whereas it presents at a significantly lower frequency (0.15) in Caucasians [8,10,11]. For the *RANTES* gene, we observed more *RANTES-28G* (0.09) than in European and African–Americans [3,4,6]. Although our study group was not a sero-conversion cohort, we found a statistically significant association of the homozygous *IL4-589T* with a lower viral load, a higher CD4 cell count and a better survival during the untreated period. Individuals with *RANTES-28G* allele were also had a better survival rate.

The presence of *IL4-589T* allele was reported to be associated with an increased promoter activity [12]. Thus one expects the down-regulation of CCR5 and the up-regulation of CXCR4 in the CD4 T cell population in individuals with this allele [13]. Consequently, in individuals with *IL4-589T*, levels of replication of R5 viruses would be lower than in those without *IL4-589T*. It has been shown that R5 viruses were generally resistant to neutralizing antibodies whereas X4 viruses are sensitive, and X4 viruses are rarely detected until the advanced stage of HIV disease [14]. If it is the R5 viruses that are mainly responsible for driving immune deficiency in HIV infection, it is plausible that a higher expression of IL-4 associates with a better prognosis.

Two previous studies based on two independent cohorts in France showed a protective effect of *IL4-589T* on

HIV disease progression [8,9]. One paper based on the Amsterdam cohort showed an association of this allele with the delay of acquisition of X4 virus and the decline of CCR5 expressing memory CD4 T cell numbers, though this study did not show effects of *IL4-589T* on overall disease progression [10]. Our present study based on a cohort in Thailand is the third example to demonstrate the significant protective effect of *IL4-589T* on HIV disease progression. Only one previous report, which analyzed five USA natural history cohorts including several ethnic groups, failed to show such an association [11]. At present, the reason for the discrepancy among different studies is unclear, but may be due to the differences in study design (sero-conversion/cross-sectional, marker/endpoint of disease progression, duration of follow-up, etc.) or difference in the frequency of this allele. In addition, interactive effects may differ among different populations due to haplotype structure and allele frequency differences. As our study population was not a sero-conversion cohort, a considerable number of individuals had already progressed to end-stage HIV disease by the time of study enrollment. It is possible that individuals at the end stage of diseases might cause certain biases that we could not realize at present.

Compared with the most common *RANTES* haplotype (*RANTES In1.1T* and *RANTES-28C*), our study showed that individuals carrying *RANTES In1.1C* with *RANTES-28G* had better survival, while individuals carrying *RANTES In1.1C* without *RANTES-28G* had poorer survival. Our present finding on *RANTES-28G* was concordant with the previous study on Japanese patients [12], in whom the frequency of *RANTES-28G* was higher than in Thais. On the other hand, our result on *RANTES In.1C* was concordant with the previous results that *RANTES In1.1C* was associated with an increased rate of HIV-1 disease progression in African–Americans [3], in whom *RANTES-28G* is absent. Since most of *RANTES In1.1C* are associated with *RATNES-403A* in Thai patients (Table 2), our finding on *RANTES In1.1C* was also concordant with the previous results that *RANTES-403A* was associated with an increased rate of HIV-1 disease progression in European Americans [4], in whom the frequency of *RANTES-28G* was much lower than in Thais. Therefore, the discrepancy of the effect of *RANTES* polymorphisms in different studies may be due to the difference in *RANTES-28G* allele frequency in different ethnic groups. However, the differences in study design and effects of other genetic polymorphism background could not be excluded.

In contrast to the *IL4-589T* allele, the effect of *RANTES* polymorphisms on HIV-1 diseases was more clearly observed in the survival analysis (Fig. 1) than in baseline viral load and CD4 cell counts (Table 4). Analysis by the Cox proportional hazard models suggested that the protective effect of *IL4-589T* was mediated through a lower virus load and higher CD4 cell count, whereas effects of *RANTES* polymorphisms were totally independent from those factors. The reason for this discrepancy is not clear at present. One possible explanation for this discrepancy is that the *RANTES* polymorphisms affect HIV-1 disease progression through the mechanisms other than the direct suppressive effect of RANTES on HIV-1 entry. RANTES also affects trafficking and proliferation of cells expressing CCR1, CCR3 or CCR5 [15]. It is possible that, in individuals with different *RANTES* genotypes, certain types of cells expressing those chemokine receptors can be modulated to different extents, leading to the different degree of susceptibility to disease progression and/or opportunistic infections. It is noteworthy that numbers of CD8 cells, which are known to express more CCR5 than CD4 cells, also tended to be higher in patients with *RANTES-28G* than those without this allele in 246 drug-free Thai females (941 and 862 cells/μl, $P = 0.178$). Further studies are necessary to elucidate precise roles of RANTES in HIV-1 disease courses.

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A Specific Region of 37 Amino Acid Residues in the SPRY (B30.2) Domain of African Green Monkey TRIM5α Determines Species-Specific Restriction of Simian Immunodeficiency Virus SIVmac Infection

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Human immunodeficiency virus type 1 (HIV-1) efficiently enters cells of Old World monkeys but encounters a block before reverse transcription. This restriction is mediated by a dominant repressive factor. Recently, a member of the tripartite motif (TRIM) family proteins, TRIM5α, was identified as a blocking factor in a rhesus macaque cDNA library. Among Old World monkey cell lines, the African green monkey kidney cell line CV1 is highly resistant to not only HIV-1 but also simian immunodeficiency virus SIVmac infection. We analyzed TRIM5α of CV1 cells and HSC-F cells, a T-cell line from a cynomolgus monkey, and found that both CV1- and HSC-F-TRIM5αs could inhibit CD4-dependent HIV-1 infection, as well as vesicular stomatitis virus glycoprotein-mediated infection. CV1-TRIM5α could also inhibit SIVmac infection, whereas HSC-F-TRIM5α could not. In the SPRY (B30.2) domain of CV1-TRIM5α, there was a 20-amino-acid duplication that was not present in HSC-F-TRIM5α. A chimeric TRIM5α containing 37 amino acid residues from CV1-TRIM5α, which spanned the 20-amino-acid duplication, in the background of HSC-F-TRIM5α fully gained the ability to inhibit SIVmac infection. Conversely, the mutant CV1-TRIM5α lacking the 20-amino-acid duplication completely lost the ability to restrict SIVmac infection. These findings clearly indicated that a specific region of 37 amino acid residues in the SPRY domain of CV1-TRIM5α contained a determinant of species-specific restriction of SIVmac.

Human immunodeficiency virus type 1 (HIV-1) is thought to have been introduced into the human population from chimpanzees (9) and shows a very narrow host range limited only to humans and chimpanzees. HIV-1 does not experimentally infect Old World monkeys, such as rhesus and cynomolgus monkeys, and fails to replicate in activated CD4-positive T lymphocytes obtained from these monkeys (13, 31). In contrast, simian immunodeficiency virus (SIV) isolated from a macaque monkey (SIVmac) can replicate well in rhesus (13, 31) and cynomolgus monkeys (2, 3). The restricted host range of HIV-1 has greatly hampered its use in animal experiments and, hence, caused difficulty in developing prophylactic vaccines against HIV-1 infection.

Several studies have suggested that the block of HIV-1 replication in Old World monkey cells occurred at a postentry step (7, 13, 31) and appeared to result from a failure to initiate reverse transcription (13). The block was still observed when CD4-negative monkey cells were infected with HIV-1 pseudotyped with vesicular stomatitis virus glycoprotein (VSV-G) and was overridden by a high multiplicity of infection (MOI) with VSV-G-pseudotyped virus or virus-like particles lacking genomic RNA (5, 10, 16, 19). Importantly, resistance against HIV-1 infection was shown to be dominant in heterokaryons between human and Old World monkey cells, suggesting the presence of inhibitory factor(s) against HIV-1 infection in Old World monkey cells (19). Studies on chimera of HIV-1 and SIVmac have suggested that restriction determinants lie within the HIV-1 P24 capsid protein (CA) (11, 23, 24, 29).

Recently, the screening of a rhesus monkey cDNA library identified tripartite motif 5α (TRIM5α), a component of cytoplasmic bodies, as a factor that confers resistance to HIV-1 infection (33). Shortly after, TRIM5α of African green monkey, another Old World monkey, was also shown to restrict HIV-1 infection, whereas human TRIM5α was reported to restrict N-tropic murine leukemia virus (12, 14, 25, 36).

An African green monkey kidney cell line, CV1, was shown to be highly resistant to SIVmac infection, as well as to HIV-1 infection. We analyzed TRIM5α of CV1 cells and HSC-F cells, a T-cell line from a cynomolgus monkey, and report here that the ability of CV1-TRIM5α to suppress SIVmac infection was determined by a small region composed of 37 amino acid residues in the SPRY (B30.2) domain of CV1-TRIM5α.

MATERIALS AND METHODS

Cloning and expression of TRIM5α. TRIM5α cDNA was amplified by reverse transcription-PCR from the human T-cell line MT4, cynomolgus monkey T-cell line HSC-F (2, 3), and African green monkey cell lines CV1 and Vero by using 5′-GCGGCCGCTACTATGGCTTCTGG-3′ as a forward primer and 5′-GAA TTCTCAAGAGCTTGGTGA-3′ as a reverse primer. Amplified products were then cloned into the vector pCR-2.1TOPO (Invitrogen), and the nucleotide sequence of 10 clones for each TRIM5α was determined.

The entire coding regions of selected clones were transferred to pcDNA3.1 (Invitrogen) by using NotI and EcoRI sites, which were introduced by primers used in the PCR step. Hamster TK-ts13 cells (4) were transfected with pcDNA3.1 carrying TRIM5α cDNA and cultured in the presence of 0.75 mg of

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G418 (Gibco)/ml for 14 days. The colonies obtained were examined for the expression of TRIM5α by using the TaqMan PCR method according to the manufacturer's instructions (Applied Biosystems). Sequences of the probe and primers used to specifically detect each TRIM5α were as follows: MT4-TRIM5, forward primer (5′-AACCTGGAGAAGGAGGAGGAAGAC-3′), reverse primer (5′-CTGGGTCTGCTGCACCAT-3′), and probe (5′-FAM-TCAGTTTCAGAG TTCG-TAMRA-3′); HSC-F-TRIM5, forward primer (5′-AACCTGGAGAAG GAGAAAGAAGAC-3′), reverse primer (5′-CTGGGTCTGCTGCACCAT-3′), and probe (5′-FAM-TTCGTTTCAGACTTTG-TAMRA-3′); and CV1-TRIM5, forward primer (5′-AACCTGGAGAAGGAGGAAGAAGA-3′), reverse primer (5′-CTGGGTCTGCTGCACCAT-3′), and probe (5′-FAM-TCCGTTTCAGAC TTCG-TAMRA-3′). These primers amplify the coiled-coil region of TRIM5 genes. Plasmid DNA used for transfection served as a standard to determine the number of copies of TRIM5α transcripts. The parental TK-ts13 cells were totally negative for the primate TRIM5α expression. Clones expressing each TRIM5α at comparable levels (ca. 4 to 6×10^7 copies/μg of total RNA) were used for subsequent experiments.

To generate CV1-TRIM5α and HSC-F-TRIM5α cDNAs carrying a hemagglutinin (HA) tag (YPYDVPDYAA) at their C termini, cloned CV1-TRIM5α and HSC-F-TRIM5α cDNAs in pcDNA3.1 were used as templates for PCR amplification with a primer containing a nucleotide sequence corresponding to the HA tag fused with the C-terminal portion of TRIM5α. The C-terminal portion of TRIM5α fused with the HA tag (BamHI to NotI) and the N-terminal portion of TRIM5α (NotI to BamHI) were assembled on a pCEP4 vector (Invitrogen). To generate chimeric TRIM5α HSC-F+60tag, the 182-bp SphI-BamHI fragment of HSC-F-TRIM5α-tag was replaced with the corresponding 242-bp SphI-BamHI fragment of CV1-TRIM5α in the background of HSC-F-TRIM5α-tag. Conversely, the 242-bp SphI-BamHI fragment of CV1-TRIM5α was replaced with the 182-bp SphI-BamHI fragment of HSC-F-TRIM5α-tag in the background of CV1-TRIM5α-tag to generate CV1-60tag. PCR-based mutagenesis of HSC-F-TRIM5α-tag was performed to generate HSC-delete-tag, which possessed the 5′-proximal 84 bp of the SphI-BamHI fragment of CV1-TRIM5α in the background of HSC-F-TRIM5α-tag, and HSC-insert-tag, which possessed 3′-proximal 158 bp of the SphI-BamHI fragment of CV1-TRIM5α in the background of HSC-F-TRIM5α-tag. Similarly, CV1-delete-tag, which possessed the 3′-proximal 98 bp of the SphI-BamHI fragment of HSC-F-TRIM5α in the background of CV1-TRIM5α-tag, was generated by a PCR-based mutagenesis of CV1-TRIM5α-tag. The entire coding sequences of these TRIM5α-tags were then transferred to the NotI site of pSeV18+b(+). Recombinant Sendai viruses (SeVs) carrying various TRIM5α-tags were recovered according to a previously described method (32). The viruses passaged a second time in embryonated chicken eggs were used as stock for all experiments. The wild-type Z strain of SeV served as a control in all of the experiments.

To establish human cell lines which constitutively express primate TRIM5αs or their chimeras, human osteosarcoma C143 cells were transfected with pCEP4 containing cDNA of CV1-TRIM5α-tag, HSC-F-TRIM5α-tag, CV1-60tag, or HSC-F+60tag, and cells were cultured in the presence of 0.3 mg of hygromycin B (Gibco)/ml for 14 days.

Immunoprecipitation and Western blot analysis. When we performed Western blot analysis of cells expressing HA-tagged TRIM5α proteins, we consistently observed nonspecific binding of anti-HA antibody to a protein that comigrated with HSC-F-TRIM5α. Therefore, we analyzed the expression of each HA-tagged TRIM5α protein in the hygromycin B-resistant C143 cells or MT4 cells infected with recombinant SeVs by immunoprecipitation, followed by Western blot analysis as described previously (20) to reduce nonspecific background. Briefly, cell lysate was first adsorbed with protein A-agarose before the addition of anti-HA antibody to avoid nonspecific protein binding to protein A-agarose. TRIM5α proteins in the cell lysate were then precipitated with anti-HA high-affinity rat monoclonal antibody (Roche) by using a protein A-immunoprecipitation kit (Roche). Precipitated materials were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 4 to 12% NuPAGE Bis-Tris gel (Invitrogen). Proteins in the gel were then electronically transferred to a polyvinylidene difluoride membrane (Immobilon; Millipore). Blots were blocked and probed with anti-HA antibody overnight at 4°C and then incubated with peroxidase-conjugated anti-rat immunoglobulin G (American Qualex) and developed by using the Immun-Star HRP chemiluminescence kit (Bio-Rad). Visualized image was recorded by LAS1000 (Fuji) and quantified by ImageGauge (Fuji). At least three independent experiments were performed, and the means and standard deviations (SD) for the data were calculated.

Viruses and HIV-1 lentivirus vector. VSV-G-pseudo typed HIV-1-NL43, SIVmac239, or HIV-2-GH123 was prepared by transfection of 293T cells with a combination of pMD.G (17, 18) and pNL432 (1), pBRmac239 (15), or pGH123 (30), respectively. HIV-1 vector expressing green fluorescence protein (GFP) was prepared as described previously (17, 18). Two days after transfection, culture supernatants of 293T cells were collected and assayed for reverse transcriptase activity using a reverse transcriptase colorimetric assay (Roche).

Viral infection. Assays for the HIV-1 vector expressing GFP were performed in 24-well plates containing 4×10^4 Tk-ts13-derived target cells. Serially diluted VSV-pseudotyped HIV-1 vectors encoding GFP were inoculated, and infected cells were enumerated by using a flow cytometer (FACScan; Becton Dickinson) 40 h after infection. For VSV-pseudotyped HIV-1, SIVmac239, and HIV-2 infection assays, we inoculated viruses containing 1 ng of reverse transcriptase into 4×10^4 C143 cells. For CD4-dependent infection assays, 2.5×10^5 MT4 cells were infected with SeV expressing CV1-TRIM5α-tag, HSC-F-TRIM5α-tag, or the parental Z strain of SeV at a MOI of 10 PFU per cell, followed by incubation at 37°C for 9 h. Cells were then superinfected with 30 ng of p24 of an X4 HIV-1 strain, NL43, or 30 ng of p27 of SIVmac239. The culture supernatants were collected periodically, and the level of p24 or p27 was measured by using a RETROtek antigen ELISA kit (ZeptoMetrix).

Data deposition. The sequences described here have been deposited in the GenBank database under accession numbers AB210050 to AB210052.

RESULTS

Variation in TRIM5α. We cloned TRIM5α cDNA from the human T-cell line MT4, cynomolgus monkey T-cell line HSC-F, and African green monkey kidney cell lines CV1 and Vero. The predicted amino acid sequences of TRIM5αs are compared in Fig. 1A and B.

Human TRIM5α from MT4 cells differed at an amino acid position 249 (G249D) from the previously published sequence (33) and was designated MT4-TRIM5α. The cynomolgus monkey TRIM5α from HSC-F (HSC-F-TRIM5α) was two amino acids shorter than the rhesus monkey TRIM5α (33) and two amino acids longer than the human TRIM5α. All 10 clones derived from Vero cells had the same sequence as the previously published one (36). On the other hand, we found at least two distinct TRIM5α sequences in CV1 cells. The two major TRIM5α sequences obtained from CV1 were designated CV1-TRIM5α-type1 and CV1-TRIM5α-type2, and five amino acids were found to differ between the two sequences (I259V, L337S, R351L, G359R, and G438S). Among 10 cDNA clones obtained from CV1, there were four type 1 clones and four type 2 clones. The remaining two clones were most likely chimeric artifacts. Two recently published sequences of TRIM5α from CV1 cells (AY593973 and AY625002) showed differences at three positions—L7V, I259V, and G438S—and have S, L, and R at positions 337, 351, and 359, respectively (14, 36), whereas both the type 1 and 2 clones had leucines at the seventh position. All sequences obtained from Vero and CV1 contained a 20-amino-acid duplication within the SPRY domain, which was not observed in human MT4 and cynomolgus monkey HSC-F (Fig. 1B).

The phylogenetic tree of various TRIM5α sequences showed that cynomolgus and rhesus monkey TRIM5αs are similar to each other, a finding consistent with the fact that these two monkeys belong to the genus *Macaca* (Fig. 1C).

African green monkey and cynomolgus monkey TRIM5α inhibit HIV-1 infection in nonprimate cells. We first sought to determine whether or not each TRIM5α can inhibit HIV-1 infection in the context of nonprimate cells because human and primate cells express endogenous TRIM5α that could complicate a functional analysis of TRIM5α-mediated restriction. The hamster cell line TK-ts13 was used, because it is very susceptible to a VSV-G-pseudotyped, HIV-1-based GFP-expressing lentivirus vector, HIV-1-GFP. Cell clones stably ex-

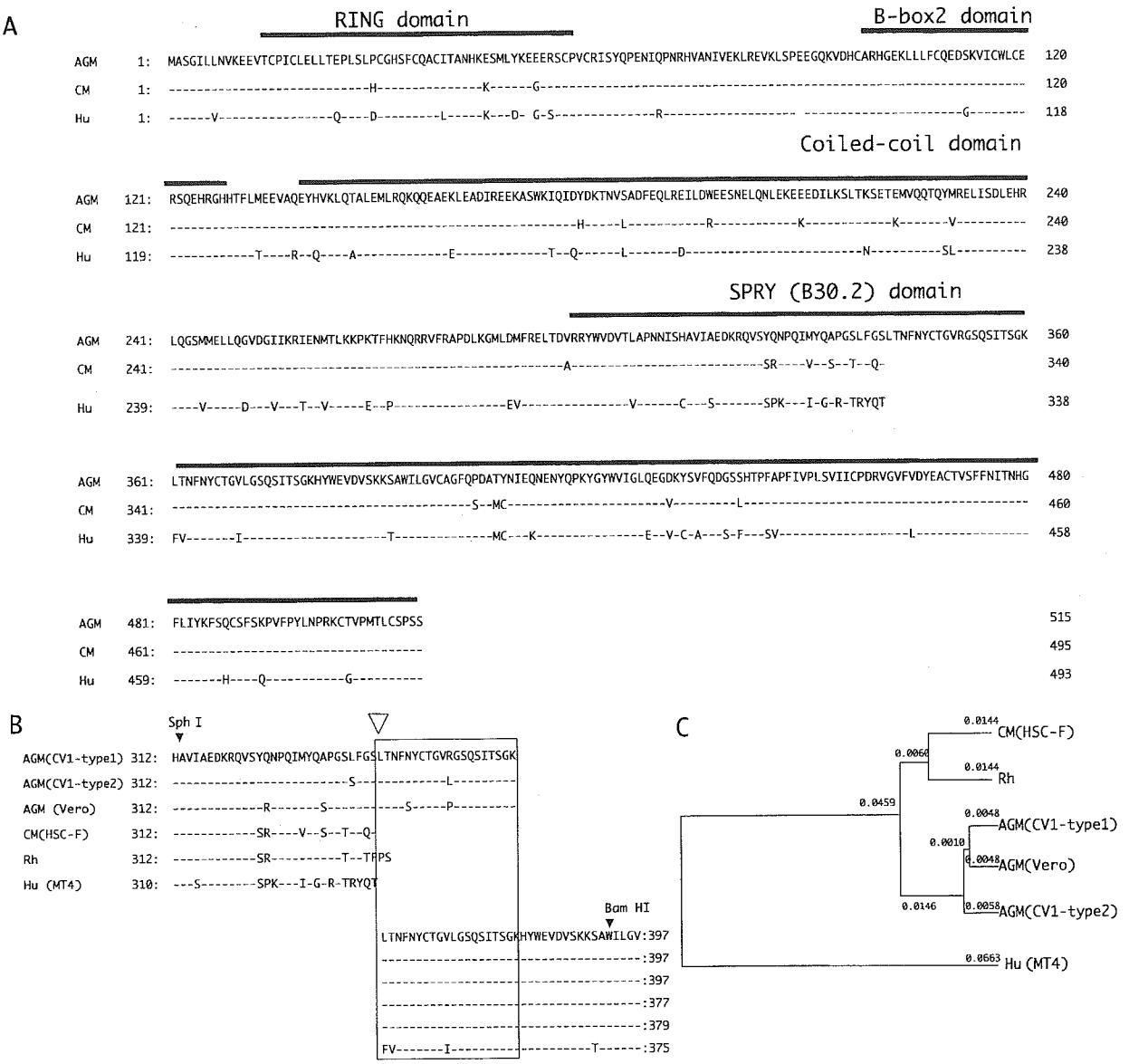

FIG. 1. (A) Alignment of amino acid sequences of African green monkey (AGM, CV1-TRIM5α-type1), cynomolgus monkey (CM, HSC-F-TRIM5α), and human TRIM5α (Hu, MT4-TRIM5α) predicted from the sequences of the cDNAs used in the present study with key domains indicated. (B) Alignment of amino acid sequences of the highly variable region within the SPRY (B30.2) domain of TRIM5α. The rhesus monkey TRIM5α (Rh) sequence published by Stremlau et al. (33) was added. A box indicates the 20-amino-acid duplication within African green monkey TRIM5α. An open arrowhead denotes the recombination point in chimeric TRIM5αs, HSC-delete-tag, HSC-insert-tag, and CV1-delete-tag (see Fig. 4A). (C) Phylogenetic tree of various TRIM5α sequences produced by the UPGMA (unweighted pair-group method with arithmetic averages) method.

pressing MT4-, CV1-, and HSC-F-TRIM5α were selected according to the method described in Materials and Methods. The levels of expression of TRIM5α were determined by using a real-time PCR, and cells expressing comparable amounts of TRIM5α (4×10^6 to 6×10^6 copies/μg of total RNA) were used for subsequent study. Restriction can be quantified by comparing the percentage of GFP-positive cells with or without TRIM5α.

As can be seen in Fig. 2A, MT4-TRIM5α had a very weak anti-HIV-1 effect (~1.2-fold), a finding consistent with a previous study (33). In contrast, restriction of HIV-1 was clearly evident over a wide range of initial MOIs in cells expressing HSC-F-TRIM5α, CV1-TRIM5α-type1, and CV1-TRIM5α-type2 (ca. 5- to 10-fold). There was no significant difference between CV1-TRIM5α-type1 and type2; therefore, we chose CV1-TRIM5α-type1 to be representative of CV1-TRIM5α in the subsequent experiments. We obtained the same results as described above when we used an HA-tagged version of TRIM5α (data not shown).

African green monkey and cynomolgus monkey TRIM5α inhibit CD4-dependent HIV-1 infection in human cells. To test the restriction properties of TRIM5α in CD4-dependent

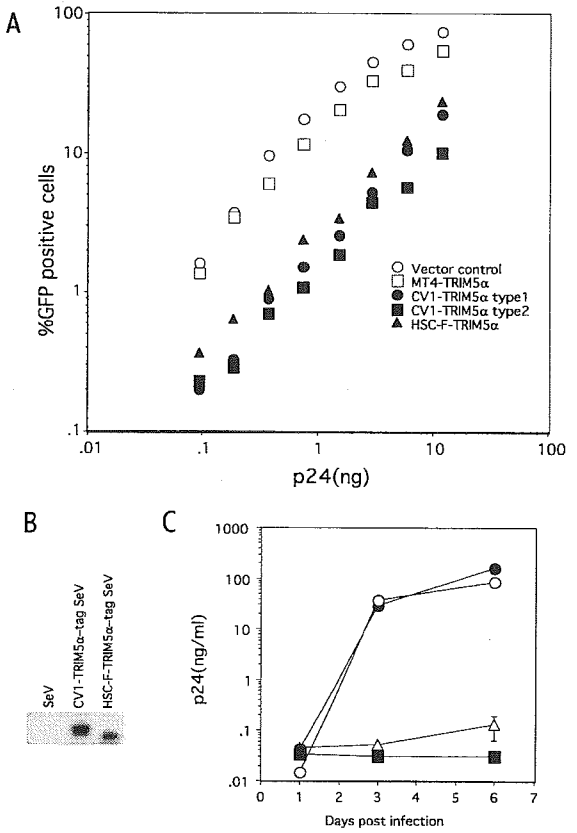

FIG. 2. (A) TK-ts13 cell clones expressing MT4-TRIM5α (□), CV1-TRIM5α-type1 (●), CV1-TRIM5α-type2 (■), HSC-F-TRIM5α (▲), or empty vector (○) were exposed to the indicated GFP-expressing HIV-1 vector. GFP-positive cells were counted with a flow cytometer. Data typical of at least three independent clones for each TRIM5α are shown. (B) Lysates of MT4 cells infected with recombinant SeV expressing CV1-TRIM5α-tag, HSC-F-TRIM5α-tag, or the parental Z strain were immunoprecipitated by anti-HA antibody. Resultant immunoprecipitates were visualized by Western blotting with an antibody to HA. A representative result of four independent experiments is shown. (C) MT4 cells were mock infected (○), or infected with SeV expressing CV1-TRIM5α-tag (■), HSC-F-TRIM5α-tag (△), or the parental Z strain (●). At 9 h after infection, cells were inoculated with an HIV-1 strain, NL43, and culture supernatants were periodically assayed for levels of p24. The datum points are means for triplicate samples with the SD.

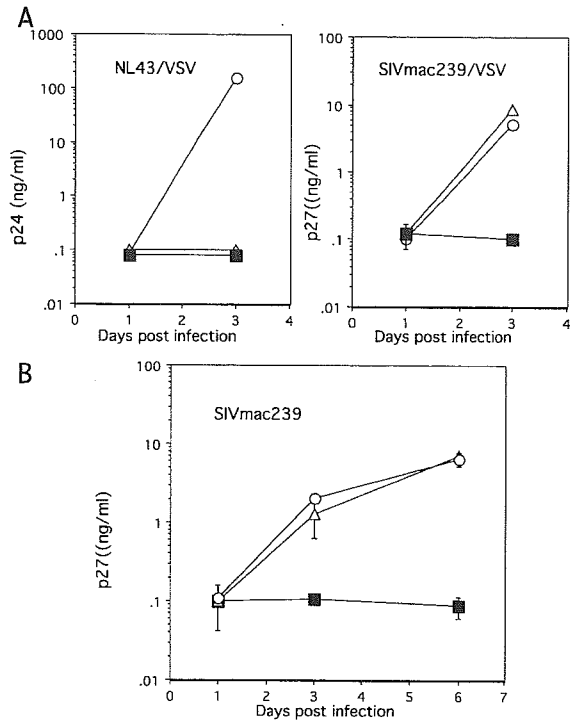

FIG. 3. (A) MT4 (○), HSC-F (△), or CV1 (■) cells were infected with VSV-pseudotyped NL43 or VSV-pseudotyped SIVmac239, and culture supernatants were periodically assayed for levels of p24 or p27. The datum points are means for triplicate samples with the SD. (B) MT4 cells were mock infected (○) or infected with SeV expressing CV1-TRIM5α-tag (■) or HSC-F-TRIM5α-tag (△). At 9 h after infection, cells were inoculated with SIVmac239, and culture supernatants were periodically assayed for levels of p27. The datum points are means for triplicate samples with the SD.

HIV-1 infection, we constructed a recombinant SeV expressing TRIM5α fused with the HA tag in the C-terminal of HSC-F-TRIM5α or CV1-TRIM5α (HSC-F-TRIM5α-tag SeV or CV1-TRIM5α-tag SeV). Human T-cell line MT4 cells were first infected with the SeV expressing TRIM5α-tag (Fig. 2B), incubated at 37°C for 9 h, and then infected with an X4-tropic HIV-1 strain NL43. As can be seen in Fig. 2C, both HSC-F-TRIM5α-tag and CV1-TRIM5α-tag completely inhibited HIV-1 replication, whereas MT4 cells infected with SeV empty vector fully supported HIV-1 replication.

Distinct patterns of restriction for SIVmac among nonhuman primate TRIM5α. In African green monkey CV1 cells, both HIV-1 and SIVmac239 were restricted, whereas only HIV-1 was restricted in cynomolgus monkey HSC-F cells (Fig. 3A). Therefore, we examined whether or not CV1-TRIM5α also could inhibit the replication of SIVmac. In MT4 cells infected with recombinant SeV expressing HSC-F-TRIM5α-tag, the replication of SIVmac239 was not suppressed at all (Fig. 3B), indicating that the HSC-F-TRIM5α showed a similar specificity to rhesus monkey TRIM5α (33). In MT4 cells infected with recombinant SeV expressing CV1-TRIM5α-tag, in contrast, the replication of SIVmac239 was completely suppressed. These results suggested that the distinct sensitivity of African green monkey and cynomolgus monkey cells to HIV-1 and SIVmac infection was, at least partly, determined by TRIM5α.

A small region of 37 amino acid residues in the SPRY domain of CV1-TRIM5α determines SIVmac restriction. A comparison of the human and nonhuman primate TRIM5α sequences showed the presence of a highly variable region in the N-terminal portion of the SPRY domain (Fig. 1A and B). In this region, CV1 and Vero TRIM5α had a 20-amino-acid repetition that was totally absent in HSC-F-TRIM5α. These findings prompted us to test whether this highly variable region of TRIM5α determined the species-specific inhibition of SIVmac infection. We constructed chimeric TRIM5αs from HSC-F-TRIM5α-tag and CV1-TRIM5α-tag by using SphI and BamHI restriction enzyme digestion (Fig. 4A). HSC-F+60tag contained the 242-bp fragment of CV1-TRIM5α in the background of HSC-F-TRIM5α-tag. The reciprocal chimera, CV1-60tag, contained a 182-bp fragment of HSC-F-TRIM5α in the

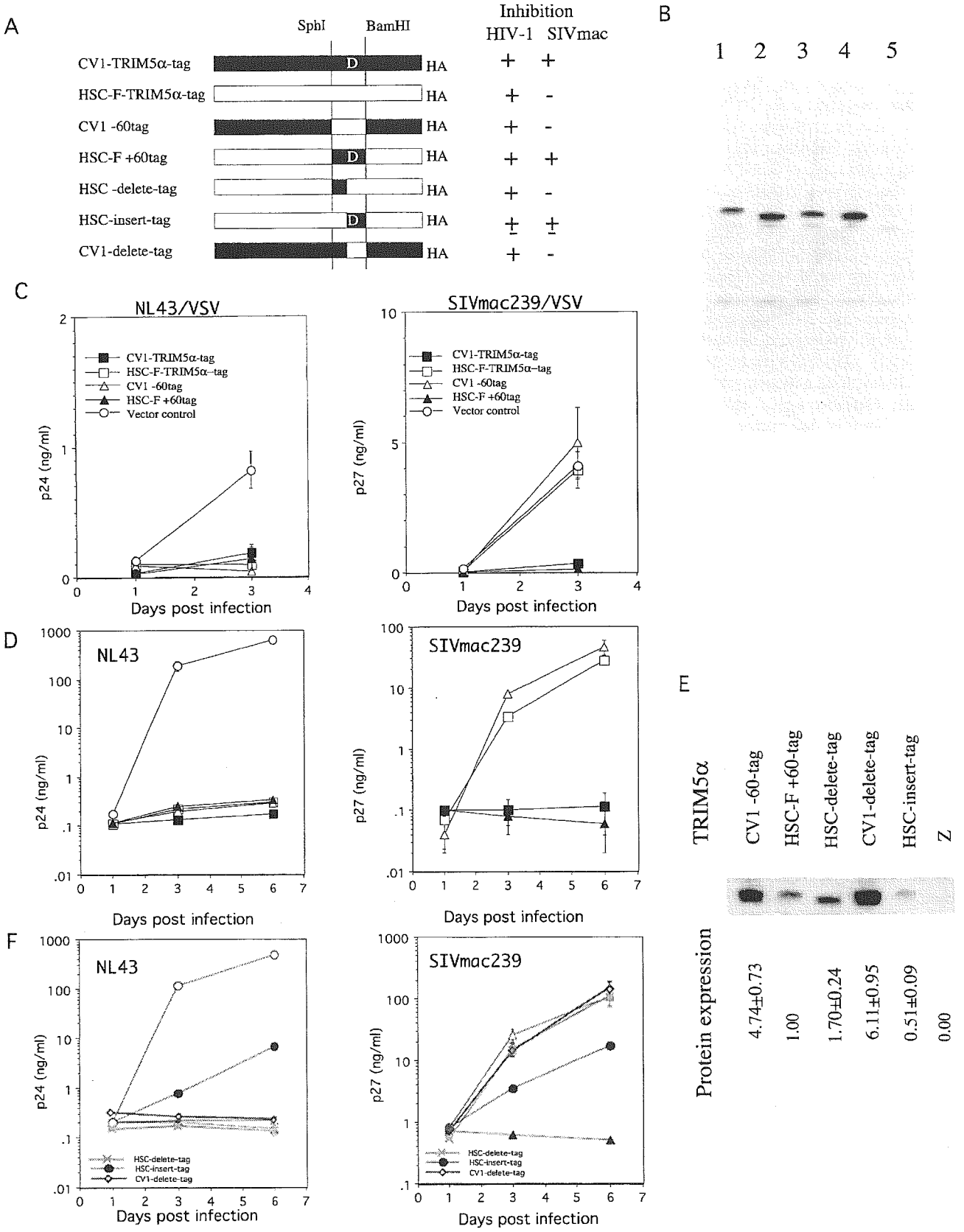

FIG. 4. (A) Schematic representation of chimeric TRIM5α and summary of the results. Filled and open bars denote CV1 and HSC-F sequences, respectively. D, the CV-1-TRIM5α-specific 20-amino-acid duplication. The +, ±, and − symbols denote full, partial, and no

background of the CV1-TRIM5α-tag. In this fragment, the differences between CV1 and HSC-F TRIM5α, including the 20-amino-acid repetition, were located in a small region of 37 amino acid residues (Fig. 1B). Human osteosarcoma C143 cells stably expressing various TRIM5αs (Fig. 4B) were infected with VSV-G-pseudotyped HIV-1 NL43, and levels of P24 in the culture supernatants were assayed periodically. As expected, both chimeric TRIM5αs and parental TRIM5αs clearly inhibited the replication of HIV-1 NL43 (Fig. 4C). When these cells were infected with VSV-G-pseudotyped SIVmac239, the parental CV1-TRIM5α-tag could also inhibit the replication of SIVmac239, whereas CV1-60tag, which contained the 182-bp fragment of HSC-F-TRIM5α in the background of CV1-TRIM5α-tag, could not. Conversely, the parental HSC-F-TRIM5α-tag did not inhibit the replication of SIVmac239 at all, whereas HSC-F+60tag, which contained the 242-bp fragment of CV1-TRIM5α in the background of HSC-F-TRIM5α-tag, clearly inhibited SIVmac239 (Fig. 4C). We obtained the same results as described above when we used SeVs to express parental TRIM5α-tag or chimeras (Fig. 4D). These results indicated that the determinant of the species-specific inhibition of SIVmac239 replication is located in 37 amino acid residues in the SPRY domain of CV1-TRIM5α.

To narrow the SIVmac restriction determinant more precisely, we generated two more chimeric TRIM5αs (Fig. 4A). HSC-delete-tag contained a CV1-derived 17-amino-acid fragment without the 20-amino-acid duplication in the background of the HSC-TRIM5α-tag. HSC-insert-tag contained the HSC-F-derived 17-amino-acid fragment with the CV-1 derived 20-amino-acid duplication in the background of HSC-TRIM5α-tag. Recombinant SeVs expressing these chimeric TRIM5αs were generated and used in the subsequent experiments. Although the expression levels of mRNA of each chimericTRIM5α was virtually identical to each other (data not shown), we observed considerable variations in the levels of TRIM5α protein expression among chimeras (Fig. 4E). The HSC-delete-tag showed slightly higher levels of protein expression than those of HSC-F+60tag. However, HSC-delete-tag failed to inhibit SIVmac replication, whereas it restricted HIV-1 replication as completely as HSC-F+60tag did (Fig. 4F). These results clearly indicated that the CV1-derived 17-amino-acid region alone was not sufficient for SIVmac restriction. On the other hand, HSC-insert-tag partially restricted both HIV-1 and SIVmac, although this chimera showed lower levels of protein expression than other chimeras did (Fig. 4E and F). These results indicated that the CV1-specific 20-amino-acid duplication was important in SIVmac restriction.

To determine whether the CV1-specific 20-amino-acid duplication was indispensable for SIVmac restriction, we generated CV1-delete-tag, which lacked the 20-amino-acid duplication in the CV1-TRIM5α-tag (Fig. 4A). The protein expression level of CV1-delete-tag was comparable to that of CV1-60tag (Fig. 4E), and CV1-delete-tag inhibited HIV-1 replication as completely as CV1-60tag did. However, CV1-delete-tag was shown to lose the ability to inhibit SIVmac infection (Fig. 4F). Taken together, our data clearly indicated that the 20-amino-acid duplication of CV1-TRIM5α was necessary for SIVmac restriction and suggested that the adjacent 17-amino-acid region of CV1-TRIM5α was also necessary to fully restrict SIVmac infection.

HIV-2 GH123 is sensitive to cynomolgus monkey TRIM5α, as well as African green monkey TRIM5α. HIV-2 is closely related to SIVmac (9). We tested whether or not the sensitivity of HIV-2 to various TRIM5αs was similar to that of SIVmac239. C143 cells expressing CV1, HSC-F, and their chimeric TRIM5αs were infected with the VSV-G-pseudotyped HIV-2 strain GH123. Surprisingly, HSC-F-TRIM5α-tag inhibited HIV-2 replication as CV1-TRIM5α-tag had done (Fig. 5). Both chimeric TRIM5αs, CV1-60tag and HSC-F+60tag, also inhibited HIV-2 replication to a similar extent (Fig. 5). These results indicated that HIV-2 strain GH123 was sensitive to cynomolgus monkey TRIM5α despite its high level of sequence homology to SIVmac239.

DISCUSSION

In the present study, we showed that both cynomolgus and African green monkey TRIM5αs could inhibit HIV-1 infection. African green monkey TRIM5α could also inhibit SIVmac infection, whereas cynomolgus monkey TRIM5α could not. Experiments on chimeras of the cynomolgus and African green monkey TRIM5αs unequivocally demonstrated that a small region composed of 37 amino acid residues in the SPRY domain of African green monkey TRIM5α was responsible for restricting the SIVmac infection.

A previous study showed that rhesus monkey TRIM5γ, a splicing variant lacking the SPRY domain, did not suppress HIV-1 infection (33). In the case of TRIM7, the SPRY domain alone was sufficient for binding to its ligand glycogenin (38). Deletion of the entire SPRY domain from TRIM11 also abol-

suppression, respectively. (B) Lysates of C143 cells expressing CV1-TRIM5α-tag (lane 1), HSC-F-TRIM5α-tag (lane 2), HSC-F+60tag (lane 3), CV1-60tag (lane 4), or empty vector (lane 5) were immunoprecipitated by using anti-HA antibody. The resultant immunoprecipitates were visualized by Western blotting with an antibody to HA. A representative result of three independent experiments is shown. (C) C143 cells expressing CV1-TRIM5α-tag (■), HSC-F-TRIM5α-tag (□), CV1-60tag (△), HSC-F+60tag (▲), or empty vector (○) were infected with VSV-pseudotyped NL43 or SIVmac239, and culture supernatants were periodically assayed for levels of p24 or p27. The datum points are means for triplicate samples with the SD. (D) MT4 cells infected with SeV expressing CV1-TRIM5α-tag (■), HSC-F-TRIM5α-tag (□), CV1-60tag (△), HSC-F+60tag (▲), or empty vector (○) were infected with NL43 or SIVmac239, and culture supernatants were assayed for levels of p24 and p27. The datum points are means for triplicate samples with the SD. (E) Lysates of MT4 cells infected with recombinant SeVs expressing CV1-60tag, HSC-F+60tag, HSC-delete-tag, CV1-delete-tag, HSC-insert-tag, or parental Z strain were immunoprecipitated by using anti-HA antibody. Resultant immunoprecipitates were visualized by Western blotting with an antibody to HA. A representative result of three independent experiments is shown. The relative amounts of TRIM5α protein to that of HSC-F+60tag were calculated, and means and SD values of three independent experiments are shown. (F) MT4 cells infected with SeV expressing CV1-60tag (△), HSC-F+60tag (▲), HSC-delete-tag (asterisks with blue lines), HSC-insert-tag (●), CV1-delete-tag (diamonds with red lines), or empty vector (○) were infected with NL43 or SIVmac239, and culture supernatants were assayed for levels of p24 and p27. The datum points are means for triplicate samples with the SD.

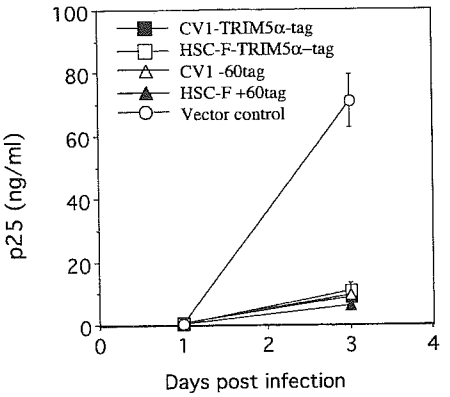

FIG. 5. C143 cells expressing CV1-TRIM5α-tag (■), HSC-F-TRIM5α-tag (□), CV1-60tag (△), HSC-F+60tag (▲), or empty vector (○) were infected with VSV-pseudotyped GH123, and culture supernatants were periodically assayed for levels of p25. The datum points are means for triplicate samples with the SD.

ished its ability to bind Humanin (21). Therefore, it is reasonable to assume that the variable N-terminal region of the SPRY domain of TRIM5α binds to HIV-1 or SIVmac CA protein. This assumption is consistent with the recent findings that in owl monkey cells, HIV-1 infection was restricted by a TRIM5-cyclophilin A fusion protein in which the SPRY domain was replaced with cyclophilin A, since cyclophilin A is a well-known ligand of HIV-1 CA protein (22, 28).

In the attempt to further narrow the SIVmac restriction determinant more precisely, we were able to demonstrate that the African green monkey-specific 20-amino-acid duplication was indispensable for SIVmac restriction and that the adjacent 17-amino-acid region of African green monkey alone was not sufficient. However, HSC-insert-tag carrying the cynomolgus monkey-derived 17-amino acid region with African green monkey-specific 20-amino-acid duplication showed low levels of protein expression and only partial suppression of HIV-1 and SIVmac replication. It is possible that an artificial combination of African green monkey-specific 20-amino-acid duplication with the cynomolgus monkey-derived 17-amino-acid region made TRIM5α molecules unstable. Further studies, including mutational analysis of the African green monkey-specific 17-amino-acid region, are necessary to determine the precise role of this region in SIVmac restriction.

Despite its close similarity to SIVmac, HIV-2 strain GH123 was restricted by cynomolgus monkey TRIM5α, as well as by African green monkey TRIM5α. Although both HIV-2 and SIVmac were considered to come from SIVsm (9), it is possible that HIV-2 has been replicating in the human population in the absence of TRIM5α restriction for a certain period and has lost its ability to escape from cynomolgus monkey TRIM5α. However, it has also been reported that there was a considerable degree of variation in the ability to grow in monkey cells among HIV-2 strains (6, 8, 26). Therefore, it is necessary to examine various HIV-2 strains for their sensitivity to human and monkey TRIM5αs before we can draw a definite conclusion. It would also be interesting to identify specific amino acid changes determining the sensitivity to cynomolgus monkey TRIM5α in viral CA proteins, since nearly 90% of the amino acid residues in SIVmac239 CA protein are conserved in HIV-2GH123.

In CV1 cells, the level of TRIM5 gene expression was ca. 3 $\times 10^6$ copies/μg of total RNA, a level similar to that observed in other human cell lines examined (data not shown). Although HIV-1 infection was suppressed in hamster TK-ts13 or human C143 cells expressing CV1-TRIM5α, relatively high levels of TRIM5α (nearly 5×10^7 copies/μg of total RNA) appeared to be required for a level of suppression similar to that observed in CV1 cells. One possible explanation for this discrepancy is that certain molecules cooperating with TRIM5α also showed species specificity, and CV1-TRIM5α was not fully supported in hamster and human cells. Because TRIM5 gene products are suspected to be an E3 ubiquitin ligase (35), it is important to identify the E2 ubiquitin-conjugating enzyme interacting with TRIM5α. Alternatively, restriction factors other than TRIM5α may exist in CV1 cells, or certain molecules required for efficient lentivirus infection may be absent in CV1 cells.

After we submitted these findings for publication, small amino acid differences in the SPRY domain between human and rhesus monkey TRIM5αs were reported to determine HIV-1 restriction (27, 34, 37). Our findings are in good agreement with the results of these studies.

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Influence of Glycosylation on the Efficacy of an Env-Based Vaccine against Simian Immunodeficiency Virus SIVmac239 in a Macaque AIDS Model

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The envelope glycoprotein (Env) of human immunodeficiency viruses (HIVs) and simian immunodeficiency viruses (SIVs) is heavily glycosylated, and this feature has been speculated to be a reason for the insufficient immune control of these viruses by their hosts. In a macaque AIDS model, we demonstrated that quintuple deglycosylation in Env altered a pathogenic virus, SIVmac239, into a novel attenuated mutant virus (Δ5G). In Δ5G-infected animals, strong protective immunity against SIVmac239 was elicited. These HIV and SIV studies suggested that an understanding of the role of glycosylation is critical in defining not only the virological properties but also the immunogenicity of Env, suggesting that glycosylation in Env could be modified for the development of effective vaccines. To examine the effect of deglycosylation, we constructed prime-boost vaccines consisting of Env from SIVmac239 and Δ5G and compared their immunogenicities and vaccine efficacies by challenge infection with SIVmac239. Vaccination-induced immune responses differed between the two vaccine groups. Both Env-specific cellular and humoral responses were higher in wild-type (wt)-Env-immunized animals than in Δ5G Env-immunized animals. Following the challenge, viral loads in SIVmac239 Env (wt-Env)-immunized animals were significantly lower than in vector controls, with controlled viral replication in the chronic phase. Unexpectedly, viral loads in Δ5G Env-immunized animals were indistinguishable from those in vector controls. This study demonstrated that the prime-boost Env vaccine was effective against homologous SIVmac239 challenge. Changes in glycosylation affected both cell-mediated and humoral immune responses and vaccine efficacy.

Primate lentiviruses, human immunodeficiency viruses (HIVs), and simian immunodeficiency viruses (SIVs) share common genetic and biological properties. As SIVmac, originally isolated from macaques in primate research centers in the United States, causes AIDS in macaques with remarkable similarities to HIV type 1 (HIV-1) infection in humans, this AIDS monkey model has been utilized to study vaccine development and the pathogenesis of HIV infection (for reviews, see references 10, 14, 17, 43, and 47).

HIV/SIV infection in the host consists of two phases, the primary infection and chronic infection. During the primary infection, extensive viral replication and dissemination of the infection occur. In chronic infection, viral replication continues for a long period, eventually leading to AIDS. Due to the host immune response against the infection, these two phases are separated by a set point at which the viral load reaches its lowest level. The viral loads of the set point and chronic infection are inversely correlated with the control of SIV/HIV infection and predict disease progression (25, 31); however, it remains unclear which host responses determine the viral loads of the set point and chronic infection. Nevertheless, virus-specific immune responses have been implicated in the host's control of the infection. Cellular immunity, such as that shown by cytotoxic T lymphocytes (CTL) and helper T cells, has been reported to correlate with the control of HIV/SIV infection (for reviews, see references 2, 24, 28, and 39). The role of the neutralizing antibody (NAb) in the control of infection and the

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emergence of escape mutants has also been reported previously (7, 16, 51).

Despite these immune responses against HIV/SIV infection, humans and macaques fail to contain the infection due to the virus properties. HIV/SIV infects major target cells, such as $CD4^+$ T cells and macrophages, by binding viral envelope glycoproteins (Env) to cellular surface proteins and CD4 and chemokine receptors (CCR5, CXCR4, or others) on target cells (5, 32). Since viral entry consists of multiple steps (virion binding to these viral receptors, conformational change of Env, and fusion between the virion and the cellular membrane) and the critical parts of Env used in these steps are exposed only during each step, naturally generated antibodies are only partly effective in preventing HIV/SIV infection in their hosts (7, 8). Primary isolates can be neutralized to various degrees by HIV-infected patient serum but not by contemporaneous autologous samples. Consequently, escape mutants against preexisting NAb are selectively replicated (51). Thus, effective NAb is rarely induced in HIV/SIV infection (8, 10). This could partly explain the failure of Env-based vaccine trials against HIV-1 (8, 50).

The heavy glycosylation of Env is a unique feature of HIV/SIV that is distinctive from features of other enveloped viruses and is significantly related to their neutralization-resistant property (8, 29, 44). We therefore assumed that the insufficient immune containment of HIV/SIV might be due to heavy glycosylation in Env and that the removal of some glycans might allow the host to mount a protective immune response against the infection. Thus, we studied the influence of deglycosylation on the replication of SIVmac239 in a T-cell line and created a quintuple deglycosylation mutant of SIVmac239 (Δ5G), which has maximal removal of N-glycans at amino acid residues 79, 146, 171, 460, and 479 in Env and retains a replication capability similar to that of SIVmac239 in phytohemagglutinin-stimulated rhesus peripheral blood mononuclear cells (PBMCs) (36, 40). We then examined the infection of rhesus macaques with Δ5G; although Δ5G was replicated as extensively as SIVmac239 during the primary infection, the subsequent Δ5G infection was restricted to a level less than the detection sensitivity of a plasma viral load assay by 8 weeks postinfection (p.i.), in contrast to high chronic viral replication in SIVmac239 infection. Furthermore, an almost sterilizing immunity against SIVmac239 was induced in Δ5G-infected animals (36). Interestingly, another quintuple-deglycosylation-mutation strain with mutations at amino acid residues 146, 156, 184, 244, and 247 in Env was created (44) and was demonstrated to share common features with Δ5G in viral replication in animals and in functions as an attenuated vaccine (20). Since these two viruses share only one deglycosylation mutation and other mutations distributed differently in surface envelope protein gp120 (SU), these two studies suggest that heavily glycosylated Env determines the pathogenicity of HIV/SIV.

To dissect the mechanism for notable containment of Δ5G infection after primary infection, we hypothesized that the Env of Δ5G, a viral protein that differs from that in SIVmac239, might elicit protective immunity against SIVmac239, because deglycosylation in Env might alter antigenic properties such as B-cell and T-cell epitopes and enhance the protective immunity against SIVmac239. For this purpose, we immunized animals with Env of Δ5G (Δ5G Env) or Env of SIVmac239 (the wild type; wt Env), and examined the effect of these vaccinations against SIVmac239 infection.

MATERIALS AND METHODS

Generation of SU DNA vaccines. DNA vaccine plasmids expressing SIV mac239 SU or Δ5G SU, pJWSUmac239 and pJWSUmacΔ5G, were constructed using the expression vector pJW4303 (45). To produce secreted SU efficiently, the native signal sequence in the SIVmac239 SU gene was replaced with the human tissue plasminogen activator signal in plasmid pJW4303, and a termination codon was created at the cleavage site for SU transmembrane (TM) protein (9). An SIVmac239 SU or Δ5G SU DNA sequence was amplified with a pair of primers, SUmacA (5′-TGT**GCTAGC**TATGTCACAGTCTTTTATGGTGTAC-3′) and SUmacB (5′-CC**AGGATCC**TATTACCTCTTCACATCTGTGGGGG C-3′). The SUmacA primer consisted of nucleotides (nt) 6923 to 6955 of the SIVmac239 sequence (GenBank accession number M33262) and the boldface nucleotides, which were changed to create a NheI site; primer SUmacB consisted of nt 8412 to 8381 and the boldface nucleotides, which were changed to create a BamHI site, and the underlined nucleotides, which generated tandem termination codons. The PCR-amplified fragments were digested with NheI and BamHI and cloned into the NheI- and BamHI-digested eukaryotic expression vector pJW4303 to yield pJWSUmac239 and pJWSUmacΔ5G. These plasmids were prepared using a Plasmid Mega kit (QIAGEN, Tokyo, Japan).

Generation of Env vaccinia vaccines. Recombinant vaccinia viruses expressing Env of SIVmac239 or Δ5G, WRvvmac239 or WRvvΔ5G, respectively, were constructed using a vaccinia virus WR strain (WRvv) as described previously (15). To excise the entire coding region of the *env* gene from the cloned SIV plasmid, BamHI and SmaI sites were introduced by in vitro mutagenesis at 5′- and 3′-end-flanking sites of the *env* gene, respectively. Primer B-6808 (5′-GAA AGAGAAGAAGGATCCCGAAAAAGG-3′) consisted of nt 6796 to 9822 and the underlined mutations of the BamHI site; S-9537 (5′-TATGAATACTCCC GGGAGAAACCC-3′) consisted of nt 9527 to 9550 and the underlined mutations of the SmaI site. DNA fragments containing the *env* gene of SIVmac239 or Δ5G were isolated by digesting the mutated plasmids with BamHI and SmaI and were cloned into the SmaI- and BamHI-digested vaccinia virus vector plasmid pNZ68K2. To transfer the *env* gene from a recombinant plasmid to WRvv, the standard homologous recombination method using CV-1 cells was performed. Env expression in the recombinant vaccinia virus was confirmed by immunoprecipitation. The function of Env was confirmed by CD4- and CCR5-dependent fusion activity. The recombinant Env-expressing vaccinia viruses obtained were propagated and titrated in CV-1cells. The two recombinant viruses were propagated with similar kinetics in CV-1 cells.

Expression of SU-expressing plasmids and Env-expressing vaccinia virus in vitro. CV-1 cells were transfected with equal amounts of the following SU-expressing plasmids: pJWSUmac239, pJWSUmacΔ5G, or the vector pJW4303. Secreted SU metabolically labeled with ^{35}S protein labeling mix (PerkinElmer, Boston, MA) in culture supernatant was concentrated, immunoprecipitated with plasma from SIVmac239-infected monkeys, and then analyzed by sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) as described previously (40). To examine Env-expressing vaccinia viruses, CV-1 cells were infected with WRvvmac239, WRvvΔ5G, or WRvv at a multiplicity of infection of 10, metabolically labeled with ^{35}S protein labeling mix overnight, lysed, immunoprecipitated with plasma from SIVmac239-infected monkeys, and then analyzed by SDS-PAGE as described for the expression of SU-expressing plasmids.

Animals, immunization, and challenge. Twelve juvenile rhesus macaques from Myanmar or Laos that were seronegative for SIV, simian T-cell lymphotropic virus, B virus, and type D retroviruses were used. As the polymorphism of major histocompatibility complex (MHC) genes influenced cellular immune responses against SIV/HIV infection, MHC II haplotypes and alleles of the macaques were determined (data not shown). All animals were housed in individual cages and maintained according to the rules and guidelines for experimental animal welfare stated by the National Institute of Infectious Diseases. As shown in Fig. 1, the 12 animals were divided into three immunization groups of four animals each: the SIVmac239 (wt)-Env immunization group (Mm0005, Mm0007, Mm0010, Mm0012), the Δ5G Env immunization group (Mm0001, Mm0002, Mm0003, Mm0009), and the vector control immunization group (Mm0004, Mm0006, Mm0008, Mm0011). All animals were inoculated with 1 mg of plasmid DNA in 1 ml of saline, one into each quadriceps femoris at 0, 4, and 8 weeks after the initial prime immunization (weeks p.p.). The boost consisted of 5×10^7 PFU of vaccinia virus in 1 ml of phosphate-buffered saline (PBS), administered in two 0.1-ml intradermal inoculations, one into the skin of each femur, and two 0.4-ml inoculations, one into each quadriceps femoris at 21 weeks p.p. All animals were

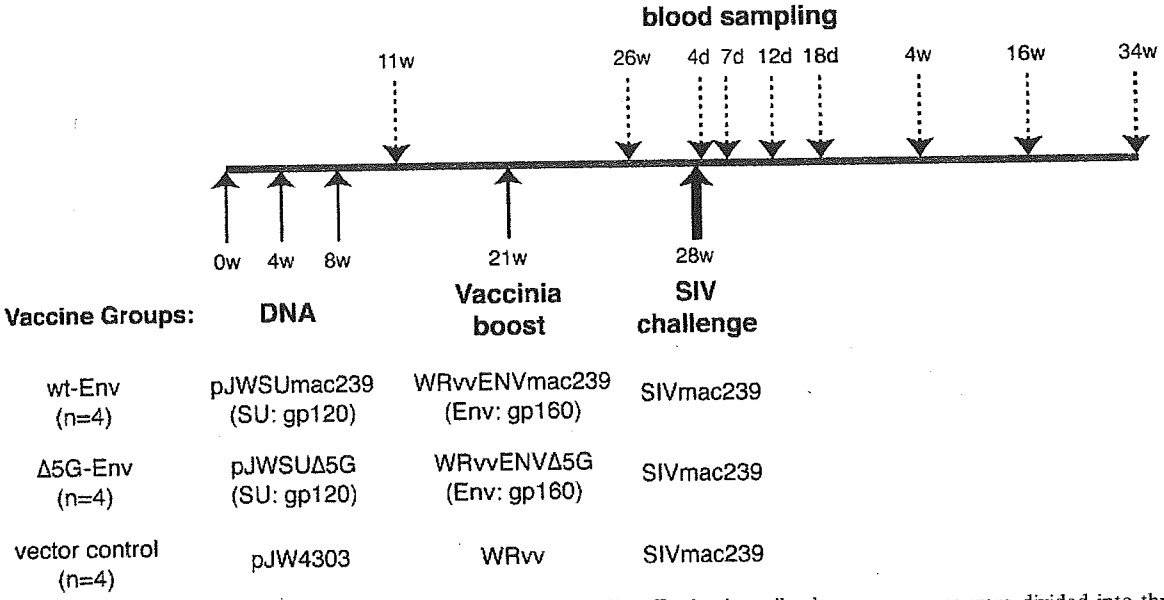

FIG. 1. Outline of immunization, challenge infection, and blood sampling. Twelve juvenile rhesus macaques were divided into three immunization groups of four animals each: the wt-Env immunization group (Mm0005, Mm0007, Mm0010, and Mm0012), the Δ5G Env immunization group (Mm0001, Mm0002, Mm0003, and Mm0009), and the vector control immunization group (Mm0004, Mm0006, Mm0008, and Mm0011). Animals were inoculated with a DNA vaccine (pJWSUmac239 for the wt-Env vaccine group, pJWSUΔ5G for the Δ5G Env vaccine group, and pJW4303 for the vector control group) at 0, 4, and 8 weeks p.p. The boost vaccine consisted of vaccinia virus (WRvvENVmac239 for the wt-Env vaccine group, WRvvENVΔ5G for the Δ5G Env vaccine group, and the WR strain for the vector control group) administered at 21 weeks p.p. All animals were challenged with 10 $TCID_{50}$ of SIVmac239 intravenously at 28 weeks p.p. w, weeks; d, day.

challenged with 10 50% tissue culture infective doses ($TCID_{50}$) of SIVmac239 intravenously at 28 weeks p.p.

Viral load measurement. To monitor SIV infection, the plasma viral load was measured by the real-time-PCR method described previously (36). Viral RNA was isolated from plasma from the infected animals using a commercial viral-RNA isolation kit (PE Applied Biosystems, Urayasu, Japan). SIV *gag* RNA was amplified and quantified using a commercial RNA reverse transcription (RT)-PCR kit (TaqMan EZ RT-PCR; PE Applied Biosystems) with the two *gag* primers, namely, the forward primer 1224F (5′-AATGCAGAGCCCCAAGAA GAC-3′), the reverse primer 1326R (5′-GGACCAAGGCCTAAAAAACCC-3′), and TaqMan probe 1272T (6-carboxyfluorescein-5′-ACCATGTTATGGCC AAATGCCCAGAC-3′-6-carboxymethylrhodamine). Purified viral RNA (10 μl) was reverse transcribed and amplified in a MicroAmp optical 96-well reaction plate (PE Applied Biosystems) according to the manufacturer's instructions and with the following thermal cycle conditions: 1 cycle of three sequential incubations (50°C for 2 min, 60°C for 30 min, and 95°C for 5 min) and then 50 cycles of amplification (95°C for 5 s, 62°C for 30 s) in a 7000 Prism sequence detection system (PE Applied Biosystems). In vitro RNA transcripts were quantified by optical density at 260 nm (OD_{260}) measurement and branched DNA assay for SIV viral RNA (Bayer Diagnostics, Tarrytown, N.Y.). RNA equivalent to 10 to 10^7 copies per reaction was used as the standard for each assay. The detection sensitivity of plasma viral RNA using this method was 1,000 copies/ml.

Flow cytometry. CD4 depletion was monitored by measuring the percentage of $CD4^+$ T cells, memory cells (CD29 high $CD4^+$) T cells (48) in PBMCs. PBMC samples were purified from a citrate anticoagulant containing blood using standard Ficoll-Hypaque gradient centrifugation. For flow cytometry, 2×10^5 PBMCs were reacted with fluorescein isothiocyanate or phycoerythrin-labeled antibodies (anti-human CD4, Nu-Th/I [Nichirei, Tokyo, Japan]; anti-human CD8, Leu2a [Becton Dickinson, San Jose, CA]; anti-human CD29, 4B4 [Coulter, Miami, FL]; anti-monkey CD3, FN-18 [Biosource, Camarillo, CA]; and anti-human CD20, Leu16 [Becton Dickinson, San Jose, CA]) as previously described (36, 37, 48).

Peptides. Overlapping peptides were synthesized by Emory University, Microchemical Facility, Winship Cancer Center (Atlanta, GA.). All SIVmac239 viral proteins except Env, Gag, Pol, Vif, Vpr, Vpx, Tat, Rev, and Nef were covered by consecutive 20-mer peptides overlapped by 12 amino acids. Env of SIVmac239 was covered by 72 consecutive 25-mer peptides overlapped by 13 amino acids. Peptides were dissolved in PBS with 10% dimethyl sulfoxide (Sigma Chemical, St. Louis, Mo.).

rSeV. Recombinant Sendai viruses (rSeV) expressing SIVmac239 Gag, SU, or Δ5G SU were used to infect herpesvirus papio-transformed B-lymphoblastoid cell lines (B-LCLs) to prepare autologous B-LCLs presenting these viral antigens. rSeV Gag expressing unprocessed SIVmac239 Gag and p55 (22, 23) and rSeV SU and rSeV/Δ5G SU expressing wt SU and Δ5G SU were constructed as described previously (52) and were also used to infect autologous B-LCLs.

Anti-SIV ELISA. A 1:100 dilution of each plasma sample in PBS (pH 7.4) containing a blocking reagent (Dainippon Seiyaku, Osaka, Japan) was assayed for SIV-specific antibody by using a standard enzyme-linked immunosorbent assay (ELISA) technique with 96-well plates precoated with SIVmac239 virion lysate. The OD_{492} was measured using a microplate reader (range of absorbance with linearity, 0 to 3.0; Tecan Japan, Tokyo, Japan) and utilized as a relative measurement of the antibody titer.

ELISPOT assay. Virus-specific $CD4^+$ T cells and $CD8^+$ T cells in PBMCs were measured using a monkey γ-IFN ELISPOT assay kit (U-CyTech, Utrecht, The Netherlands).

Cryopreserved PBMCs were thawed and cultured overnight in R-10 medium (RPMI 1640 [Sigma] supplemented with 10% heat-inactivated, defined fetal bovine serum [HyClone, Logan, Utah], 55 μM 2-mercaptoethanol, 50 U/ml penicillin, and 50 μg/ml streptomycin). PBMCs were subjected to the depletion of $CD4^+$ cells with magnet beads coated with anti-human CD4 Ab (Dynal ASA, Oslo, Norway) or subjected to the depletion of $CD8^+$ cells with magnet beads coated with anti-human CD8 Ab (Miltenyi Biotec, Bergisch Gladbach, Germany). Depletion of $CD4^+$ or $CD8^+$ cells from PBMCs was confirmed by flow cytometry. Using this depletion method, more than 95% of $CD4^+$ or CD8 cells were removed from PBMCs. These PBMCs were used for ELISPOT assay for virus-specific $CD8^+$ T cells and virus-specific $CD4^+$ T cells. Virus-specific stimulation of T cells was performed with autologous B-LCLs pulsed with pooled peptides for Pol, Vif, Vpx, Vpr, Tat, Rev, and Nef or B-LCLs infected with an rSeV for Gag, wt Env, and Δ5G Env. B-LCLs were incubated with pooled peptides corresponding to each viral protein at a final concentration of 2 μg/ml or infected with rSeV at a multiplicity of infection of 10 at 37°C overnight. Peptide-pulsed or infected B-LCLs were inactivated with long-wave UV irradiation (19) in the presence of 10 μg/ml psoralen (Sigma) for 10 min at a distance of 3.5 cm from a UV light, washed three times with R-10, and then used as stimulators in an ELISPOT assay. $CD4^+$ or $CD8^+$ cell-depleted PBMCs were cultured with these stimulators in an anti-γ-IFN Ab-coated ELISPOT plate (U-CyTech) overnight according to the protocol for the kit. Spots on the ELISPOT plate were imaged using an Olympus model SZX12 microscope

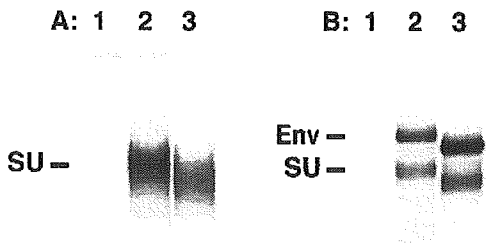

FIG. 2. Expression of SU and Env by SU-expressing DNA vaccines and Env-expressing vaccinia viruses. A: SU secreted in supernatant from CV-1 cells transfected with SU-expressing plasmids. Lane 1, pJW4303 vector; lane 2, pJWSUmac239; lane 3, pJWSUmacΔ5G. B: Env in cell lysates of CV-1 cells infected with recombinant vaccinia viruses. Lane 1, WRvv; lane 2, WRvvmac239; lane 3, WRvvΔ5G.

(Olympus, Tokyo, Japan) equipped with a digital camera, PDMCIe/OL (Polaroid, Cambridge, MA), and analyzed using a personal computer with MAC SCOPE version 2.61 (Mitani Corporation, Toyama, Japan). The results were calculated as numbers of spot-forming cells (SFC) per million PBMCs after subtraction of the background.

Neutralization assay. The original protocol of this neutralization assay was reported by Means et al. (29). Plasma that was heat inactivated at 56°C for 30 min was serially diluted and incubated with a fixed concentration of SIVmac239, Δ5G, or a macrophage-tropic SIV, 239/envMERT, at room temperature for 1 h. CEMx174/SIVLTR-SEAP cells were added to the mixture and then incubated at 37°C for 3 days. Secreted alkaline phosphatase activity in the culture supernatant was measured using a Phospha-Light System (Applied Biosystems). Chemiluminescence was detected with a Wallac Microbeta plate reader.

Statistical analysis. Statistical analysis was based on the Mann-Whitney test and performed using GraphPad Prism 4.0 software.

RESULTS

Experimental design. We adopted a DNA prime-vaccinia virus boost regimen to immunize rhesus macaques with wt Env or Δ5G Env as shown in Fig. 1. Twelve macaques were immunized at 0, 4, and 8 weeks after the initial prime immunization (weeks p.p.) with one of three different DNA expression plasmids ($n = 4$): pJWSUmac239 expressing SU of SIVmac239, pJWSUΔ5G expressing SU of Δ5G, or the vector pJW4303. At 21 weeks p.p., all animals were boosted with recombinant WR vaccinia viruses expressing the respective Env proteins: vaccinia virus expressing Env of SIVmac239, vaccinia virus expressing Env of Δ5G, or vaccinia virus (Fig. 1).

Expression of SU DNA plasmids and Env vaccinia viruses in vitro and in animals. Although Δ5G replicated similarly to wild-type SIVmac239 in animals (36), quintuple deglycosylation might affect the expression of SU in a plasmid vector and the expression of Env in the vaccinia virus vector. Thus, we examined the expression of these vaccines in CV-1 cells. SU expressions in the wild-type plasmid (pJWSUmac239) and in the deglycosylated SU plasmid (pJWSUmacΔ5G) were at similar levels (Fig. 2A). The expression and processing of Env in the wild type (WRvvENVmac239) and in the deglycosylated Env mutant vaccinia virus (WRvvENVΔ5G) were also at similar levels (Fig. 2B). The reduced molecular size of the proteins due to deglycosylation was confirmed by PAGE (Fig. 2). As the amount of secreted SU in the supernatant by DNA transfection was comparable to that of Env in the cell lysate from CV-1 cells infected with WRvvEnv, a high expression of SU was achieved in a *rev*-independent manner by the pJW403 expression plasmid as described previously (9).

The expression of Env vaccines in the immunized animals was indirectly estimated by Env-specific antibody responses measured by a peptide ELISA using overlapping Env peptides. Env peptide-specific Ab was detected from 11 weeks p.p. after immunization with DNA vaccines, whereas there was no significant difference in the titers and the specificity of the responses between the two vaccine groups (data not shown), suggesting similar amounts of Env expressed in animals immunized with either Env vaccine. To examine the protective effect of the Env vaccines, all animals were challenged with 10 $TCID_{50}$ of SIVmac239 intravenously at 28 weeks p.p.

Cellular immune responses elicited by Env vaccines. The DNA prime-vaccinia virus boost regimen has been used in many studies, has successfully induced a high frequency of virus-specific $CD8^+$ T cells in macaques, and has conferred protective immunity against chimeric simian/human immunodeficiency virus (SHIV) (3, 27, 45). We therefore examined the vaccine-induced Env-specific T-cell responses by IFN-γ ELISPOT assay. Since deglycosylation in Env might change T-cell epitopes in SIVmac239, we measured the wt-SU and Δ5G SU-specific T-cell response by using autologous B-LCLs infected with recombinant Sendai viruses expressing either wt SU and/or Δ5G SU, respectively.

Although there was a tendency for more ELISPOT-positive cells to be observed by homologous SU than heterologous SU, comparable results were obtained by both assays (Fig. 3A and B). As vaccinated animals were challenged with SIVmac239, the results from the wt-SU assay were subsequently used to assess the SU-specific immune response. Immunization with the DNA vaccine induced only marginal SU-specific $CD8^+$ T cells or $CD4^+$ T cells at 11 weeks p.p.; however, boost immunization with recombinant WR vaccinia virus significantly increased SU-specific $CD8^+$ T cells and $CD4^+$ T cells in PBMCs at 26 weeks p.p. (Fig. 3A, B, and C). Notably, SIVmac239 Env (wt Env) induced twofold more SU-specific CD8 T cells (mean, 770 SFC per million PBMCs; range, 540 to 880) responding to wt SU than Δ5G Env (mean, 320; range, 110 to 400) ($P = 0.029$) (Fig. 3A and C). Similarly, twofold more SU-specific $CD4^+$ T cells were observed in wt-Env vaccinees (mean, 1,260; range, 840 to 1,710) than in Δ5G Env vaccinees (mean, 680; range, 150 to 1,260) at 26 weeks p.p. ($P = 0.11$) (Fig. 3B and C). Thus, a twofold-greater number of both SU-specific $CD4^+$ T cells and $CD8^+$ T cells were induced in SIVmac239 Env vaccinees than in Δ5G Env vaccinees at 26 weeks p.p. In vector controls, only negligible SU-specific $CD4^+$ T cells and $CD8^+$ T cells were detected in PBMCs at 26 weeks p.p. (Fig. 3A and B).

Humoral immune response elicited with Env vaccines. The anti-Env Ab titer was examined by SIVmac239 virion lysate ELISA. Anti-SIV Ab was detected in both wt-Env vaccinees and Δ5G Env vaccinees after an rVV boost (Fig. 4) (26 weeks p.p.). Anti-SIV Ab titers were comparable between the two vaccine groups.

Next, we examined the NAb against either SIVmac239, Δ5G, or a macrophage-tropic mutant, 239env/MERT (33, 35), in the two vaccine groups. Macrophage-tropic SIVs were highly susceptible to neutralization by plasma from most SIV-infected macaques (29), whereas SIVmac239 was highly resistant to neutralization as were most clinical isolates of HIV-1

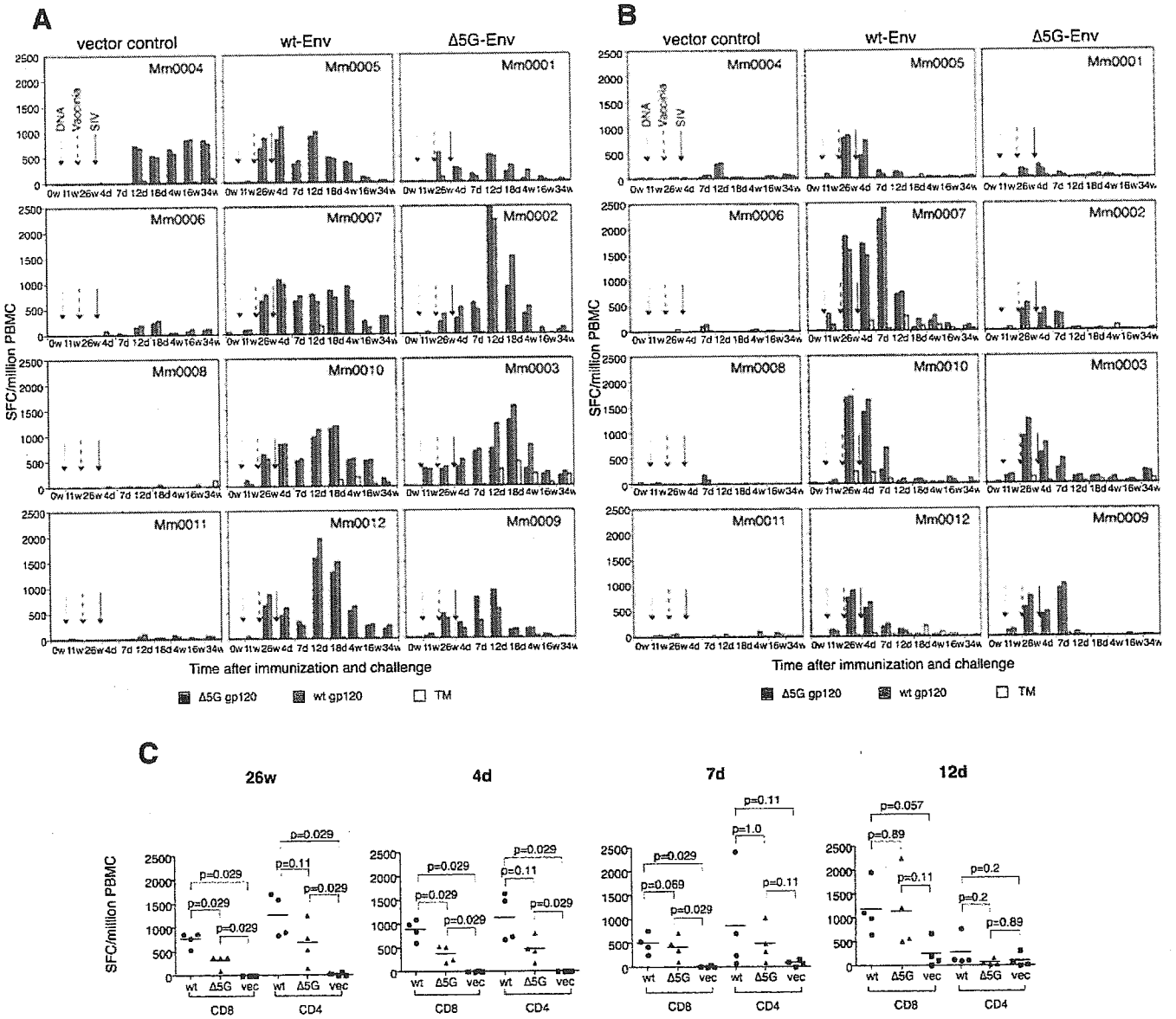

FIG. 3. Env-specific $CD4^+$ T-cell and $CD8^+$ T-cell responses in 12 macaques. A: Env-specific $CD8^+$ T cells in PBMCs were measured by ELISPOT assay for IFN-γ in three groups. B: Env-specific $CD4^+$ T cells in PBMCs were measured by ELISPOT assay for IFN-γ in three groups. ELISPOT results are colored as follows: Δ5G SU-specific T cells (red), wt-SU-specific T cells (green), and TM-specific T cells (yellow). Arrows with a dotted line, arrows with broken line, and arrows with a solid line indicate the time of the third DNA vaccination at 8 weeks p.p., the time of the vaccine boost at 21 weeks p.p., and the time of SIVmac239 challenge at 28 weeks p.p., respectively. C: Comparison of SU-specific $CD8^+$ T cells and $CD4^+$ T cells in PBMCs among the wt-Env vaccine group, the Δ5G Env vaccine group, and the vector control group at 26 weeks p.p. and 4, 7, and 12 days p.i. The numbers of SFC responding to SIVmac239 SU were used to compare the effects of the two vaccines. w, weeks; d, days.

(21, 29, 30). Plasma at 26 weeks p.p. from all immunized animals failed to neutralize not only SIVmac239 but also a multiple-deglycosylation-mutation strain, Δ5G (Table 1); in contrast, these plasma specimens did neutralize 239env/MERT. Furthermore, a marked difference was observed between the two vaccine groups. The NAb titer in the wt-Env vaccine group was eightfold higher than in the Δ5G Env vaccine group (Table 1). The difference of this immune response between the two vaccine groups was significant ($P = 0.029$).

SIV replication in Env-immunized animals. As described above, wt-Env vaccine and Δ5G Env vaccine induced different magnitudes of virus-specific cellular and humoral immunity in macaques. To examine the effect of the two vaccines, we challenged the vaccinated animals with SIVmac239. Viral loads in vector controls were mostly consistent with our previous results with SIVmac239-infected rhesus macaques (36, 48). The mean peak viral load at 2 weeks p.i. was 1.4×10^7 copies/ml, with a range of 0.5×10^7 to 2.2×10^7 copies/ml. Viral loads in chronic infection diverged into two patterns (Fig. 5A). Subsequent to the set point at 20 weeks p.i., the viral loads in three animals increased more than 10^4 copies/ml. In contrast, viral loads in one animal (Mm0011) remained as low as 1,000 copies/ml up to 45 weeks p.i.

Compared with the vector controls, viral loads in wt-Env