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Effects of human interleukin 7 on HIV-1 replication in monocyte-derived human macrophages

Running head: human interleukin 7 and HIV-1 replication in monocyte derived human macrophages.

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Summary

Interleukin 7 (IL-7) contributes to development and proliferation of T cells. We investigated the effect of IL-7 on HIV-1 infected monocyte-derived human macrophages. IL-7 treatment for macrophages at a concentration of 10ng/ml reduced replication of the R5 HIV-1 strain by approximately 50%. Meanwhile, HIV-1-infected macrophages themselves could excrete IL-7 approximately 20% more than uninfected macrophages. These results suggested the advantage of IL-7 as a therapeutic modality to recover CD4+ T cells.

Text

Macrophages and CD4+ T cells are the predominant cell types for human immunodeficiency virus-1 (HIV-1) infection [1, 14]. Macrophages can be infected by HIV-1 in many tissues, including the brain, lung and lymph nodes [16]. Unlike infected CD4+ T cells, which have a short half-life of 1-1.5 days, macrophages are quite resistant to the cytopathic effect of the virus, and may thus provide a reservoir for persistent infection and virus dissemination. It is known that cytokines play an important role in HIV-1 infection. Interleukin 7 is a cytokine produced by stromal cells of the thymus and bone marrow [2-4]. It has capacity to induce growth of immature B lymphocytes [5], and contributes to development and proliferation of T cells [6-9]. With respect to HIV-1 infection, there is a reverse correlation between CD4+ T cell number and IL-7 serum levels in HIV-1-infected patients [17-19]. After patients started to receive antiretroviral drugs, the elevated IL-7 in the serum decreased to normal levels [17]. On the other hand, IL-7 increases HIV-1 replication in thymic organ cultures [10-12] and to induce latent HIV-1 in resting CD4+

T cells [13]. However, effects of IL-7 on HIV-1 replication in macrophages remain unclear. In the present study, we evaluated levels of HIV-1 proliferation in monocyte-derived human macrophages treated with or without exogenous IL-7.

Peripheral blood mononuclear cells (PBMC) from blood buffy coats of healthy donors were isolated by centrifugation through Ficoll-Hypaque and plated on a 24 well MULTIWELL™ PRIMARIA™ plate (Becton Dickinson, Franklin Lakes, New Jersey, USA) with RPMI 1640 supplemented with 10% fetal calf serum (FCS). After incubation at 37℃ for one day, the floating cells were removed by washing the plate with phosphate-buffered saline four times and the adherent cells were incubated at 37℃ for 11 days with 0.5ml of RPMI 1640 supplemented with 10% FCS plus 100ng/ml of granulocyte macrophage colony stimulating factor (GM-CSF).

To determine whether or not differentiated macrophages express the IL-7 receptor, we analyzed total RNA extracted from macrophages. PBMC stimulated with PHA and 10ng/ml of IL-2 for three days and monkey kidney CV-1 cells served as positive and negative controls, respectively. RNA was reverse-transcribed into cDNA, and levels of IL-7 receptor cDNA were measured by an ABI 7500 Real-time PCR System. As shown in Fig. 1a, IL-7 receptor mRNA in macrophages was clearly detected. It was also detected in PHA and IL-2-stimulated PBMC. Levels of IL-7 receptor mRNA in macrophages were approximately 1/10 of those in PHA and IL-2-stimulated PBMC, while CV-1 cells were totally negative for expression of the IL-7 receptor.

Macrophages were infected with 6.5ng of p24 of HIV-1 SF162 strain for two hours on day 12. Cells were then incubated with 1ml of RPMI 1640 supplemented with 10% FCS plus 100ng/ml of GM-CSF

and 0, 3 or 10ng/ml of IL-7 (Techne, Minneapolis, Minnesota, USA). Infected macrophages were fed on days 3, 6, 9 and 12 (after infection) by exchanging one half of the culture supernatant with fresh media containing the same amount of cytokines. Levels of HIV-1 p24 antigen in culture supernatants on days 1, 3, 6, 9 and 12 were measured by using an HIV-1 P24 Antigen ELISA Kit (Fig. 1b). The p24 antigen level in the culture supernatant of macrophages treated with 3ng/ml of IL-7 was apparently less than that of macrophages without IL-7 treatment (Fig. 1b). This difference was evident on day 3 and became greater with time. On day 12, the p24 level of macrophages treated with 3ng/ml of IL-7 was 2220 pg/ml while that of untreated macrophages was 3542 pg/ml. That is, the suppression with 3ng/ml of IL-7 on HIV-1 replication was 37.4% on day 12. Treatment of macrophages with 10ng/ml of IL-7 showed a greater suppressive effect on HIV-1 replication (Fig. 1b). On day 12, the p24 level of macrophages treated with 10ng/ml of IL-7 was 1760 pg/ml. That is, suppression with 10ng/ml of IL-7 on HIV-1 replication was 50.3% on day 12.

Monocyte-derived human macrophages were also infected with the Z strain of the Sendai virus. Hemagglutination assay (HA) titers were measured on days 1, 3 and 6 after infection. As shown in Fig. 1c, there was no difference in HA titers between IL-7-treated and untreated macrophages, indicating no apparent effect of IL-7 on Sendai virus replication in macrophages.

Levels of IL-7 in supernatants of macrophages were measured on days 1 and 3 after HIV-1 infection (Fig. 1d). On day 1, there was no difference in levels of IL-7 between HIV-1 infected and uninfected macrophages. On day 3, IL-7 levels in HIV-1-infected macrophages slightly increased, while those in uninfected macrophages did not.

The mechanisms of the suppressive effect of IL-7 on R5 HIV-1 replication in macrophages are still not clear. A previous study showed that IL-7 treatment for PBMC enhanced excretion of β chemokines including MIP-1 β, which can suppress R5 HIV-1 replication [15]. We pretreated macrophages with 10ng/ml of IL-7 for one day, and then inoculated them with HIV-1. However, we failed to detect any further suppression of HIV-1 replication compared with macrophages treated with IL-7 only after HIV-1 infection (data not shown). Therefore, it is unlikely that IL-7 could suppress HIV-1 replication by up regulation of MIP-1 β. It is possible that IL-7 could affect HIV-1 replication after HIV-1 enters macrophages.

We have shown that HIV-1-infected macrophages could excrete more IL-7 than uninfected ones. Therefore, it is likely that IL-7 is a self-defense system for macrophages against HIV-1 infection. Furthermore, our finding that IL-7 could moderately suppress HIV-1 replication in macrophages suggested the advantage of IL-7 as an immune modulator which could be used to recover CD4+ T cell numbers in HIV-1-infected individuals.

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FIG. 1. (a) Expression of IL-7 receptor mRNA in macrophages measured by real-time monitoring of fluorescence signals. IL-7 receptor mRNA in macrophages (open circles), PHA-stimulated PBMC (open triangles) as the positive control and CV-1 cells (closed squares) as the negative control are shown. Rn is the ratio of the fluorescence intensity of the target cDNA to the fluorescence intensity of water instead of cDNA. Data shown are representative of two independent experiments with similar results. (b) The HIV-1 p24 antigen in the supernatant of HIV-1 infected macrophages treated with 0 (closed triangles), 3 (open circles) or 10ng/ml (closed circles) of IL-7 was measured on days 1, 3, 6, 9 and 12 after HIV-1 infection. Data show the mean of duplicate samples from the same blood donor with bars indicating the actual fluctuation between two individual measurements. Data shown are the representative of three independent experiments with similar results. (c) Growth of the Sendai virus in monocyte-derived

macrophages treated with 10ng/ml of IL-7 (open circles) or un-treated with IL-7 (closed squares). HA titers in culture supernatants were measured on days 1, 3 and 6 after infection. Data shown are representative of two independent experiments with similar results. (d) Levels of IL-7 in the supernatants of HIV-1 infected macrophages (closed squares) or uninfected macrophages (open triangles) were measured on days 1 and 3 after HIV-1 infection. Data show the mean of duplicate samples from the same blood donor with bars indicating the actual fluctuation between two individual measurements. Data shown are representative of two independent experiments with similar results.

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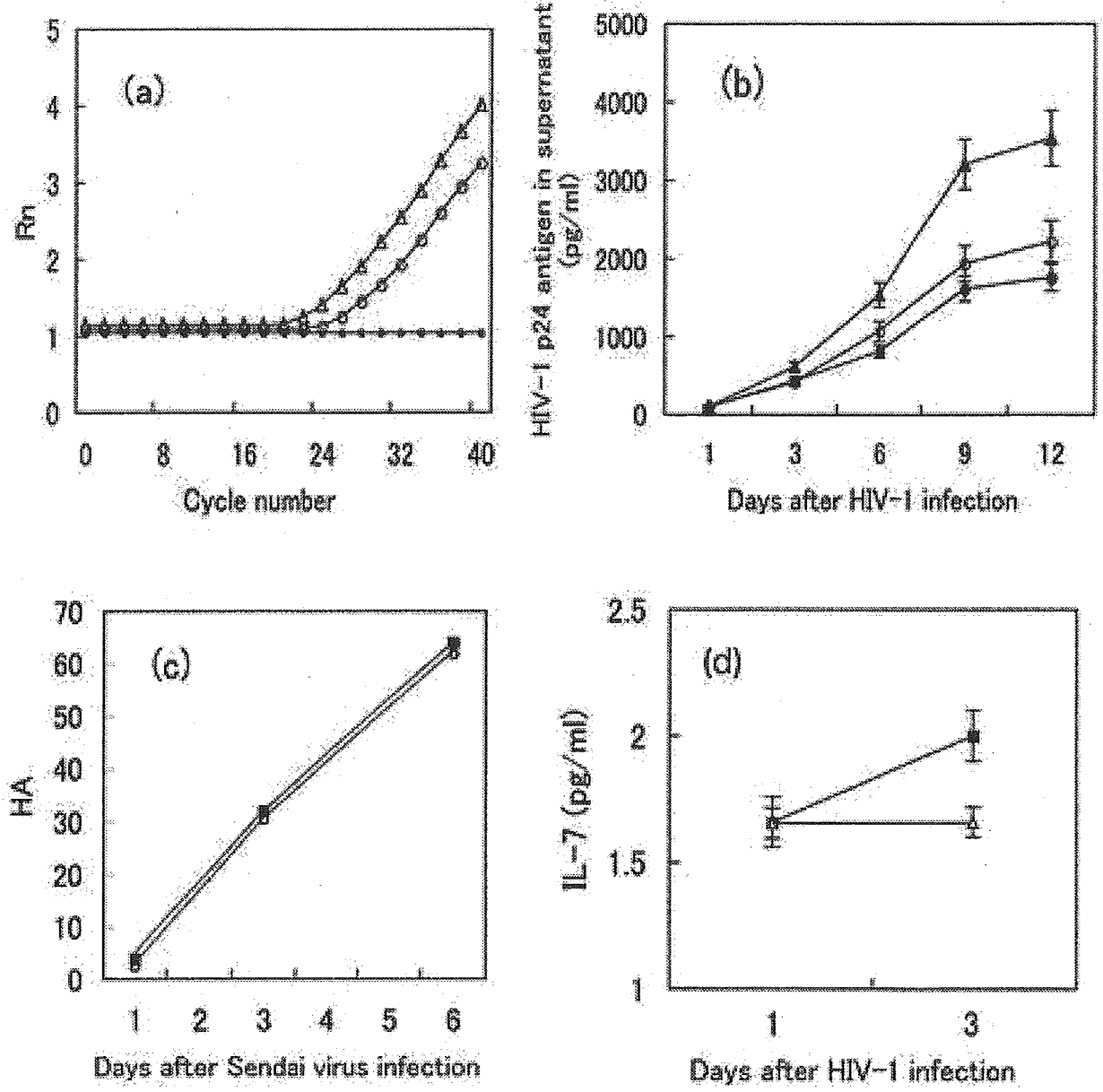

Protective Effects of *IL4-589T* and *RANTES*-28G on HIV-1 disease progression in infected Thai females

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Objective: To evaluate the effect of polymorphisms in interleukin-4 (*IL4*) and *RANTES* promoters on disease progression in HIV-1 infected Thais.

Design: Antiretroviral (ARV) drug-free HIV-1 infected females from the prospective cohort.

Methods: A total of 246 DNA samples were genotyped for *IL4* and *RANTES* promoter polymorphisms by PCR–RFLP. Associations of genotype with HIV-1 disease progression were assessed with respect to baseline clinical data including plasma HIV-1 load, CD4 cell counts, and proportion of symptomatic/AIDS, and survival status during 3 years of follow-up.

Results: Patients with homozygous *IL4-589T* allele showed a significantly lower HIV-1 viral load ($P = 0.005$) and a higher CD4 cell count ($P = 0.003$) than the other patients with heterozygous *IL4-589C/T* or homozygous *IL4-589C* allele. Kaplan–Meier analysis demonstrated an apparent but insignificant trend towards better survival in homozygous *IL4-589T* patients. On the other hand, patients with *RANTES-28G* allele showed a significantly better survival while those with *RANTES In1.1C* allele without *RANTES-28G* showed a significantly poorer survival compared with those who did not possess either *RANTES In1.1C* or *RANTES-28G* ($P = 0.02$), although those polymorphisms only weakly associated with baseline viral load and CD4 cell counts.

Conclusions: Our results implicate the significant protective effect of *IL4-589T* and *RANTES-28G* on HIV disease progression in Thais. In contrast, *RANTES In1.1C* without *RANTES-28G* had an accelerating effect on HIV disease progression.

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Keywords: *IL4-589T*, *RANTES* promoter polymorphisms, HIV disease progression, survival, Thailand, viral load, CD4 cells

Introduction

HIV-1 infected individuals have widely different rates of disease progression. Some infected individuals become symptomatic within 2–3 years while others remain asymptomatic for more than 10–15 years [1]. It is important to investigate factors modulating rates of disease progression for designing novel therapies and vaccines.

RANTES is a natural CCR5 ligand and potently inhibits cell entry of HIV-1 that uses CCR5 as a coreceptor

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(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

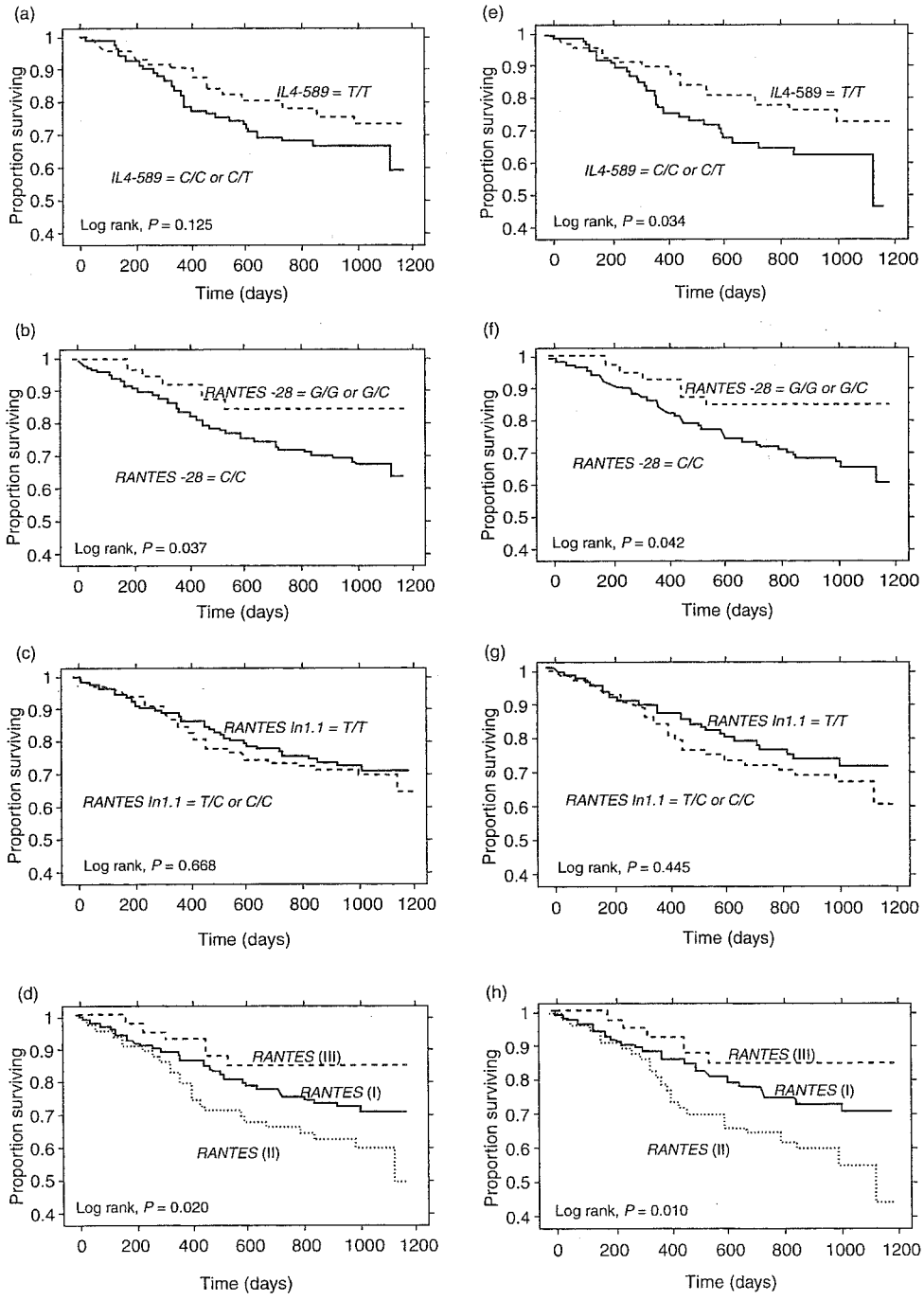

Fig. 1. Kaplan–Meier analysis of the effect of promoter polymorphism of *IL4* and *RANTES* on survival in ARV drug-free HIV-1 infected Thai females during the total observation period (a–d) or untreated period (e–h). (a and e) *IL4-589T*; (b and f) *RANTES-28*; (c and g) *RANTES-403*; and (d and h): 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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