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High frequency and proliferation of $CD4^+FOXP3^+$ Treg in HIV-1-infected patients with low CD4 counts

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The frequency of Treg is reported to be higher in patients with chronic HIV type 1 (HIV-1) infection and $CD45RA^+$ Treg exist in normal adults. In this study, we found a lower absolute number (15 cells/μL) but a higher proportion (16.2%) of $FOXP3^+$ cells (Treg) in the $CD4^+$ population in treatment-naïve HIV-1 patients with low CD4 (<200 cells/μL) counts than in those with high CD4 counts (34 cells/μL and 9.3%) or healthy adults (48 cells/μL and 7.5%). In HIV-1 patients, $CD45RA^+CCR7^+$, $CD45RA^-CCR7^+$, and $CD45RA^-CCR7^-$ subsets were identified in the Treg population, and the proportion of $CD45RA^-CCR7^-$ Treg was higher (57.9%) in patients with low CD4 than high CD4 counts (38.3%). Treg were in a high proliferation state especially in patients with low CD4 counts. HIV viral load correlated positively with the Treg proliferation rate and the proportion of $CD45RA^-CCR7^-$ Treg. Furthermore, the proliferation of Treg correlated positively with the $CD45RA^-CCR7^-$ Treg proportion but negatively with Treg numbers. Successful antiretroviral therapy resulted in a limited increase in Treg numbers, but their frequency was reduced in 1–2 months due to a rapid rebound of $FOXP3^-$ $CD4^+$cells. Our results suggest that HIV-activating Treg may be a reason for the high frequencies of Treg and $CD45RA^-CCR7^-$ Treg in the peripheral blood of late-stage HIV-1-infected patients.

Key words: Cell proliferation · HIV · Immune regulation · Treg

Supporting Information available online

Introduction

HIV type 1 (HIV-1) infection is characterized by a progressive loss and dysfunction of $CD4^+$ T cells [1, 2]. With regard to reduced T-cell functions, accumulating evidence suggests that the balance between the immune suppression function of natural Treg cells and the effector functions of other types of lymphoid cells influences the magnitude of immune reactions in various types of infections, *e.g.* those caused by *Leishmania major*, *Shistosoma mansonia*, and hepatitis C virus [3–7]. FOXP3 is not only a specific marker but also a critical lineage specification factor for Treg [8–11]. Treg are considered mainly as $CD45RA^-$ cells. However, recent studies have shown that $CD45RA^+$ cells also exist among immune-suppressing $CD25^+CTLA4^+CD4^+$ T cells in adults [12, 13].

The local interaction between Treg and other T cells plays an important role in immune suppression and the local density of Treg determines the course of immune responses to infections [4, 7, 14]. Thus, Treg can be both detrimental and beneficial to the host in response to pathogens [5, 7]. For example, in HIV-infected patients, $CD4^+CD25^+$ Treg have been reported to be proportionally increased, decreased, or highly increased in tonsils, their numbers to correlate with HIV viral load, and to exhibit suppression activity [15–23]. Furthermore, antiretroviral

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therapy (ART) has been reported to have either a negative or no influence on Treg or expression of FOXP3 [18, 23]. In HIV-1-infected individuals, immunodeficiency is often considered when the CD4 cell count falls below 200 cells/μL [1]. However, to our knowledge, there is controversy or little information about the absolute number, frequency, and status of homing markers of Treg in HIV-1-infected patients especially in those with low CD4 counts and late-stage AIDS-related diseases or not on ART [24, 25]. Little is known about the dynamic changes of Treg after ART has been introduced.

It is considered that the CCR7 molecule on T cells is an essential trafficking factor for T cells homing to lymphoid tissues as well as an important marker for defining differentiation stage of T cells with CD45RA molecule [26–28].

The present study was designed to investigate Treg in late-stage HIV-1-infected patients with CD4 count <200 cells/μL and the early impact of ART on Treg. We used the chemokine receptor CCR7 and CD45RA molecules to characterize distinct population of migratory Treg.

Results

High-frequency but low absolute numbers of Treg in HIV-1 patients with low CD4 counts

In this study, we enrolled 95 HIV-1-infected patients and 21 HIV-1-negative Japanese adults as our subjects. Because most AIDS-related diseases occur in HIV-1 patients when their CD4 count decreases to below 200 cells/μL, we classified the patients into two groups, a low CD4 group with a $CD4^+$ T cell count less than 200 cells/μL and a high CD4 group with a $CD4^+$ T cell count not less than 200 cells/μL, for some comparison analysis. Table 1 lists the demographic and clinical characteristics of HIV-1-infected patients and healthy HIV-1-negative controls.

Although FOXP3 expression is considered as the best and most specific marker of Treg, some studies have reported that CD127 and CD25 could distinguish Treg [29, 30]. Accordingly, we first compared the staining of FOXP3 with CD25 and CD127 using PBMC from HIV-1-positive individuals. As shown in Supporting Information Fig. 1A and B, $CD25^+CD127^-$ were a proportion of the CD4 cells. However, gating these cells as Treg seems difficult because of the smear staining of both CD25 and CD127. However, gating FOXP3 in CD4 cells was much easier because of the clear staining of FOXP3. Furthermore, we tested the correlation of the Treg by the two classification markers. Supporting Information Fig. 1C shows a good correlation between the proportion of $FOXP3^+$ and $CD25^+$ $CD127^-$ in CD4 cells in 18 HIV-1 patients. Therefore, in the present study, we considered the $FOXP3^+CD4^+$ cells as Treg, and called $FOXP3^-CD4^+$ cells as conventional $CD4^+$ T cells (Tcon).

In the next step, we investigated the frequency and absolute number of Treg in HIV-1-infected individuals without an ART history and compared them with those of healthy Japanese adults. Figure 1A and B shows FOPX3 expression in $CD4^+$ cells. As shown in Table 2, the proportion of Treg in CD4 cells was $16.2 \pm 2.6\%$ in HIV-1 patients with a low CD4 count and

Table 1. Demographic and clinical characteristics of subjects

	Group[a]		
	A (low CD4)	B (high CD4)	H (healthy)
Characteristics			
Numbers	27	68	21
Age (years, range)	39 (21–65)	38 (21–67)	38 (21–60)
Gender (male:female)	27:0	16:1	3:4
CD4 count (cells/μL, SD)	102 (58)	383 (164)	650 (178)
LogVL (SD)	5 (0.6)	4.2 (0.7)	N/A
AIDS-related diseases[b] (*n*, %)	23 (85)	11 (16)	N/A
Months of HIV^+ (range)[c]	12.3 (0–97)	21 (0–124)	N/A
Numbers for tests			
Frequency and subsets of Treg[d]	20	39	21
Ki67 staining *versus* FOXP3[e]	11	24	5
CCR7FOXP3 *versus* CD25[f]	3	16	
CD127CD25 *versus* FOXP3[g]	6	12	

[a] Low CD4: <200 cells/μL; high CD4: ≥ 200 cells/μL.
[b] AIDS-related diseases included: candida, herpes simplex virus infection, tuberculosis, pneumocystis jirovici pneumonia, lymphoma (kaposis sarcoma), *etc.*
[c] Months between the date of the first time of consulting the hospital and the date of blood collected.
[d] Table 2 and Fig. 1.
[e] Figure 2 and Supporting Information Fig. 2.
[f] Figure 1C.
[g] Supporting Information Fig. 1.

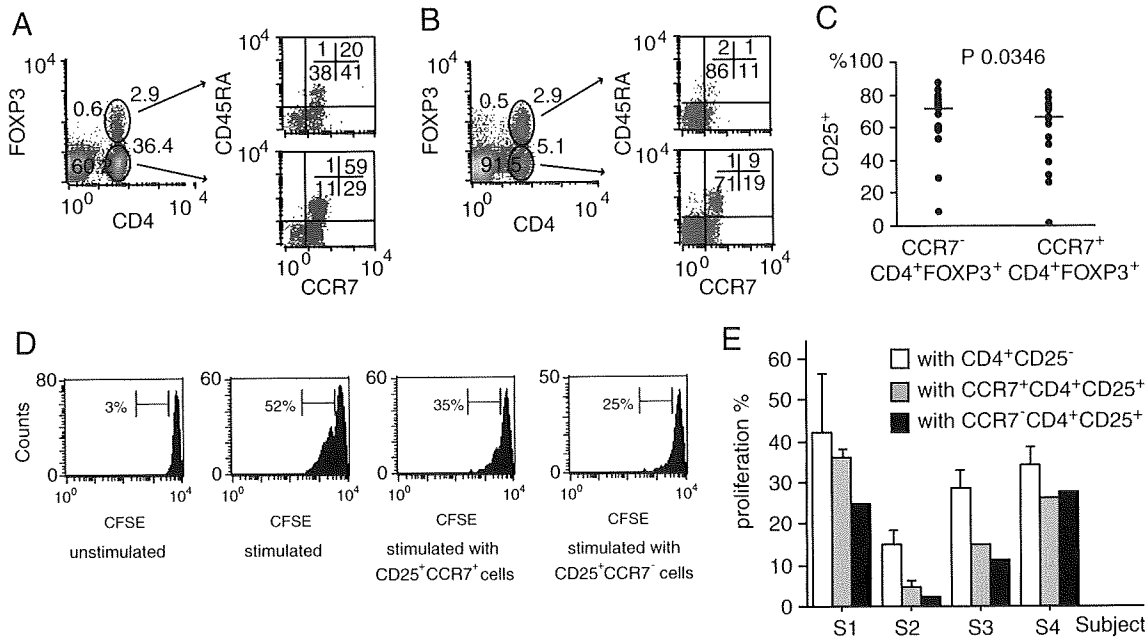

Figure 1. Subsets of Treg in healthy adults and HIV-1-infected patients. (A) Staining of a healthy adult. (B) Staining of an HIV-1-infected patient with low CD4 count. FOXP3 was mainly found in $CD4^+$ T cells both in healthy adults and HIV-1 patients. Treg ($FOXP3^+CD4^+$) cells could be subdivided into $CD45RA^+CCR7^+$, $CD45RA^-CCR7^+$, and $CD45RA^-CCR7^-$ subsets, similar to Tcon ($FOXP3^-CD4^+$, conventional CD4 cells). (C) In HIV-1 patients, the proportion of $CD25^+$ among $CCR7^-$ Treg was higher than that among $CCR7^+$ Treg ($p<0.05$, $n = 19$). (D) A representative proliferation of $CD4^+CD25^-$ responder cells cultured with $CCR7^-CD25^+CD4^+$, $CCR7^+CD25^+CD4^+$ cells, or unlabeled $CD25^-CD4^+$ cells stimulated by anti-CD3 mAb with autologous APC (the data are derived from healthy control). (E) $CCR7^+$ and $CCR7^-$ Treg suppression of responder cells in four subjects. S1–S3: healthy subjects, S4: HIV-1-positive patient (the error bars show duplicate or triplicate tests). Horizontal bars represent median values and p value represents comparison result from Wilcoxon-signed rank test.

$9.3 \pm 0.5\%$ in patients with a high CD4 count. The absolute counts of Treg in low CD4 and high CD4 groups were 15 ± 3 and 34 ± 2 cells/μL, respectively. In healthy adults, the mean CD4 count was 650 cells/μL, and the frequency of Treg among $CD4^+$ cells was $7.5 \pm 0.5\%$ with a mean absolute number of 48 ± 4 cells/μL. Therefore, HIV-1 patients with low CD4 counts had a lower absolute count but a significantly higher frequency of Treg than HIV patients with high CD4 and healthy controls.

High proportion of $CD45RA^-CCR7^-$ Treg in HIV-1 patients with low CD4

Considering the distinct homing potentials and effector functions, CD4 T cells could be subdivided into three subsets, namely naïve ($CD45RA^+CCR7^+$), central memory ($CD45RA^-CCR7^+$), and effector memory ($CD45RA^-CCR7^-$) cells, based on their surface marker and cytokine secretion [26]. Given that local interaction of Treg and Tcon plays an important role in immune suppression and the local number and/or density of Treg reflects immune suppression, we next investigated whether Treg have the same characteristic phenotype as Tcon. Figure 1A shows that Treg could be divided into three subsets, similar to Tcon, based on CD45RA and CCR7 staining in healthy controls. Interestingly, the proportion of each subset of Treg was different compared with the respective subsets of Tcon (Table 2). In healthy adults, the proportion of $CD45RA^-CCR7^-$ Treg ($39.7 \pm 2\%$) was higher than $CD45RA^-CCR7^-$ Tcon cells ($15.6 \pm 1.2\%$), but the proportion of $CD45RA^+CCR7^+$ Treg ($19.3 \pm 1.6\%$) was lower than $CD45RA^+CCR7^+$ Tcon cells ($45.8 \pm 2.4\%$).

In HIV-1-infected patients, the staining patterns of intracellular FOXP3 and surface CD4, CD45RA, and CCR7 were similar to those in healthy controls (Fig. 1A and B). Figure 1B shows a high proportion of $CD45RA^-CCR7^-$ Treg in a representative patient with a low CD4 count. As shown in Table 2, the proportion of $CD45RA^-CCR7^-$ Treg in the low CD4 group ($57.9 \pm 4.2\%$) was significantly higher than in the high CD4 ($38.3 \pm 1.8\%$) or control groups ($39.7 \pm 2\%$). In contrast, the proportion of $CD45RA^-CCR7^+$ Treg in patients with low CD4 counts was significantly lower than in those with high CD4 counts and the control groups. In all subject groups, the proportions of $CD45RA^-$ cells in Treg were higher than in Tcon. Moreover, we found that in HIV-1-infected patients, the proportion of $CD25^+$ in $CCR7^-$ Treg ($64 \pm 19\%$) was higher than in $CCR7^+$ Treg ($58.8 \pm 21\%$, Fig. 1C).

$CD45RA^+$ Treg have been reported to show suppressive function [12]. Based on the finding of a high proportion of $CCR7^-$ Treg in patients with a low CD4 count (Table 2), and considering that $CCR7^+$ cells tend to home to lymphoid tissues whereas $CCR7^-$ cells tend to move to peripheral tissues, we next investigated whether there is any difference in the suppressive activity between $CCR7^+$ and $CCR7^-$ Treg. The results showed

Table 2. Comparison of Treg and Tcon in healthy persons and HIV-1-infected patients[a)]

	Healthy (H)	HIV-1(+)ART(−)		*p* value		
		CD4<200 (A)	CD4≥200 (B)	A *versus* B	A *versus* H	B *versus* H
Number of subjects	21	20	39			
Lymphocytes (cells/μL)	1718 (381)	1028 (447)	1661 (579)	<0.0001	<0.0001	NS
CD4 (cells/μL)	650 (178)	108 (58)	395 (195)	<0.0001	<0.0001	<0.0001
CD4 (%)	38.4 (8.6)	11.4 (7.6)	20.5 (8.5)	0.0001	<0.0001	<0.0001
Treg (cells/μL)	48 (16)	15 (11)	34 (14)	<0.0001	<0.0001	0.0008
Treg (%)	7.5 (2.4)	16.2 (11.8)	9.3 (3.4)	0.0137	0.0004	0.0464
Treg (%)						
$CCR7^+$	57	40.1	59.6	0.0001	0.0029	NS
$CD45RA^+CCR7^+$	19.3	13.4	21.1	0.0109	0.0504	NS
$CD45RA^-CCR7^-$	39.7	57.9	38.3	0.0001	0.0006	NS
$CD45RA^-CCR7^+$	37.7	26.7	38.5	0.0005	0.0057	NS
$CD45RA^-$	77.4	84.6	76.8	0.0131	0.0419	NS
Tcon (%)						
$CCR7^+$	81.3	55.8	74.8	0.0178	0.0035	NS
$CD45RA^+CCR7^+$	45.8	31.9	41.1	NS	0.0217	NS
$CD45RA^-CCR7^-$	15.6	36.8	22.1	0.0283	0.0035	0.04
$CD45RA^-CCR7^+$	35.5	23.9	33.7	0.0048	0.0045	NS
$CD45RA^-$	51.1	60.7	55.8	NS	NS	NS
p Value (Treg *versus* Tcon)						
$CCR7^+$	<0.0001	0.0187	<0.0001			
$CD45RA^+CCR7^+$	<0.0001	0.0001	<0.0001			
$CD45RA^-CCR7^-$	<0.0001	0.0004	<0.0001			
$CD45RA^-CCR7^+$	NS	NS	0.005			
$CD45RA^-$	<0.0001	<0.0001	<0.0001			

[a)] Data are means (SD). NS: not significant. CD4<200, CD4≥200: 200 cells/μL. Mann–Whitney *U*-test was used for comparison between groups (A *versus* B, A *versus* H, B *versus* H). Wilcoxon-signed rank test was used for comparison in group (Treg *versus* Tcon).

that both $CCR7^+$ and $CCR7^-$ $CD25^+CD4^+$ cells suppressed the proliferation of responder cells (Fig. 1D). The suppressive activity was observed in three healthy controls and one HIV-1 patient (Fig. 1E), although no difference was found in the suppression function between the $CCR7^+$ and $CCR7^-$ Treg.

The above results demonstrated the existence of $CD45RA^+CCR7^+$, $CD45RA^-CCR7^+$, and $CD45RA^-CCR7^-$ Treg subsets, similar to Tcon. The proportion of $CCR7^+$ Treg was lower than $CCR7^+$ Tcon cells in both healthy controls and HIV-1 patients. However, the proportion of $CD45RA^-CCR7^-$ Treg was higher than $CD45RA^-CCR7^-$ Tcon, particularly in patients with low CD4 count.

High proliferation of Treg correlates with HIV-1 viral load

Immune cells are activated in HIV-infected patients and such activation is linked to CD4 cell depletion [31]. To determine the mechanism of the high frequency of Treg and $CD45RA^-CCR7^-$ Treg in advanced HIV patients, we stained CD4 cells for the proliferation markers Ki67 in 24 patients (including 11 patients with low CD4 counts and 13 patients with high CD4 counts) and five healthy controls. Figure 2A shows that there was no difference between gating the Ki67 in Treg and Tcon in a healthy control and an HIV-1-infected person. As shown in Fig. 2, the proportions of Ki67-stained cells among Treg in low CD4, high CD4, and control groups (41.7, 24.5, and 22.3%, respectively) were higher than those in Tcon cells (18.1, 11.8, and 7.4%, respectively) (Fig. 2B). The expression of Ki67 in both Treg and Tcon cells was higher in patients with low CD4 counts than in those with high CD4 counts and healthy controls. Furthermore, in the 24 HIV-1-infected patients assessed for Ki67, HIV-1 viral load showed a positive correlation with the frequency of Ki67 in Treg and the proportion of $CD45RA^-CCR7^-$ in Treg. However, the CD4 count showed a negative correlation with the frequency of Ki67 in Treg (Fig. 2C). Moreover, the frequency of Ki67 in Treg correlated negatively with the Treg count and the proportion of $CD45RA^+CCR7^+$ in Treg, but positively with the proportion of $CD45RA^-CCR7^-$ in Treg (Fig. 2D). The same correlation was also observed in Tcon cells (Supporting Information Fig. 2).

ART reduces the frequency of Treg

In HIV-1-infected patients, ART can effectively reduce the HIV viral load and improve CD4 counts. In highly active ART-treated patients, a depleted or normalized Treg was observed in

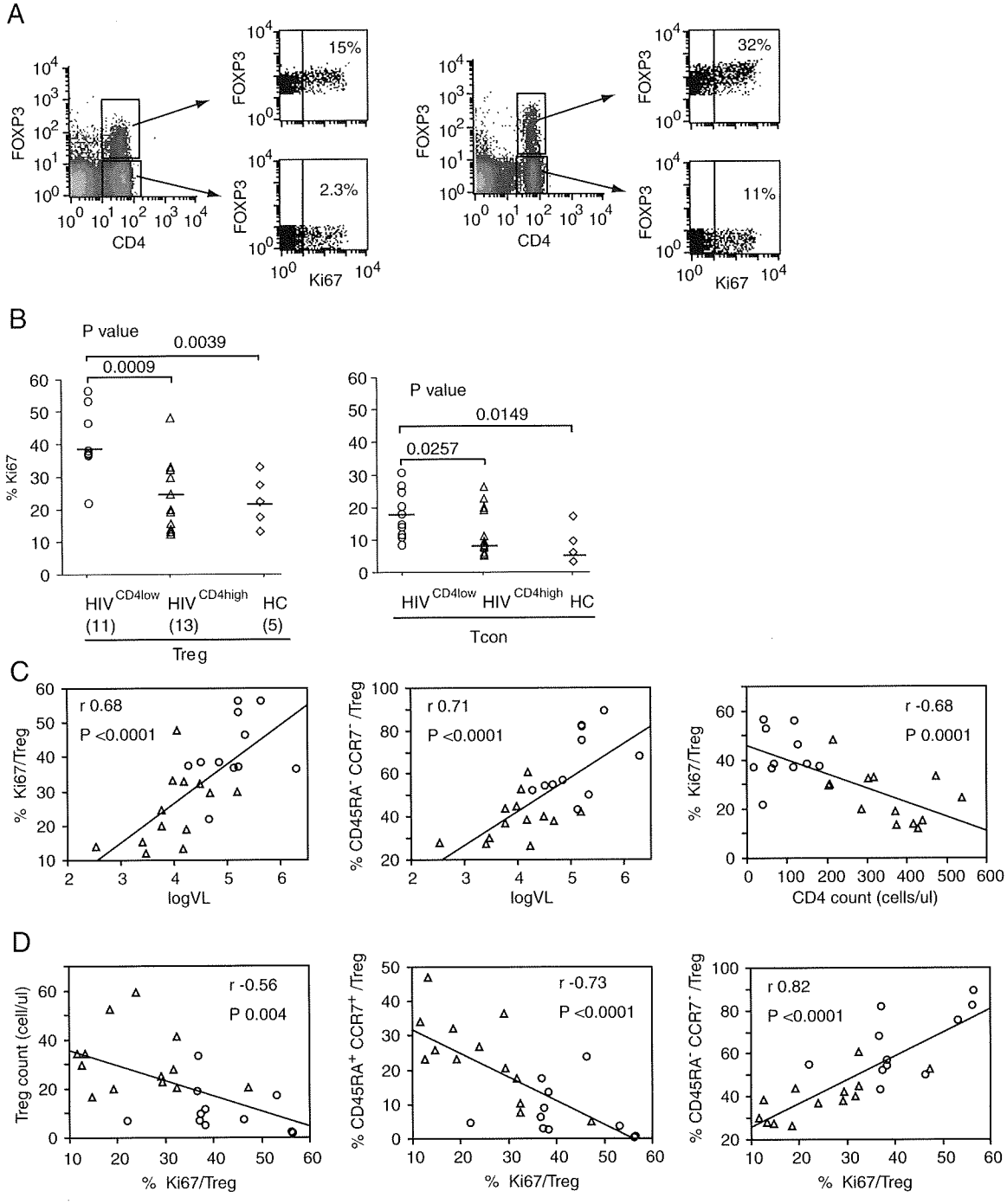

Figure 2. Ki67 staining and high proliferation rate of Treg is associated with viral load. (A) Gating of Ki67 in FOXP3$^+$ and FOXP3$^-$ CD4$^+$ cells in a healthy control (left panel) and an HIV-1-infected person (right panel). (B) Proportion of Ki67-positive Treg (left panel) is higher than that of Ki67-positive Tcon cells (right panel) in healthy controls (HC), HIV-1-infected patients with low CD4 count (HIV^{CD4low}) and HIV-1-infected patients with high CD4 count (HIV^{CD4high}) (numbers in parentheses represent the number of subjects tested). The percentages of Ki67-positive Treg and Tcon cells in the low CD4 group are higher than those in the high CD4 group and healthy control, respectively. (C) HIV-1 viral load shows a positive correlation with the percentage of Ki67 in Treg (left panel) and the proportion of CD45RA$^-$CCR7$^-$ Treg (middle panel). The CD4 count shows a negative correlation with the percentage of Ki67 in Treg (right panel). (D) The percentage of Ki67 in Treg shows correlation negatively with Treg count (left panel) and the proportion of CD45RA$^+$CCR7$^+$ Treg (middle panel) but positively with the proportion of CD45RA$^-$CCR7$^-$ Treg (right panel). Horizontal bars represent median values and p values represent results from Wilcoxon-signed rank test. Simple regression was used for correlation analysis.

PBMC and mucosal tissue [23, 32]. To investigate the impact of ART on Treg, we checked the dynamic change in Treg, their proliferation state, and subsets in nine patients until 9 months after commencement of ART (Fig. 3). The plasma viral load decreased sharply soon after commencement of ART (Fig. 3A). Associated with the decrease in viral load was a rise in the $CD4^+$ count especially in the first 2 months of ART. The CD4 count increased more than 100 cells/μL average in the first month (Fig. 3B). The absolute count of Treg increased in the first month but decreased to some extent thereafter (Fig. 3C); the frequency of Treg decreased rapidly to normal levels within 1–2 months of commencement of ART in all patients (Fig. 3D). On the other hand, the change in the proportion of Ki67 among Tcon and Treg showed a complex pattern. The proportion of Ki67 among Tcon cells increased in the first month of treatment and then decreased and fluctuated on a small scale thereafter (Fig. 3E). However, in the first 1–2 months of ART, the proportion of Ki67 among Treg decreased but maintained high levels until 9 months of ART (Fig. 3F). There was no significant change in each subset in both Treg and Tcon (Fig. 3G and H). However, the $CD45RA^-CCR7^-$ subset still accounted for a high proportion, especially in Treg (Fig. 3G and H, the right panels). The detailed change of each item in each patient is shown in Supporting Information Fig. 3. These results suggest that after initiation of ART, the slow change in the absolute number of Treg and the rapid rebound of Tcon counts resulted in a rapid normalization of the frequency of Treg in HIV-1 patients.

Discussion

Regulation of the immune response is important in maintaining self-tolerance. However, in individuals with immunodeficiency, such as patients with HIV infection, severe immune suppression may contribute to progression of AIDS. Previous studies reported activation of the immune system in HIV-1-infected patients and indicated that human $CD4^+CD25^{high}FOXP3^+$ Treg cells are derived through rapid turnover of memory populations *in vivo* [31, 33, 34].

In the present study, we found that untreated HIV-1-infected patients with low CD4 counts have a high frequency of Treg and $CD45RA^-CCR7^-$ Treg. Cell proliferation was higher in Treg than Tcon cells, especially in HIV-1 patients with low CD4 counts. In these patients, both Tcon and Treg showed a high proliferation state, particularly about 40% Treg were Ki67-positive. Ndhlovu *et al.* [22] reported that $FOXP3^+CD127^{lo}$ $CD4^+$ T cells in PBMC showed a strong negative correlation with T-cell activation during the early chronic stage of HIV infection. In our study, we also found a negative correlation between the proliferating frequency of Treg and Treg absolute count. However, we found that the proliferation of Treg correlated positively with the proportion of $CD45RA^-CCR7^-$ Treg. Furthermore, HIV viral load showed a positive correlation with both Treg proliferation and the proportion of $CD45RA^-CCR7^-$ Treg. These results suggest that HIV infection may activate Treg and result in an increased proportion of $CD45RA^-CCR7^-$ among Treg. On the other hand, Epple *et al.* [32] reported that the frequency and absolute counts of mucosal Treg were highly increased in untreated HIV patients. This finding may be considered another reason for our results because $CCR7^+$ lymphocytes tend to home to lymph nodes and lymphoid tissues. Therefore, we consider that in HIV-infected patients, HIV could simultaneously activate the differentiation of Treg as well as stimulate $CCR7^+$ Treg homing to lymph nodes and lymphoid tissues. These two effects of HIV on Treg result in the high frequency of Treg and a high proportion of $CD45RA^-CCR7^-$ Treg in peripheral blood in patients with low CD4 counts.

ART has been a great success in controlling HIV replication and aiding the recovery of CD4 T cells. However, data about its impact on Treg, especially in detail, are rare. In the current study, we observed that with the rapid decrease in viral load was a robust rebound of Tcon 1–2 months after ART initiation; however, the number of Treg increased in some patients but was almost unchanged in others. The unbalanced change in Tcon and Treg resulted in the frequency of Treg decreasing precipitously to normal levels in the first 1–2 months of therapy. Although the viral load decreased to a very low level in a short period after ART introduction, the proliferative state of Tcon and Treg did not decrease significantly. On the contrary, both Tcon and Treg maintained a high proliferation level, especially Treg. Moreover, the three subsets, *i.e.* $CD45RA^+CCR7^+$, $CD45RA^-CCR7^+$, and $CD45RA^-CCR7^-$ in Tcon and Treg did not show a robust change till 9 months. The results suggest that the recovery of phenotypes needs a much longer period, even if they can recover after ART.

Chase *et al.* [23] observed Treg depletion in highly active ART-treated HIV-1 patients but not in elite suppressors. Here, we did not observe depletion of Treg counts after ART introduction, but we indeed noticed a rapid normalization of the Treg frequency. As we know, to do the suppression assay *in vitro*, an appropriate ratio of Treg to responder cells is needed for observing significant suppression. Considering the suppressive function of both $CCR7^+$ and $CCR7^-$ Treg, we think that the high frequency of Treg, but not the low absolute number of Treg, provides a much better suppressive marker in treatment-naïve HIV-1 patients with low CD4 counts. On the other hand, ART may induce some improvement of the immune suppression because it could reduce the frequency of Treg.

In summary, our results of high frequencies of Treg and $CD45RA^-CCR7^-$ Treg, which tend to migrate to non-lymphoid tissues, in untreated HIV-1 patients with low CD4 counts, emphasize the potential role of Treg in immune deficiency in late-stage HIV-1 infection. Furthermore, anti-HIV treatment could result in a rapid rebound of conventional T cells but not a robust improvement of Treg within 9 months after ART initiation. The different response of Treg and Tcon to ART leads to a rapid decrease in the frequency of Treg. Recently, immune reconstitution syndrome (IRS) is becoming an important problem in HIV treatment. Most IRS occurs in 1–3 months after commencement

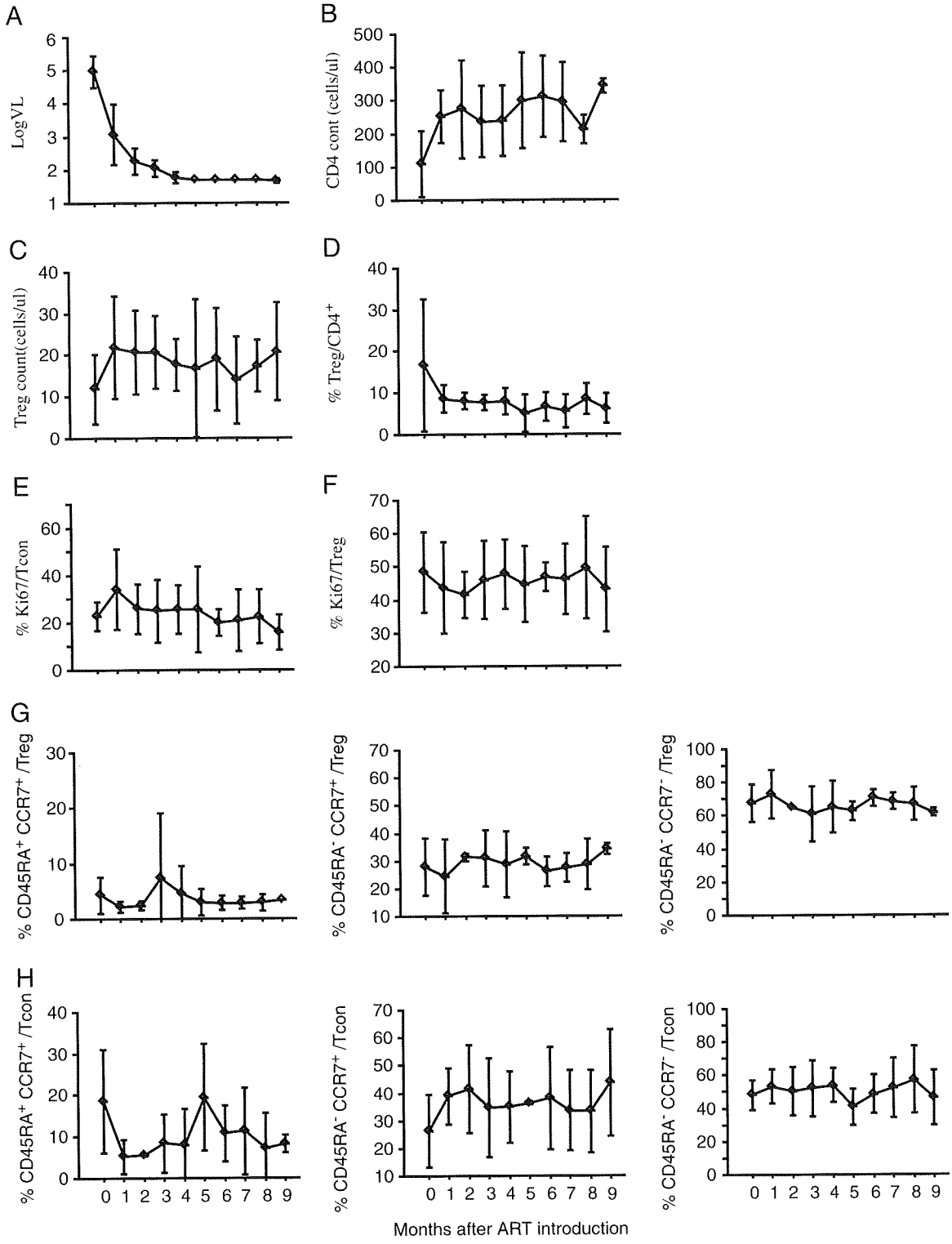

Figure 3. Serial changes in Treg and Tcon cells after commencement of ART. Commencement of ART resulted in rapid reduction in HIV viral load (A) and increase in $CD4^+$ cell count (B). Treatment caused increase in the absolute number of Treg in the first month, then fluctuated slightly thereafter (C), but resulted in a sharp decrease in their percentages in 1 month (D). The proportion of Ki67-positive Tcon increased in the first month but decreased in some extent thereafter (E), while the proportion of Ki67-positive Treg showed some change but still retained a high level at 9 months of commencement of ART (F). At 9 months after ART started, the recovery of the proportion of $CD45RA^+CCR7^+$ Treg (G, left panel) and Tcon (H, left panel) seems very slow, while the proportion of $CD45RA^-CCR7^+$ Treg (G, middle panel) and Tcon (H, middle panel) increased in some extent. However, the proportion of $CD45RA^-CCR7^-$ Treg (G, right panel) and $CD45RA^-CCR7^-$ Tcon (H, right panel) showed a small-scale change, but $CD45RA^-CCR7^-$ Treg maintained a high proportion till 9 months. (A–F) was from nine patients, while (G–H) was from six of them. Vertical bars represent mean ± 1SD.

of ART. Thus, we suppose that the unbalanced improvement of conventional CD4 cells and Treg after commencement of ART might be a factor for IRS. However, this issue needs more investigation.

Materials and methods

Subjects

The subjects were 95 HIV-1-infected patients who have not received any ART and gave written consent before enrollment in this study at the AIDS Clinical Center, International Medical Center of Japan, Tokyo. Nine patients who started ART were followed up for investigation of the impact of ART on Treg. Twenty-one HIV-1-negative adults were recruited as healthy controls. The demographic and clinical characteristics of the subjects are listed in Table 1. HIV-1 viral load was quantified by AMPLICOR HIV-1 MONITOR Test (Roche Diagnostics).

Cell preparation

PBMC were prepared from blood samples collected into EDTA-containing tubes by Ficoll-paque gradient centrifugation. Ki67 staining and evaluation of the ART-treated patients were carried out using cryopreserved PBMC.

For suppression assay, $CD4^+$ cells were isolated from freshly prepared PBMC by using $CD4^+$ T-cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to instructions provided by the manufacturer. $CD4^+$ cells were separated by anti-CD25 mAb (PE) and anti-PE Multisort Kit (Miltenyi) into $CD25^-$ and $CD25^+$ cells. After microbeads release, $CD25^+$ cells were sorted into $CCR7^+$ and $CCR7^-$ cells by using anti-CCR7 mAb (FITC, mouse IgG2a, R&D Systems, Minneapolis, MN) and Rat Anti-Mouse IgG2a+b Microbeads (Miltenyi). The $CD4^+$ $CD25^-$ cells were labeled by 2 μM 5-6-CFSE as responder cells in the suppression assay. Unlabeled $CD4^+CD25^-$ cells were used as non-Treg for cell number control. PBMC that were depleted of $CD3^+$ cells by CD3 MicroBeads (Miltenyi) and irradiated with 3000 rad were used as APC.

Cell staining and flow cytometry

Freshly isolated PBMC were surface stained and also stained intracellularly for FOXP3 (PE/APC labeled, clone PCH101, eBioscience, San Diego, CA) and other markers. The stained cells were analyzed on Becton Dickinson FACSCalibur with CellQuest software (BD Bioscience, San Jose, CA). The monoclonal antibodies used in these staining procedures included anti-CCR7-FITC, anti-CD4-perCP, anti-CD25-PE, anti-CD45RA-APC/perCP, anti-Ki67-PE (BD PharMingen, San Diego, CA), and anti-CD127-FITC (eBioscience).

In vitro suppression assay

In a 96-well, round-bottom plate coated with anti-CD3 mAb (0.25–0.5 μg/mL), 5×10^4 CFSE-labeled $CD4^+CD25^-$ cells were seeded and followed by adding autologous APC (2.5×10^4). For testing Treg suppression, the same number of $CD4^+CD25^+$ $CCR7^+$ or $CCR7^-$ cells was added as regulatory cells. In control wells, the same number of unlabeled non-Treg $CD4^+CD25^-$ cells was added in order to adjust cell numbers in each well. After 3–4 days culture in an incubator at 37°C under 5% CO_2, the cells were harvested and analyzed on FACSCalibur. Live cells were gated and the dilution of CFSE was measured as proliferation of responder cells.

Statistical analysis

Data are expressed as mean ± SD. Differences between groups or stratified groups were examined for statistical significance using Mann–Whitney *U*-test and Wilcoxon-signed rank test. Simple linear regression was used for correlation analysis. All analyses were conducted using the StatView software (version 5.0). A p value of <0.05 was considered statistically significant.

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Abbreviations: **ART:** antiretroviral therapy · **HIV-1:** HIV type 1 · **IRS:** immune reconstitution syndrome · **Tcon:** conventional $CD4^+$ T cells

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LETTERS

Adaptation of HIV-1 to human leukocyte antigen class I

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The rapid and extensive spread of the human immunodeficiency virus (HIV) epidemic provides a rare opportunity to witness host–pathogen co-evolution involving humans. A focal point is the interaction between genes encoding human leukocyte antigen (HLA) and those encoding HIV proteins. HLA molecules present fragments (epitopes) of HIV proteins on the surface of infected cells to enable immune recognition and killing by $CD8^+$ T cells; particular HLA molecules, such as HLA-B*57, HLA-B*27 and HLA-B*51, are more likely to mediate successful control of HIV infection[1]. Mutation within these epitopes can allow viral escape from $CD8^+$ T-cell recognition. Here we analysed viral sequences and HLA alleles from >2,800 subjects, drawn from 9 distinct study cohorts spanning 5 continents. Initial analysis of the HLA-B*51-restricted epitope, TAFTIPSI (reverse transcriptase residues 128–135), showed a strong correlation between the frequency of the escape mutation I135X and HLA-B*51 prevalence in the 9 study cohorts ($P = 0.0001$). Extending these analyses to incorporate other well-defined $CD8^+$ T-cell epitopes, including those restricted by HLA-B*57 and HLA-B*27, showed that the frequency of these epitope variants ($n = 14$) was consistently correlated with the prevalence of the restricting HLA allele in the different cohorts (together, $P < 0.0001$), demonstrating strong evidence of HIV adaptation to HLA at a population level. This process of viral adaptation may dismantle the well-established HLA associations with control of HIV infection that are linked to the availability of key epitopes, and highlights the challenge for a vaccine to keep pace with the changing immunological landscape presented by HIV.

The extent to which HIV is evolving at the population level in response to immune selection pressure is under debate[2–6]. Resolving the impact of HLA class I alleles on viral evolution is problematic because it can be obscured by other influences, such as founder effect[6] (polymorphisms present within the early strains establishing the epidemic in a group). In addition, most HLA alleles do not drive significant selection pressure on HIV, a proportion of escape mutations revert to wild type after transmission, and different HLA alleles may drive the identical escape mutation[7].

To test the hypothesis that the frequency of escape mutations in a given population is correlated with the prevalence of the relevant HLA allele in that population, we studied nine distinct cohorts from North America, the Caribbean, Europe, sub-Saharan Africa, Australia and Japan, in which we performed HLA typing, and defined the viral mutations arising within $CD8^+$ T-cell epitopes. We focused initially on a well-characterized mutation, I135X, within the HLA-B*51-restricted epitope, TAFTIPSI (RT 128–135)[8], because it arises in acute infection, non-HLA-B*51 alleles do not also select this mutation[7,9], and it does not revert to Ile 135 after transmission to HLA-B*51-negative subjects[9]. Thus, if highly prevalent HLA alleles drive a high frequency of escape mutations in the population, this would be most obvious in relation to HLA-B*51 and the escape mutant I135X. We then considered an additional 13 well-defined escape mutations, including those known to reduce viral fitness and therefore liable to revert after transmission.

I135X was selected in 205 of 213 (96%) HLA-B*51-positive individuals analysed (Figs 1 and 2, and Supplementary Fig. 1). The I135X variants do not significantly affect viral replicative capacity *in vitro*, other than the rare I135V mutation. This was the only variant observed to revert to wild-type *in vivo* during a 3-year follow-up of 38 HLA-B*51-negative subjects identified during acute HIV infection who carried I135X mutant viruses at transmission (Fig. 1e). The I135X mutants substantially affect HLA binding, and therefore also recognition by $CD8^+$ T cells (Fig. 1f–h). Thus, HIV transmission from HLA-B*51-positive subjects would probably involve transmission of I135X, which would persist in the new host. Newly infected HLA-B*51-positive subjects receiving an I135X mutant would be unable to generate an HLA-B*51-TAFTIPSI-specific response.

To test the hypothesis that the population frequency of I135X is correlated with HLA-B*51 prevalence, HIV sequence and HLA data were collated from the nine study cohorts. One cohort comprised subjects with acute/early HIV infection; the remaining cohorts comprised chronically infected subjects. In all cohorts the odds ratio strongly favoured I135X in the HLA-B*51-positive subjects, even in the acute cohort where I135X was selected sufficiently early to be already over-represented in HLA-B*51-positive subjects (odds ratio 1.65, $P = 0.07$, Fig. 2a). In Japan, where HLA-B*51 is highly

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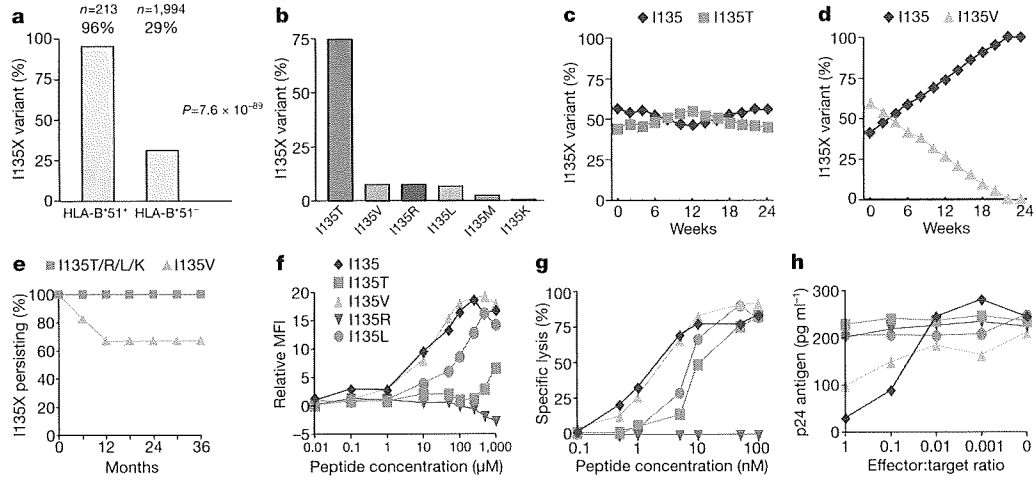

Figure 1 | Selection and fitness cost of I135X escape variants and recognition by the HLA-B*51–TAFTIPSI (RT 128–135)-specific CD8$^+$ T cells. **a**, Association between I135X and HLA-B*51 in all study cohorts. **b**, Ile 135 variation in HLA-B*51-positive subjects. **c**, **d**, *In vitro* competition assays between NL4-3 wild-type virus and I135X viral variants (I135T (**c**) and I135V (**d**)). I135R and I135L showed no fitness cost (not shown). **e**, Persistence of I135X mutants in 38 HLA-B*51-negative subjects followed from acute infection. **f**, TAFTIPSI variant binding to HLA-B*51 (see Methods). MFI, mean fluorescence intensity. **g**, **h**, Recognition of peptide-pulsed HLA-B*51-matched targets and viral variants by representative TAFTIPSI-specific CD8$^+$ T-cell clones.

prevalent[10] (21.9% of the study cohort), the frequency of I135X was >50%, and overall across all cohorts the I135X frequency was strongly correlated with HLA-B*51 prevalence ($P = 0.0001$, Fig. 2b). To control for the possibility that disproportionately more virus sequences from HLA-B*51-positive subjects were analysed, the same analysis comparing I135X frequency in HLA-B*51-negative subjects only was undertaken, with similar findings (Fig. 2c, $P = 0.0006$). These data suggest that HIV may be adapting to HLA-B*51 with respect to the HLA-B*51–TAFTIPSI response in localities where HLA-B*51 is at high prevalence.

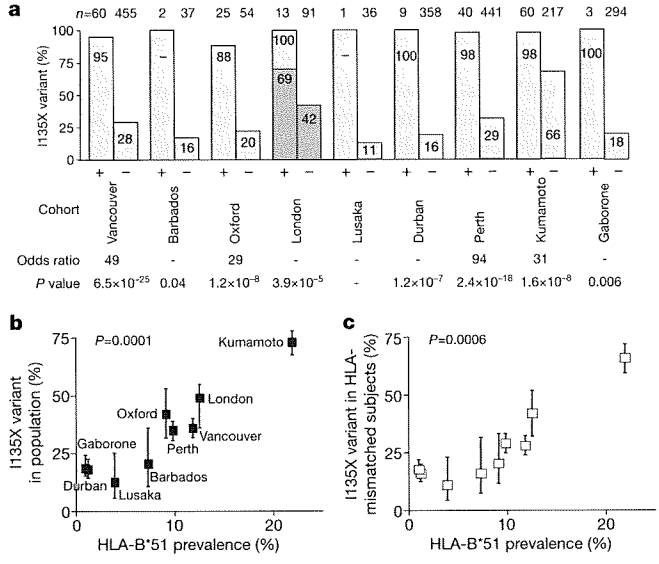

Figure 2 | Correlation between frequency of HLA-B*51-associated escape mutations and HLA-B*51 prevalence in study cohorts. **a**, Frequency of I135X mutations within TAFTIPSI (RT 128–135) in HLA-B*51-positive (+) and -negative (−) subjects within nine study cohorts. In the acute cohort (London) 69% of HLA-B*51-positive subjects expressed I135X mutant at enrolment, 100% within 2 years of baseline (Supplementary Fig. 1). **b**, Correlation between frequency of I135X mutation and HLA-B*51 prevalence in the nine study populations. Logistic regression $P = 0.0001$ (Supplementary Table 1). **c**, Correlation between I135X frequency in HLA-B*51-negative subjects and HLA-B*51 prevalence in nine study populations. Error bars represent 95% confidence limits, obtained using a binomial error distribution.

Additional evidence that I135X is accumulating in Japan comes from the observation that only 3 of 14 (21%) HLA-B*51-negative Japanese haemophiliacs infected in 1983 carried I135X, compared with 30 of 43 (70%) HLA-B*51-negative subjects infected between 1997 and 2008 ($P = 0.002$). Furthermore, HLA-B*51 does not protect against disease progression in Japanese subjects infected between 1997 and 2008, whereas HLA-B*51-positive haemophiliacs infected in 1983 had lower viraemia levels and higher CD4 counts than HLA-B*51-negative haemophiliacs (Supplementary Fig. 2). These data are consistent with fewer HLA-B*51-positive subjects targeting TAFTIPSI during 1997–2008, owing to a population-level increase in the HLA-B*51 I135X escape mutation over this 14–25-year period.

To investigate HIV adaptation to other HLA alleles, we initially examined other escape mutations shown previously to persist stably after transmission[5,7]. We selected the three non-reverting Gag polymorphisms that, from analysis of 673 study subjects in Durban, South Africa[7], were most strongly associated with the relevant restricting allele ($P < 10^{-6}$ after phylogenetic correction), namely, S357X, D260X and D312X within epitopes restricted, respectively, by HLA-B*07 (GPSHKARVL, Gag 355–363), HLA-B*35 (PPIPVGDIY, Gag 254–262) and HLA-B*44 (AEQATQDVKNW, Gag, 306–316). In addition, we analysed a non-reverting I31V variant (LPPIVAKEI, Int 28–36) previously hypothesized to increase in relation to population HLA-B*51 prevalence[5]. These additional polymorphisms show a similar relationship to that between I135X and HLA-B*51, overall showing a strongly significant correlation between variant frequency and prevalence of the restricting HLA allele (Figs 3 and 4a, and Supplementary Fig. 3).

The spectrum of HLA-associated polymorphisms also includes mutations reducing viral fitness[1]. These either revert to wild type after transmission, or persist in the presence of compensatory mutations. We extended these analyses to include epitopes restricted by HLA-B*27 and HLA-B*57, alleles strongly associated with successful immune control of HIV[11,12]. The mutations analysed themselves are associated with precipitating loss of immune control[13–16] and all inflict a documented viral fitness cost, either demonstrated by *in vitro* fitness studies and/or *in vivo* reversion[7,14,17–21] (data not shown for V168I).

Again, a strong correlation between escape mutant frequency and prevalence of the restricting HLA allele was observed (Figs 3c–f and 4b, and Supplementary Fig. 3; overall, for these nine variants affecting viral fitness, $r = 0.69$, $P < 0.0001$). Unexpectedly, this correlation

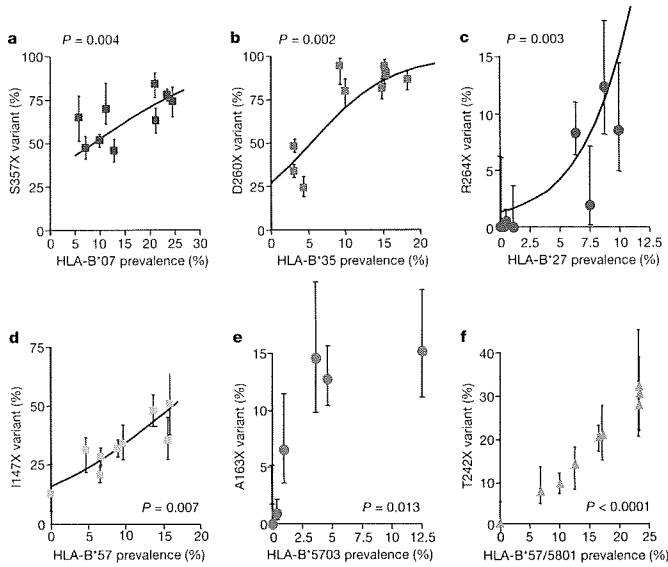

Figure 3 | Correlation between frequency of HIV sequence variant and HLA prevalence for six additional well-characterized epitopes. *P* values calculated after logistic regression analysis as shown (calculations after linear regression analysis are shown in Supplementary Table 1). **a**, Frequency of the S357X mutation within the HLA-B*07-restricted epitope GPSHKARVL (Gag 355–363). **b**, Frequency of the D260X mutation within the HLA-B*35-restricted epitope PPIPVGDIY (Gag 254–262). **c**, Frequency of the R264X mutation within the HLA-B*27-restricted epitope KRWIILGLNK (Gag 263–272). **d**, Frequency of the I147X mutation within the HLA-B*57-restricted epitope ISPRTLNAW (Gag 147–155). **e**, Frequency of the A163X mutation associated with the HLA-B*5703-restricted epitope KAFSPEVIPMF (Gag 162–172). **f**, Frequency of the T242X mutation within the B*57/5801-restricted epitope TSTLQEQIAW (Gag 240–249). Error bars represent 95% confidence limits, obtained using a binomial error distribution.

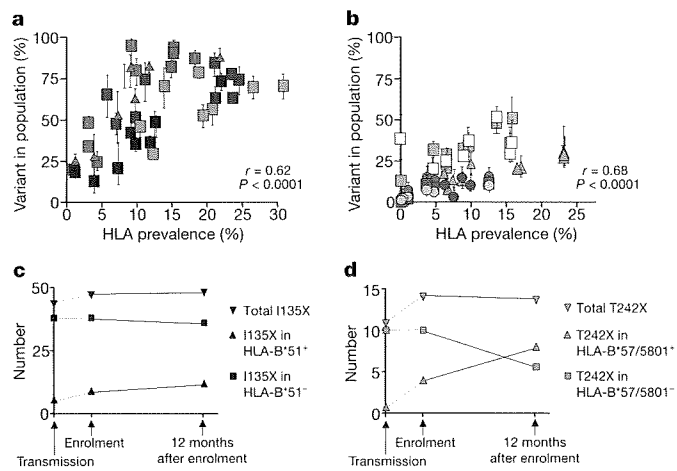

Figure 4 | Correlation between HIV variant frequency and HLA prevalence for all epitopes studied. **a**, Correlation between HLA prevalence and the five stable, non-reverting variants (symbols in Figs 2 and 3, and Supplementary Fig. 3; grey triangles, I31V; green squares, D312X). **b**, Eight variants demonstrated to reduce viral fitness (see text, Fig. 3 and Supplementary Fig. 3; turquoise triangles, L268X; yellow squares. A146X; sky-blue squares, V168I; yellow circles, I247X). **c**, **d**, Data from acute London cohort. **c**, Number of HLA-B*51-positive and HLA-B*51-negative subjects carrying the non-reverting I135X variant. The percentage of I135X in HLA-B*51-negative subjects at enrolment (42%) assumed the percentage of I135X in all subjects at transmission (I135X frequency in HLA-B*51-positive subjects at enrolment was 69%, $P = 0.07$). **d**, The reverting HLA-B*57/5801-restricted T242X mutation. T242X frequency in HLA-B*57/5801-negative subjects at enrolment was 7%, versus 33% in HLA-B*57/5801-positive subjects ($P = 0.01$). Error bars represent 95% confidence limits, obtained using a binomial error distribution.

remained significant even when comparing HLA prevalence with variant frequency in the HLA-mismatched population ($r = 0.40$, $P = 0.0004$). As anticipated, non-reverting variants such as I135X accumulate at the population level, but even rapidly reverting[18,20] mutations such as T242N can accumulate, if the selection rate exceeds the reversion rate (Fig. 4c, d).

Although frequency of the analysed HIV polymorphisms and HLA prevalence were strongly correlated overall, some anomalies were observed. For example, despite a 0% prevalence of HLA-B*57 in Japan[10], 38% of the Japanese cohort had the HLA-B*57-associated A146X variant. One potential explanation might be A146X selection by non-HLA-B*57 Japanese alleles. Analysing Gag sequences from Japanese study subjects, we observed a strong association between A146P and HLA-B*4801 ($P = 0.00035$), and then that A146P is indeed selected in HLA-B*4801-positive subjects (Supplementary Fig. 4a, b). We defined a novel HLA-B*4801-restricted epitope (Gag 138–147), showing also that A146P is an escape mutant (Supplementary Fig. 4c–f). These data illustrate that more than one HLA allele can drive the selection of a particular escape mutant (Supplementary Fig. 5). Also, in populations where HIV-specific $CD8^+$ T-cell responses are incompletely characterized, the influences of locally prevalent HLA alleles on HIV sequence variation are unknown.

These data show a strong correlation between HLA-associated HIV sequence variation and HLA prevalence in the population ($r = 0.69$, $P < 0.0001$, Supplementary Fig. 6), suggesting that the frequency of the studied variants is substantially driven by the HLA-restricted $CD8^+$ T-cell responses. Non-reverting variants[5,7], as well as those previously shown to arise at a fitness cost[7,14,16–21], were studied. The latter constitute approximately 55–65% of HLA-associated polymorphisms[7,20]. This current analysis included epitopes whose role in HIV immune control is unknown, as well as those believed to contribute significantly to containment of HIV[4,7,13–19]. Analysis of well-characterized epitopes only also served to limit potential confounding influences of epitope clustering (selection of the same variant by different HLA alleles) and of founder effect. Either would be capable of obscuring a true HLA effect on population variant frequency.

The HLA-B*57-associated A146X mutation illustrates the complexity that may result from epitope clustering. A146X is selected by at least six distinct HLA alleles (Supplementary Fig. 5). A true correlation existing between mutation frequency and individual HLA allele prevalence might thus be obscured by selection of the same mutation by other alleles.

Founder effect also has an undoubted influence on population frequencies of particular polymorphisms[6]. Phylogenetic correction of sequence data excludes founder effect as a confounder[6,7,9], and the highly significant associations between the presence of particular HLA alleles and all 14 HIV polymorphisms studied, persisting after phylogenetic correction (Supplementary Table 3), provide compelling evidence that the effects observed here are substantially HLA-driven. The large numbers of study subjects in these current studies reduce the likelihood of genuine HLA associations with HIV amino acid polymorphisms being obscured by founder effects. The relative impact of HLA and founder effect on variant frequency is harder to quantify, and is likely to differ substantially between particular populations.

The consequence of HIV adapting to certain $CD8^+$ T-cell responses is unknown. For non-reverting polymorphisms such as HLA-B*35-associated D260E, the variant approaches fixation, because even at population frequencies of 90%, D260E is still significantly selected in HLA-B*35-positive subjects (Supplementary Fig. 7b). Important questions relevant to vaccine design include the extent and rate of sequence change in populations. Relevant factors include the selection rate in subjects expressing the HLA allele, the reversion rate in HLA-mismatched subjects, the population HIV

transmission rate, and HLA allele prevalence. Models would need to include factors such as the selection of compensatory mutations to slow reversion rates, and antiretroviral therapy access that would slow transmission rates.

HLA adaptation to certain $CD8^+$ T-cell responses may also alter currently established HLA associations with slow disease progression. Data here suggest that, whereas 25 years ago HLA-B*51 was protective in Japan[11,12], this is no longer the case (Supplementary Fig. 2). The apparent increase in I135X frequency in Japan over this time supports the notion that HLA-B*51 protection against HIV disease progression hinges on availability of the HLA-B*51-restricted TAFTIPSI response. However, whether this is the case remains unknown.

For HLA-B*27 and HLA-B*57, there is more clear-cut evidence that their association with HIV control depends on the Gag-specific epitopes presented and analysed here[4,7,13–15,18,19]. For each of the HLA-B*27- and HLA-B*57-associated Gag mutations studied, an *in vitro* fitness cost or *in vivo* reversion has been observed. A strong correlation between variant frequency and HLA prevalence even for rapidly reverting variants can be explained, either by mutant acquisition exceeding reversion rate (Fig. 4D), or by selection of compensatory mutations slowing or halting reversion altogether. The clearest example of the latter is the HLA-B*27-associated R264K mutation, 'corrected' by S173A[19]. Compensatory mutations are also well described for the HLA-B*57-associated Gag mutations[14,18]. These data suggest that the escape mutations in these HLA-B*27- and HLA-B*57-restricted epitopes are accumulating over time. Several studies have now demonstrated that transmission of viruses encoding escape mutants in the critical Gag epitopes to individuals expressing the relevant MHC class results in failure to control viraemia[2,21,22]. The accumulation at the population level of these escape mutations in HLA-B*27 and HLA-B*57 Gag epitopes is therefore likely to reduce the facility of these alleles to slow HIV disease progression.

The longer-term consequences of this process for immune control of HIV are unknown. Loss of currently immunodominant epitopes would promote subdominant $CD8^+$ T-cell responses, which can be more effective[23,24]. Also, the adapted virus provides new epitopes that can be presented, potentially with beneficial effects. In hepatitis C virus, for example, HLA-A*0301 holds a particular advantage, but only against the specific strain of virus responsible for the Irish outbreak[25]. In HIV, HLA-B*1801 is associated with high viraemia in C clade but not in B clade infection[10,11,26]; the opposite applies to HLA-B*5301.

Thus, the data presented here, showing evidence that the virus is adapting to $CD8^+$ T-cell responses, some of which may mediate the well-established associations (HLA-B*57, HLA-B*27 and HLA-B*51) with immune control of HIV, highlight the dynamic nature of the challenge for an HIV vaccine. Important questions to be addressed include the speed and extent of sequence change, particularly in Gag, the most effective target for $CD8^+$ T-cell responses[1,7,13,21]. The induction of broad Gag-specific $CD8^+$ T-cell responses may be a successful vaccine strategy, but such a vaccine will be most effective if tailored to the viral sequences prevailing, and thus may need to be modified periodically to keep pace with the evolving virus. Moreover, the strong associations between certain HLA class molecules, such as HLA-B*57, HLA-B*27 and HLA-B*51, and slow disease progression may decline as the epidemic continues, particularly where these HLA alleles are highly prevalent, and where HIV transmission rates are high.

METHODS SUMMARY

Overall 2,875 subjects were studied, from 9 previously established study cohorts. These cohorts comprised subjects from North America, the Caribbean, Europe, sub-Saharan Africa, Australasia and Asia. All subjects were antiretroviral-therapy-naive. Apart from the London acute cohort ($n = 142$), all cohorts comprised chronically infected subjects. The 14 variants studied are well-defined escape mutations within well-characterized $CD8^+$ T-cell epitopes, and included those persisting after transmission and likely to have little effect on viral fitness ($n = 5$), as well as those shown previously to reduce viral fitness ($n = 9$). Autologous HIV-1 sequences, and HLA class I types, were determined for all study subjects. The replicative capacity of I135X variants selected within the HLA-B*51-restricted epitope TAFTIPSI (RT 128–135) was assessed via *in vitro* competition assays and also via longitudinal follow-up of HLA-B*51-negative subjects infected acutely with I135X variants. Polymorphism frequency in the study cohorts was compared with prevalence of the relevant HLA molecule in the study cohort using a logistic regression model taking into account the different numbers of study subjects in each cohort. Demonstration of an HLA allele driving escape at Gag 146 in the Japanese cohort was undertaken first by identification of an association between HLA-B*4801 and A146P, subsequent definition of an HLA-B*4801-restricted $CD8^+$ T-cell response to a novel epitope Gag 138–147 (LI10), and finally demonstration that A146P reduced viral recognition by LI10-specific $CD8^+$ T cells.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Author Information Accession numbers for newly determined viral sequences are included in Supplementary Information. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to P.G. (philip.goulder@paediatrics.ox.ac.uk).

ORIGINAL RESEARCH ARTICLE

High-risk status of HIV-1 infection in the very low epidemic country, Mongolia, 2007

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Summary: Thirty-six HIV-1 cases had been reported by December 2007 in Mongolia. Therefore, Mongolia has been regarded as a very low HIV-1 epidemic country, although the surveillance system is not fully developed. The aim of this study was to evaluate the risk status of HIV-1 infection in Mongolia. A total of 1415 blood samples from high-risk populations including female sex workers, men who have sex with men, mobile men, tuberculosis patients and male sexually transmitted infection (STI) clinic clients and 1050 samples from healthy controls were collected. The seroprevalences of anti-HIV-1/2, anti-*Treponema pallidum*, hepatitis B surface antigen (HBs Ag), anti-hepatitis C virus and hepatitis B surface antibody in the high-risk populations were 0%, 23.1%, 15.5%, 8.0% and 48.2%, and those in the controls were 0%, 3.1%, 14.7%, 4.4% and 44.4%, respectively. HIV-1 prevalence is currently low. However, according to the high prevalence of STIs in the high-risk populations, the risk status for HIV-1 infection is estimated to be high.

Keywords: seroprevalence of HIV, syphilis, HCV and HBV, high-risk population, Mongolia

INTRODUCTION

Mongolia is located in Central Asia bordered by Russia and China. The population of Mongolia is 2635 million, of which 61.0% live in cities and the remaining are nomadic.[1] Geographical conditions and a very low population density make communication, transport and health service provision difficult. Mongolia has witnessed radical changes in its economic and social policies since the democratic revolution of 1990. Along with independence from the former Soviet Union and loss of Soviet support, there has been an increase in unemployment, alcoholism and prostitution and a steady increase in the prevalence of sexually transmitted infections (STIs) and other communicable diseases.[2-8] A recent study demonstrated that syphilis, gonorrhoea and trichomonas were detected in 57 (43%), 18 (14%) and 37 (28%) subjects, respectively, among 132 low-income female commercial sex workers (FSWs) in Mongolia.[9] Mongolia also has a high prevalence of hepatitis B and C viral infection. In a previous study, hepatitis B surface antigen (HBs Ag) and antibodies to hepatitis C virus (anti-HCV) were detected in 24 (10%) and 41 (16%) subjects, respectively, among 249 apparently healthy individuals in Mongolia.[10] However, most of these data were obtained from convenient or non-generalized samples. There is a lack of information regarding exposures and the burden of diseases in the high-risk populations for HIV and STIs. High-risk populations such as FSWs and their sexual contacts with high rates of STIs are important populations contributing to the transmission of HIV and other STIs in developing countries.[11-13]

Since 1992, when data on HIV/AIDS began to be compiled in Mongolia, there had been only five cases reported as of December 2004. Mongolia is considered as an HIV/AIDS low-prevalence country. However, annual new cases of HIV/AIDS have been increasing in recent years. For example, 11, 9 and 11 new cases were detected in 2005, 2006 and 2007, respectively. Among them, 22 (61.1%) cases were men who have sex with men (MSM), seven (19.4%) were heterosexually transmitted and six (16.7%) were FSWs (Mongolian National Center for Communicable Diseases [NCCD], unpublished data). Owing to the lack of a sound surveillance system, the actual situation is uncertain. The primary objective of this study was to evaluate the current risk status of HIV-1 among high-risk populations in Mongolia, examining the seroprevalence of other STIs concomitantly. These data are crucial for taking future preventive measures against HIV-1 infection.

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METHODS

Study design and study population

This study was conducted from September through December 2007. The study protocol was approved by the ethics committees of the International Medical Center of Japan (H19-448) and of the Ministry of Health, Mongolia. After explaining this study and obtaining informed consent, blood samples were

Figure 1 A map of Mongolia. Blood samples were collected from the capital city Ulaanbaatar and four aimags (provinces), such as Darkhan-Uul, Huvsgul, Dornod and Dornogobi. Asterisks indicate the sites where blood samples were obtained

collected anonymously from both high-risk and healthy control populations in Ulaanbaatar (the capital city of Mongolia) and four aimags including Dornod, Huvsgul (borders of Russia), Dornogobi (a border of China) and Darkhan-Uul (Figure 1). A total of 2465 samples were collected: 1415 samples from high-risk populations and 1050 samples from healthy control populations. The high-risk populations included FSWs, MSM, mobile men, tuberculosis (TB) patients and male STI clinic clients. The number of samples in each population and demographic characteristics are listed in Table 1.

Cluster sampling was used for FSWs in locations such as bars, nightclubs, sauna and massage parlours serving as clusters. MSM were sampled only from Ulaanbaatar city, due to the limited data on MSM in other areas of the country. Mobile men were sampled from Ulaanbaatar city and Dornogobi aimag (province), along major road and rail networks and areas such as truck stops and checkpoints at borders. As for TB patients, those who were diagnosed with TB for the first time during the sampling period were enrolled. A male STI clinic client was defined as one who attended public STI clinics during the sampling period. A healthy control group included youth and blood donors. Youth was defined as unmarried, 15–35 years old students in college or university of both sexes. The blood donors were selected in health facilities during the sampling period.

Specimen collection and serology

All sera were stored below −20°C until use. Sera were tested for antibodies to HIV-1/2 (anti-HIV-1/2), *Treponema pallidum* (anti-TP), hepatitis B surface antiboby (HBs Ab), and hepatitis C virus (anti-HCV) and HBs Ag by using the chemiluminescent enzyme immunoassay (CLEIA) (Lumipalus, Fujirebio, Tokyo, Japan) according to the instructions provided by the manufacturer. Seropositive samples for anti-HIV-1/2 by CLEIA were further confirmed by chemiluminescent immunoassay (Architect, Abbott Laboratories, Abbott Park, IL, USA) and a Western blot for the final diagnosis. All laboratory analyses were performed at the AIDS Clinical Center, International Medical Center of Japan.

Statistical analyses

Differences among high-risk and/or healthy control populations were examined by the Fisher's exact test. Univariate logistic analyses were used to determine the odds ratios (OR) with corresponding 95% confidence intervals (CI). All analyses

Table 1 Demographic characteristics of persons who gave blood samples

Populations	No.	Sex M:F	Age	
			Range	Mean ± SD
High risk				
FSWs	410	0:410	17–52	25.3 ± 6.8
MSM	50	50:0	19–48	28.5 ± 6.3
Male STI clients	545	545:0	15–64	29.2 ± 7.9
TB patients	110	41:69	16–70	34.8 ± 12.5
Mobile men	300	300:0	17–57	30.9 ± 8.8
Subtotal	1415	936:479	15–70	28.8 ± 8.7
Healthy controls				
Blood donors	150	101:49	18–49	28.2 ± 9.3
Youth	900	450:450	15–35	19.9 ± 2.6
Subtotal	1050	551:499	17–49	21.0 ± 4.9

FSW = female commercial sex worker; MSM = men who have sex with men; TB = tuberculosis; STI = sexually transmitted infection

Table 2 Seroprevalence between high-risk and healthy controls in Mongolia

	% Positive in each high-risk group					High risk (%)	Healthy controls (%)	OR (95% CI)	*P* value
	FSWs	MSM	Male STI clients	TB patients	Mobile men				
Anti-HIV-1/2	0	0	0	0	0	0	0	–	–
HBs Ag	11.5	18	15.4	16.4	20.7	15.5	14.7	1.1 (0.9–1.3)	0.570
HBs Ab	48.5	42	48.9	50.9	46.7	48.2	44.4	1.2 (1.0–1.4)	0.060
Anti-HCV	6	18	7.5	15.4	7	8	4.4	1.9 (1.3–2.7)	<0.001
Anti-TP	39.5	30	17.2	10.9	14.7	23.1	3.1	9.3 (6.4–13.4)	<0.0001

FSW = female commercial sex worker; MSM = men who have sex with men; TB= tuberculosis; STI = sexually transmitted infection; OR = odds ratio; CI = confidence interval; HBs Ag = hepatitis B surface antigen; HBs Ab = hepatitis B surface antibody; TP = *Treponema pallidum*; HCV = hepatitis C virus

were conducted using the *Stat View* software version 5.0 (SAS Institute, Cary, NC, USA). A P value of <0.05 was considered statistically significant.

RESULTS

The seroprevalences of anti-HIV-1/2, HBs Ab and Ag, anti-HCV and anti-TP of each group of the high-risk and healthy populations are presented in Table 2. None of the anti-HIV-1/2-positive samples was detected in this study. The prevalences of HBs Ag and HBs Ab in the high-risk population, including among each high-risk group, were not different compared with those in the healthy control. In contrast, the prevalences of anti-HCV (8%) and anti-TP (23.1%) in the high-risk population were significantly higher than those in the healthy control. The ORs of anti-HCV and anti-TP comparing between the high-risk population and the healthy control were 1.9 (95% CI: 1.3–2.7, $P < 0.001$) and 9.3 (95% CI: 6.4–13.4, $P < 0.0001$), respectively. The prevalences of anti-HCV in MSM and TB patients were higher than those of other risk groups. The prevalences of anti-TP in FSWs (39.5%) and MSM (30%) were surprisingly high.

Geographical differences of seroprevalence are shown in Table 3. Again, there were no significant differences of the prevalence of HBs Ab and Ag in different regions of specific high-risk groups. However, incidences of anti-HCV and anti-TP had some differences in different regions of the specific groups. A striking feature was that the prevalence of anti-TP in Ulaanbaatar FSWs was 54.7%.

The prevalence of HBs Ab was high. However, there were no differences in the prevalence between high-risk and healthy control populations over the country. One reason was that a hepatitis B virus (HBV) vaccination programme in childhood has been implemented 18 years ago. Therefore, we divided the subjects into two age-related groups (below 18 years and over 20 years) and analysed the seroprevalence of HBs Ab (Figure 2). There were no differences between the high-risk and healthy control populations in both age-related groups. However, in both high-risk and healthy control populations, higher age groups had significantly higher prevalence.

DISCUSSION

Since 1992 when the first case of HIV-1 infection was reported in Mongolia, the number of reported cases remained low until 2005. However, the number has been increasing sharply since 2005, and 36 cases have been reported as of February 2008 (Ministry of Health, Mongolia, unpublished data). By the estimated report of the Global Fund for AIDS, Tuberculoses and Malaria ('Impact of AIDS in Mongolia' 2004), without prevention measures, Mongolian HIV/AIDS prevalence will be doubled every two years and 2500 people will die of AIDS by 2014. Our result supports this estimation. A current prevalence of HIV-1 infection is still low but the risk status of HIV-1 infection must be high because of the very high prevalence of syphilis in FSWs (39.5%), especially in Ulaanbaatar (54.7%). Another report also presented similar prevalence among low-income FSWs in Mongolia (43%).[9] Schwebke *et al.* [7] reported the prevalence rate (8.6%) of syphilis among 137 male STI clients in

Table 3 Seroprevalence of HBV, HCV and syphilis among a high-risk population by residence

		% positive for				
	No.	Anti-HIV-1/2	HBs Ag	HBs Ab	Anti-HCV	Anti-TP
Ulaanbaatar						
FSWs	150	0	8.7	48.7	8.7	54.7
MSM	50	0	18	42	18	30
Male STI clients	200	0	10	51.5	5.5	16.5
TB patients	50	0	18	56	12	12
Mobile men	150	0	22	48	5.3	14.7
Subtotal	600	0	14	49.5	7.8	26.3
Darkhan – Uul						
FSWs	200	0	14.5	47	4	31.5
Male STI clients	100	0	28	44	7	26
TP patients	30	0	13.3	46.7	16.7	13.3
Subtotal	330	0	18.5	46	6	28.2
Dornogobi						
FSWs	20	0	5	55	5	30
Male STI clients	45	0	26.7	48.9	13.3	15.6
TB patients	10	0	20	70	10	10
Mobile men	150	0	19.3	45.3	8.7	14.7
Subtotal	225	0	19.6	48	9.3	16
Dornod						
FSWs	10	0	10	40	10	20
Male STI clients	100	0	15	46	5	17
TP patients	10	0	10	40	10	0
Subtotal	120	0	14.2	45	5.8	15.8
Huvsgul						
FSWs	30	0	10	56.7	6.7	30
Male STI clients	100	0	9	51	12	11
TP patients	10	0	20	30	40	10
Subtotal	140	0	10	50.7	12.9	15

HBV= hepatitis B virus; HCV = hepatitis C virus; TP= *Treponema pallidum*; FSW = female commercial sex worker; MSM = men who have sex with men; TB = tuberculosis; STI = sexually transmitted infection; OR = odds ratio; CI = confidence interval; HBs Ag = hepatitis B surface antigen; HBs Ab = hepatitis B surface antibody

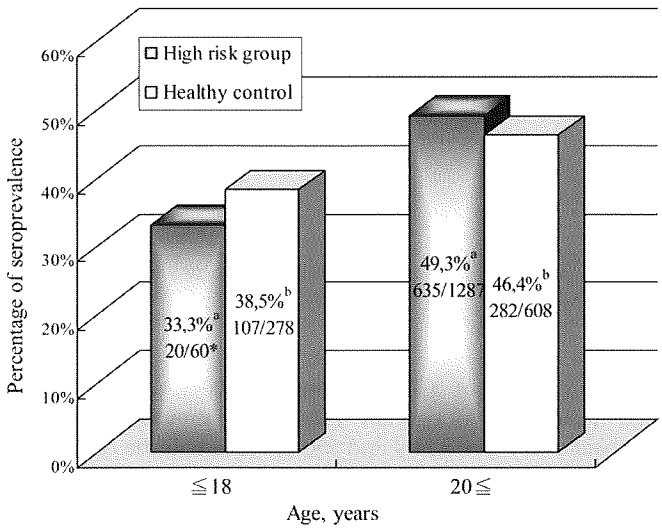

Figure 2 **Age-related seroprevalence of hepatitis B surface antibody (HBs Ab). *N-positive for HBs Ab/N-tested. [a]$P < 0.05$. [b]$P < 0.05$**

Ulaanbaatar in 1998. In our study conducted in 2007, this rate in Ulaanbaatar was 16.5%, suggesting that the prevalence of syphilis is increasing. It is true that these rates were anti-TP. Therefore, it did not mean active syphilis. However, these rates document that the exposure to syphilis is very high. A 100% condom programme is strongly recommended.

According to the unpublished data by NCCD, another risk factor for HIV-1 acquisition is that the predominant route of HIV-1 transmission in Mongolia is through sexual intercourse in MSM. The present study showed higher rates of anti-TP (30%) and anti-HCV (18%) in MSM than those in neighbouring countries: for example, 7% in Beijing (China) and 10% in St Petersburg (Russia) for syphilis and 0.8% or 5.2% in Beijing (China) for HCV.[14-16] These results indicate active high-risk sexual intercourse in Mongolian MSM. There is strong prejudice and discrimination against MSM in Mongolia. Hence, access to the MSM group was very difficult in this study. This barrier makes the delivery of information to MSM difficult. A quick countermeasure to MSM is crucial and a larger serological survey is necessary to grasp the actual prevalence of HIV-1 in Mongolian MSM.

Compared with other STIs, evaluation of hepatitis B was not simple because of the high-prevalence rate in the general population. A hepatitis B vaccination programme has been conducted 18 years ago. Around 35% of people below 18 years have HBs Ab. In contrast, those over 20 years had a significantly higher rate of HBs Ab in both high-risk and healthy control populations. Analysis of HBc-Ab could make it possible to discriminate between HBV-vaccinated and HBV-exposed individuals, which unfortunately we could not perform in this study. This result also suggests the frequent exposure to hepatitis B in Mongolians. Takahashi *et al.*[10] reported a comparable rate of HBs Ab prevalence, indicating a low selection bias of subjects in this study except for MSM and drug abusers.

The present study demonstrates that HIV prevalence is currently low. However, according to the high prevalence of syphilis and HCV in high-risk populations and the social stigma against MSM, the risk status for HIV-1 infection is estimated to be high. Close monitoring of the HIV epidemic is important in order to take quick measures for the high-risk populations and consequently keep the prevalence of HIV low in Mongolia.

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Strong Ability of Nef-Specific CD4$^+$ Cytotoxic T Cells To Suppress Human Immunodeficiency Virus Type 1 (HIV-1) Replication in HIV-1-Infected CD4$^+$ T Cells and Macrophages$^\nabla$

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A restricted number of studies have shown that human immunodeficiency virus type 1 (HIV-1)-specific cytotoxic CD4$^+$ T cells are present in HIV-1-infected individuals. However, the roles of this type of CD4$^+$ T cell in the immune responses against an HIV-1 infection remain unclear. In this study, we identified novel Nef epitope-specific HLA-DRB1*0803-restricted cytotoxic CD4$^+$ T cells. The CD4$^+$ T-cell clones specific for Nef187-203 showed strong gamma interferon production after having been stimulated with autologous B-lymphoblastoid cells infected with recombinant vaccinia virus expressing Nef or pulsed with heat-inactivated virus particles, indicating the presentation of the epitope antigen through both exogenous and endogenous major histocompatibility complex class II processing pathways. Nef187-203-specific CD4$^+$ T-cell clones exhibited strong cytotoxic activity against both HIV-1-infected macrophages and CD4$^+$ T cells from an HLA-DRB1*0803$^+$ donor. In addition, these Nef-specific cytotoxic CD4$^+$ T-cell clones exhibited strong ability to suppress HIV-1 replication in both macrophages and CD4$^+$ T cells in vitro. Nef187-203-specific cytotoxic CD4$^+$ T cells were detected in cultures of peptide-stimulated peripheral blood mononuclear cells (PBMCs) and in ex vivo PBMCs from 40% and 20% of DRB1*0803$^+$ donors, respectively. These results suggest that HIV-1-specific CD4$^+$ T cells may directly control HIV-1 infection in vivo by suppressing virus replication in HIV-1 natural host cells.

Human immunodeficiency virus (HIV)-specific CD8$^+$ cytotoxic T cells (CTLs) play a central role in the control of HIV type 1 (HIV-1) during acute and chronic phases of an HIV-1 infection (5, 29, 34). However, HIV-1 escapes from the immune surveillance of CD8$^+$ CTLs by mechanisms such as mutations of immunodominant CTL epitopes and downregulation of major histocompatibility complex class I (MHC-I) molecules on the infected cells (9, 11, 12, 49). Therefore, most HIV-1-infected patients without highly active antiretroviral therapy (HAART) develop AIDS eventually.

HIV-1-specific CD4$^+$ T cells also play an important role in host immune responses against HIV-1 infections. An inverse association of CD4$^+$ T-cell responses with viral load in chronically HIV-1-infected patients was documented in a series of earlier studies (8, 36, 39, 41, 48), although the causal relationship between them still remains unclear (23). Classically, CD4$^+$ T cells help the expansion of CD8$^+$ CTLs by producing growth factors such as interleukin-2 (IL-2) or by their CD40 ligand interaction with antigen-processing cells and CD8$^+$ CTLs. In addition, CD4$^+$ T cells provide activation of macrophages, which can professionally maintain CD8$^+$ T-cell memory (17). On the other hand, the direct ability of virus-specific cytotoxic CD4$^+$ T cells (CD4$^+$ CTLs) to kill target cells has been widely observed in human virus infections such as those by human cytomegalovirus, Epstein-Barr virus (EBV), hepatitis B virus, Dengue virus, and HIV-1 (2, 4, 10, 19, 30, 31, 38, 50). Furthermore, one study showed that mouse CD4$^+$ T cells specific for lymphocytic choriomeningitis virus have cytotoxic activity in vivo (25). These results, taken together, indicate that a subset of effector CD4$^+$ T cells develops cytolytic activity in response to virus infections.

HIV-1-specific CD4$^+$ CTLs were found to be prevalent in HIV-1 infections, as Gag-specific cytotoxic CD4$^+$ T cells were detected directly ex vivo among peripheral blood mononuclear cells (PBMCs) from an HIV-1-infected long-term nonprogressor (31). Other studies showed that up to 50% of the CD4$^+$ T cells in some HIV-1-infected donors can exhibit a clear cytolytic potential, in contrast to the fact that healthy individuals display few of these cells (3, 4). These studies indicate the real existence of CD4$^+$ CTLs in HIV-1 infections.

The roles of CD4$^+$ CTLs in the control of an HIV-1 infection have not been widely explored. It is known that Gag-specific CD4$^+$ CTLs can suppress HIV-1 replication in a human T-cell leukemia virus type 1-immortalized CD4$^+$ T-cell line (31). However, the functions of CD4$^+$ T cells specific for other HIV-1 antigens remain unclear. On the other hand, the abilities of CD4$^+$ CTLs to suppress HIV-1 replication in infected macrophages and CD4$^+$ T cells may be different, as in the case of CD8$^+$ CTLs for HIV-1-infected macrophages (17). In this study, we identified Nef-specific CD4$^+$ T cells and investigated their ability to kill HIV-1 R5 virus-infected macrophages and HIV-1 X4 virus-infected CD4$^+$ T cells and to suppress HIV-1 replication in the infected macrophages and

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$CD4^+$ T cells. The results obtained in the present study show for the first time the ability of HIV-1-specific $CD4^+$ CTLs to suppress HIV-1 replication in natural host cells, i.e., macrophages and $CD4^+$ T cells.

MATERIALS AND METHODS

Patients. Informed consent was obtained from all subjects, in accordance with the Declaration of Helsinki. Plasma and PBMCs were separated from heparinized whole blood. The patients were sampled at the AIDS Clinical Center, International Medical Center of Japan, and the HLA types of the patients were determined by standard sequence-based genotyping. Patients with active opportunistic infections or psychological disorders and those treated with immunomodulatory agents were excluded.

Synthetic peptides. Peptides (17-mer) derived from the consensus sequence of the Nef protein of HIV-1 clade B were synthesized. These 17-mer peptides overlapped each other by 11 residues. For the feasibility of screening for T-cell epitopes, eight peptides were pooled in a cocktail. Peptides were prepared by using an automated multiple peptide synthesizer. The purity of the synthesized peptides was examined by mass spectrometry, and the peptides with >90% purity were used in the present study.

Cell surface and intracellular cytokine staining. For detection of intracellular cytokines of $CD4^+$ T cells, PBMCs or Nef-specific $CD4^+$ T-cell clones (effector cells) bulk cultured with peptides were stimulated with autologous EBV-transformed B-lymphoblastoid cells (B-LCLs) prepulsed with Nef-derived peptides or peptide cocktails (10^{-6} M) at an effector-to-stimulator (E/S) ratio of 1:4. The pulsed stimulator cells were washed twice in RPMI 1640–10% fetal calf serum (FCS) before use. The mixed cells were incubated for 6 h at 37°C in 5% CO_2. Brefeldin A (Sigma-Aldrich) was added at a concentration of 10 μg/ml after the first 2 h of incubation to inhibit secretion of cytokines. In order to determine the MHC-II restriction of the $CD4^+$ T-cell epitopes, we also employed peptide-pulsed allogeneic B-LCLs with the HLA-DR allele partially matched or mismatched as stimulators in some assays. After a 6-hour incubation, the cells were stained with phycoerythrin (PE)-conjugated anti-human CD4 monoclonal antibody (MAb) (BD Biosciences, San Jose, CA). Then the cells were fixed, made permeable, stained with fluorescein isothiocyanate (FITC)-conjugated anti-human gamma interferon (IFN-γ) MAb (BD Biosciences, San Jose, CA), and analyzed by flow cytometry as previously described (16).

In order to determine the expression of cytotoxic effector molecules, we directly stained PBMCs or Nef-specific $CD4^+$ T-cell clones with allophycocyanin (APC)-conjugated anti-human CD4 or PE-conjugated anti-human CD4 MAb (BD Biosciences, San Jose, CA) without any stimulation of the cells. Then the cells were fixed, made permeable, stained with FITC-conjugated anti-human perforin, PE-conjugated anti-human granzyme A, or Alexa 647-conjugated anti-human granzyme B MAb (BD Biosciences, San Jose, CA), and analyzed by flow cytometry as previously described (44).

To detect the degranulation of Nef-specific $CD4^+$ T cells following antigen stimulation directly ex vivo, we incubated PBMCs with PE-conjugated anti-human CD107a MAb or PE-conjugated isotype control MAb in RPMI 1640–10% FCS containing the corresponding peptide (10^{-6} M), as previously described by Casazza et al. (10). Negative controls containing the PBMCs from the same individual but without peptides were also prepared. Cells were incubated for 6 h at 37°C in 5% CO_2. Brefeldin A was added at a concentration of 10 μg/ml after the first 2 h of incubation. Then, the cells were stained with APC-conjugated anti-human CD4 MAb and FITC-conjugated anti-human IFN-γ MAb and analyzed as described above.

Generation of Nef-specific $CD4^+$ T-cell clones. Peptide-specific $CD4^+$ T-cell clones were generated from an established peptide-specific bulk $CD4^+$ T-cell culture by limiting dilution in U-bottom 96-well microtiter plates (Nunc, Roskilde, Denmark) together with 200 μl of cloning mixture (RPMI 1640 medium supplemented with 10% human serum from healthy donors and 200 U/ml recombinant human IL-2, 5×10^4 irradiated allogeneic PBMCs from a healthy donor as feeders, and 1×10^5 irradiated autologous EBV-transformed B-LCLs prepulsed with a 10^{-6} M concentration of the corresponding peptide). Wells positive for growth after 2 to 3 weeks were transferred to 48-well plates together with 1 ml of the cloning mixture. The clones were examined for specific IFN-γ-producing ability by intracellular cytokine staining. All $CD4^+$ T-cell clones were cultured in RPMI 1640–10% human serum from healthy donors supplemented with 200 U of recombinant human IL-2/ml and were stimulated weekly with irradiated autologous B-LCLs prepulsed with the appropriate epitope peptide.

Blocking of $CD4^+$ T-cell responses. To determine the MHC-II restriction of Nef-specific $CD4^+$ T-cell responses, we blocked the T-cell receptor–MHC-II interaction by using human MHC-II molecule-specific MAbs L243 (anti-HLA-DR), B7/21 (anti-HLA-DP), and Hu-11 and Hu-18 (anti-HLA-DQ4+5+6 and anti-HLA-DQ7+8+9, respectively), which were kindly donated by Y. Nishimura. Autologous B-LCLs prepulsed with the Nef epitope were incubated with the appropriate antibody (10 μg/ml) for 1 h on ice. Subsequently, the cells were washed in RPMI 1640–10% FCS and then incubated with Nef-specific $CD4^+$ T-cell clones (effector cells) at an E/S ratio of 1:2 for 6 h. Brefeldin A was added to the cultures (10 μg/ml) 4 h prior to termination of the cultures. To evaluate the ability of the effector cells to produce IFN-γ under blocking conditions, we stained the cells after stimulation with PE-conjugated anti-human CD4 MAb. Then the cells were fixed, made permeable, and stained with FITC-conjugated anti-human IFN-γ, as described above.

Intracellular cytokine production (ICC) assays for stimulator cells infected with recombinant vaccinia virus. Autologous B-LCLs were infected with 10 PFU per cell of recombinant vaccinia virus expressing HIV-1 Nef (rVac-Nef) or wild-type vaccinia virus (Vac-WT) and cultured for 16 h at 37°C in 5% CO_2. The infected cells were washed twice with RPMI 1640–10% FCS and then incubated with Nef-specific $CD4^+$ T-cell clones (effector cells) at an E/S ratio of 1:4 for 6 h. Brefeldin A was present in the cultures (10 μg/ml) for the last 4 h. To evaluate the ability of the effector cells to produce IFN-γ, we stained the cells with PE-conjugated anti-human CD4 MAb after stimulation. Then the cells were fixed, made permeable, and stained with FITC-conjugated anti-human IFN-γ, as described above.

ICC assays for stimulator cells pulsed with heat-inactivated HIV-1 particles. The virus particles of HIV-1 NL-432 and its Nef-defective mutant were generated by the HIV-1 clones and were heat inactivated at 56°C for 30 min. Autologous B-LCLs were incubated with the inactivated virus particles at 0.5 μg/ml (p24 antigen concentration) for 16 h at 37°C in 5% CO_2. The pulsed cells were washed twice with RPMI 1640–10% FCS and then incubated with Nef-specific $CD4^+$ T-cell clones (effector cells) at an E/S ratio of 1:4 for 6 h. Brefeldin A was present in the cultures (10 μg/ml) for the last 4 h. To evaluate the ability of effector cells to produce IFN-γ after stimulation, we sequentially stained the cells with PE-conjugated anti-human CD4 MAb, fixed them, made them permeable, and then stained them with FITC-conjugated anti-human IFN-γ MAb, as described above.

ICC assay for stimulator cells transfected with Nef-GFP fusion mRNA. For stimulator cells endogenously expressing Nef-green fluorescent protein (GFP) fusion proteins, m7GpppG-capped and poly(A)-tailed Nef-GFP fusion mRNA or GFP mRNA was delivered to autologous B-LCLs by electroporation, as previously described (46). Briefly, B-LCLs were suspended in a serum-free medium (Opti-MEM; Invitrogen Life Technologies) at the cell density of 2×10^6 cells/ml, mixed with 10 μg of mRNA, and electroporated by using a Gene Pulser device (Bio-Rad). The cells were immediately transferred to RPMI 1640–10% FCS, incubated at 37°C for 1.5 to 3 h, and then mixed with Nef-specific $CD4^+$ T-cell clones (effector cells) at an E/S ratio of 1:4. B-LCLs transfected with GFP mRNA were prepared as negative controls. Flow cytometry revealed that more than 60% of the viable B-LCLs expressed GFP. The cell mixtures were incubated for 6 h, and brefeldin A (10 μg/ml) was present for the last 4 h of the incubation. To evaluate the ability of the effector cells to produce IFN-γ after stimulation, we performed surface and intracellular cytokine staining to the cells, as described above.

Isolation and culture of macrophages and $CD4^+$ T cells. Monocytes and $CD4^+$ T cells were isolated from PBMCs of an HLA-DRB1*0803-positive or HLA-DRB1*0403-positive healthy donor by using anti-human CD14 MAb-coated and anti-human CD4 MAb-coated magnetic beads (magnetically activated cell sorting beads; Miltenyi Biotec, Bergisch Gladbach, Germany), respectively. The isolated monocytes were cultured in complete medium containing macrophage colony-stimulating factor (50 ng/ml) for 1 week before use. The isolated $CD4^+$ T cells were cultured for 1 week in complete medium containing IL-2 (200 U/ml) and IL-4 (2.5 ng/ml) and stimulated with OKT3 anti-CD3 MAb (10 μg/ml) every 3 days during the culture period. These cultured macrophages and $CD4^+$ T cells were infected with HIV-1 as previously described (17, 45).

HIV-1 clones. Infectious proviral clones of an X4 HIV-1, pNL-432, and its Nef-defective mutant, pNL-Xh, which has a frameshift at a XhoI site (44th amino acid of the Nef protein), were kindly donated by Y. Koyanagi (Kyoto University, Kyoto, Japan). The infectious proviral clone of $pJRFL_{NL\text{-}432Nef}$ was previously constructed by exchanging the Nef region of R5 strain JRFL with that of NL-432 (17).

CTL assay. The cytotoxicity of Nef-specific $CD4^+$ T-cell clones against B-LCLs or HIV-1-infected target cells was measured by a standard ^{51}Cr release assay as previously described (17). Briefly, target cells (2×10^5) were incubated for 60 min with 100 μCi of $Na_2{}^{51}CrO_4$ in saline and washed three times with RPMI 1640 medium containing 10% NCS. Labeled target cells (2×10^3/well)