Although the extent to which CD4$^-$ cells are infected *in vivo* is unclear, it has been widely thought to be low. Nonetheless, recent studies [11,12] utilizing the novel approach of laser capture microscopy have revealed HIV-1 sequences in isolated CD4$^-$ cells of kidney epithelium and neuronal cells, indicating that latent infection might occur in such cells or tissues *in vivo*. The mechanism of viral entry into CD4$^-$ cells remains unclear, but as we show here the evidence of emergence of CD4-independent strains *in vivo* must be kept in mind.

End-stage liver disease is now becoming a frequent cause of death in HIV-1-infected hospitalized patients. HCV and HBV coinfection with HIV-1 has been shown to enhance the progression of liver damage [16]. However, little attention has been given to the direct virological interaction between HIV and HCV/HBV in the liver, as HIV has been thought not to infect hepatocytes directly. Nonetheless, a number of reports have documented that histological liver abnormalities occurred solely as a result of HIV-1 infection. In our study, we clearly demonstrated that SDA-1 efficiently enters and replicates in both proliferating and static hepatocytes through CXCR4. To our knowledge, this is the first report that HIV-1 can efficiently replicate in normal hepatocytes. Furthermore, we have shown that HIV-1 infection did not induce significant cytocidal effects in the hepatocytes. It is noteworthy that the liver is a continuously regenerating organ. Therefore, if HIV-1 enters and integrates its DNA into the host genome, liver cells containing HIV-1 DNA will be continuously generated by the division of the infected cells. Thus, the expression of HIV-1 proteins on the infected cell surface might result in chronic damage of the liver cells by inducing host immune responses. Direct virological interaction between HIV, HCV and HBV in the liver or enhanced production of HIV-1 by inflammatory cytokines produced by the HCV and HBV-activated immune cells might also exacerbate the liver injury. At present, however, we have no definite information concerning the extent to which patients' hepatocytes harbor HIV-1 and CD4-independent HIV-1 variants.

Finally, a particularly important area of vaccine research is to take advantage of gp120 structural information to guide the design of novel envelope immunogens. As has been reported, CD4-dependent viruses hide neutralizing epitopes and only CD4 binding to gp120 induces conformational changes in gp120 to fully expose epitopes for broadly neutralizing antibodies. The CD4-independent strain we isolated here seems particularly important, as it can efficiently replicate in CD4$^-$ hepatocytes. Therefore, the gp120 structural alterations, which might expose the coreceptor binding site without binding to CD4, may also open up other sites that could yield neutralizing antibodies. Nevertheless, evidence of a clinical CD4-independent R5X4 HIV-1 virus should have important implications concerning the range of mutability and tropism of HIV-1 and the pathogenesis of AIDS.

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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 $16.2\pm2.6\%$ 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.

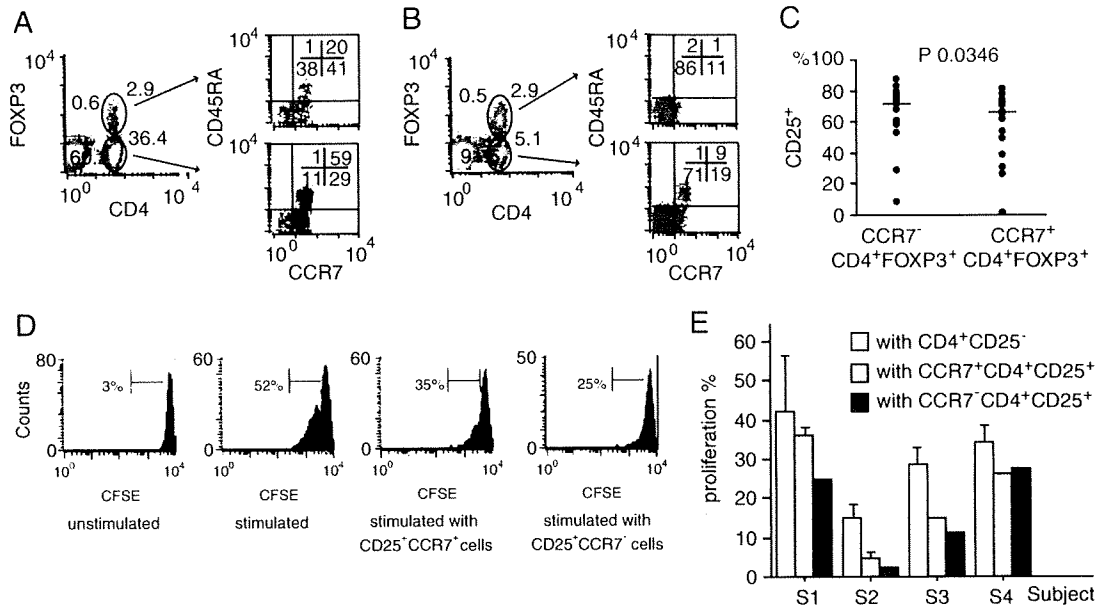

Figure 1. Subsets of Treg in healthy adults and HIV-1-infected patients. (A) Staining of a healthy adult. (B) Staining of an HIV-1-infected patient with low CD4 count. FOXP3 was mainly found in $CD4^+$ T cells both in healthy adults and HIV-1 patients. Treg ($FOXP3^+CD4^+$) cells could be subdivided 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

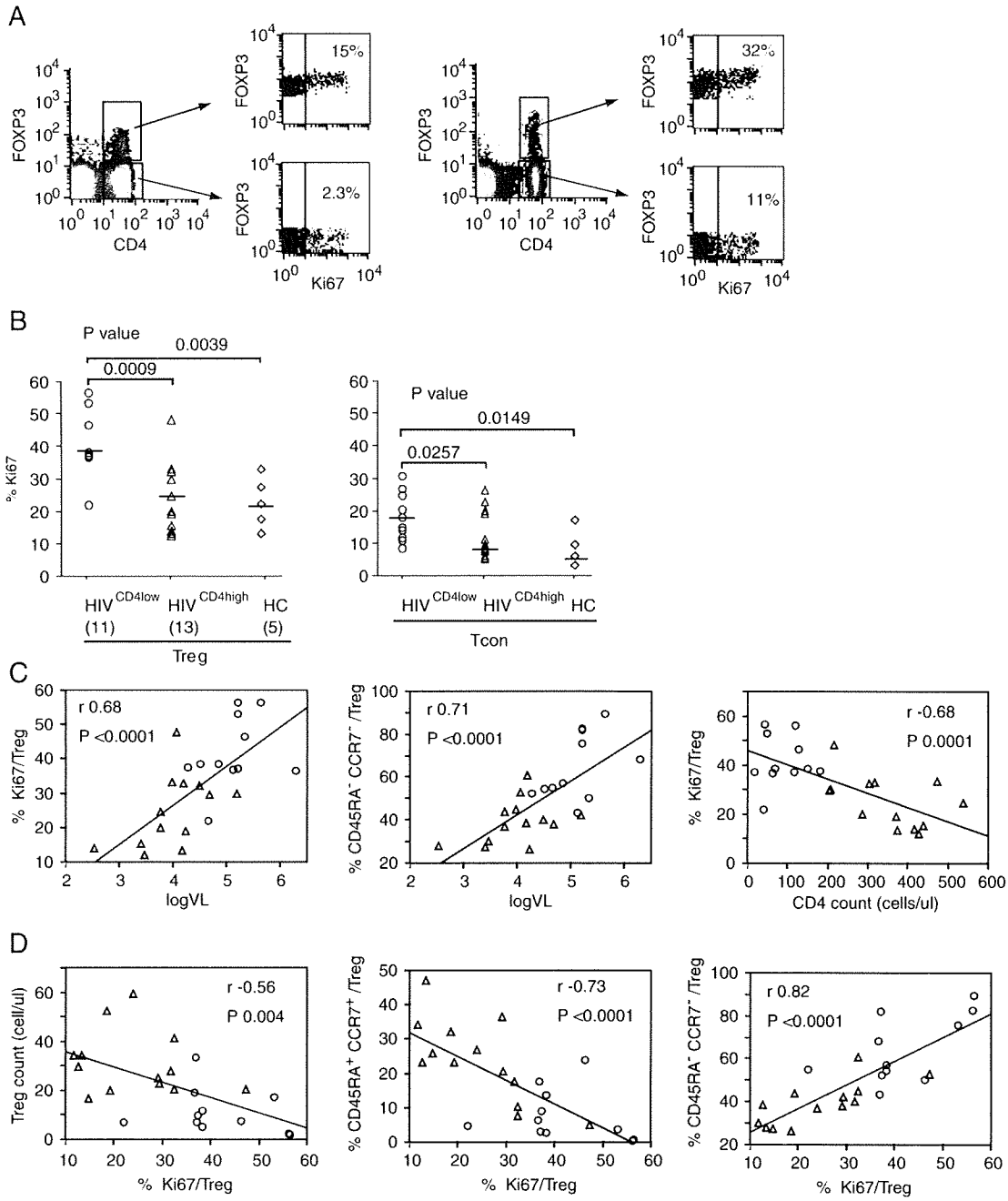

Figure 2. Ki67 staining and high proliferation rate of Treg is associated with viral load. (A) Gating of Ki67 in FOXP3$^+$ and FOXP3$^-$ CD4$^+$ cells in a healthy control (left panel) and an HIV-1-infected person (right panel). (B) Proportion of Ki67-positive Treg (left panel) is higher than that of 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

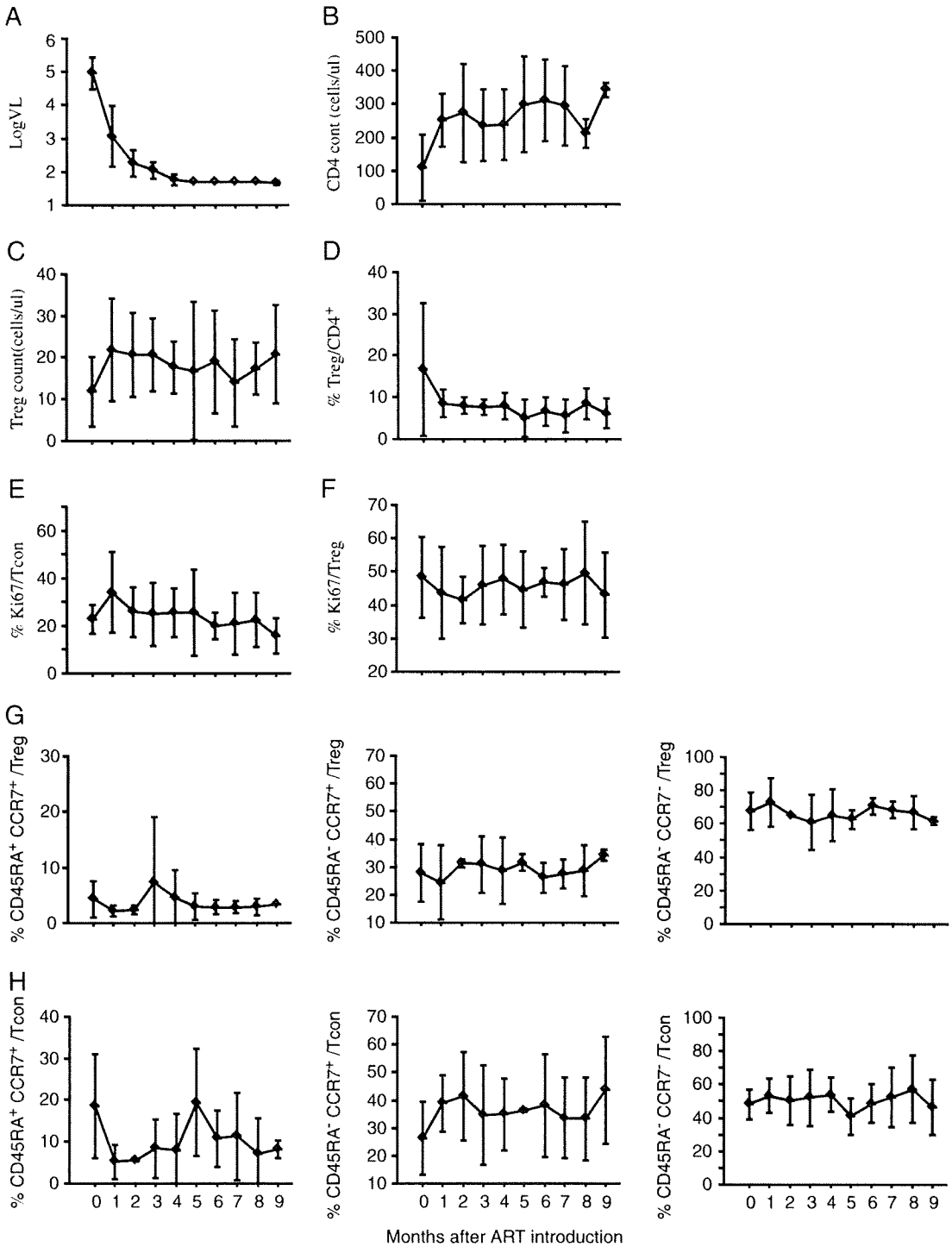

Figure 3. Serial changes in Treg and Tcon cells after commencement of ART. Commencement of ART resulted in rapid reduction in HIV viral load (A) and increase in $CD4^+$ cell count (B). Treatment caused increase in the absolute number of Treg in the first month, then fluctuated slightly thereafter (C), 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 $\pm$ 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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ART: antiretroviral therapy · **HIV-1:** HIV type 1 · **IRS:** immune reconstitution syndrome · **Tcon:** conventional $CD4^+$ T cells

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Human Herpesvirus 8 DNA Load in the Leukocytes Correlates with the Platelet Counts in HIV Type 1-Infected Individuals

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Abstract

Human herpes virus 8 (HHV-8) is known to be reactivated in immunocompromised situations and it is associated with Kaposi's sarcoma (KS) and some hematological diseases. The aim of this study was to analyze the effect of HHV-8 on HIV-1 infection, especially on thrombocytopenia complicated with HIV infection. The HHV-8 DNA load was determined by a quantitative real-time PCR, using leukocytes from 125 HIV-1-infected individuals. HHV-8 DNA was detected in 37 individuals. The increased HIV-1 load and reduced percentage of CD4-positive T cells were significantly associated with the presence of HHV-8. The prevalence and load for HHV-8 are higher in patients with KS than in patients without KS, but the difference is not significant. The increased HHV-8 DNA load was significantly correlated with thrombocytopenia, and platelet counts were significantly lower in individuals with HHV-8 than in individuals without HHV-8. We also obtained the negative correlations between changes in platelet counts and changes in HHV-8 DNA loads. The association between thrombocytopenia and HHV-8 has never been reported previously, apart from some case reports of Castleman's disease and KS. Various cytokines or chemokines are produced by HHV-8-infected cells, some of which have been reported to inhibit hematopoiesis. This may be one of the mechanisms by which HHV-8 infection induces thrombocytopenia. These results indicate that HHV-8 DNA in leukocytes may provide useful information for the assessment of the clinical appearance of HIV-1 infection.

Introduction

HHV-8, A NEW MEMBER OF THE GAMMAHERPESVIRINAE, was identified as the etiologic agent of Kaposi's sarcoma (KS). The main transmission routes of human herpes virus 8 (HHV-8) seem to be sexual contact,[1] but transmission by saliva,[2] blood products, and organ graft[3,4] has also been proposed. The distribution of HHV-8 is related to a combination of geographic and behavioral risk factors. Serological studies have shown that HHV-8 seroprevalence is high in Africa and the Middle East and low in Europe and the United States. In Japan, it is reported that HHV-8 seroprevalence among healthy controls is 0.2–1.4% and HHV-8 seroprevalence among HIV-1-positive homosexual men is 11.6–63.6%.[5,6]

HHV-8 can infect circulating B cells, monocytes, macrophages, T cells, and KS-like spindle cell progenitors,[7–9] and usually persists in a latent state in these cells. The reactivation of this latent HHV-8 infection can be induced by a number of conditions, including superinfection by other viruses, stress, chronic illnesses, malignancies, and immunosuppressive disorders, such as HIV infection. HHV-8 contains more than 80 open reading frames, including several homologues of oncogenes, cytokine, and cytokine response genes. During latent and lytic infection, some viral genes are expressed and play a causative role in the genesis of some diseases, such as AIDS and non-AIDS-related KS, multicentric Castleman's disease, body cavity-based lymphoma, and some lymphoproliferative diseases. Our previous study reported a case of Castleman's disease with HIV-1 infection in which repeated episodes of thrombocytopenia were correlated with an increase in HHV-8 DNA loads in leukocytes.[10] The aim of this study was to determine the prevalence and loads of HHV-8 DNA in peripheral blood leukocytes in HIV-1-positive individuals and to investigate the correlation with the clinical appearance of HIV-1 infection, especially with thrombocytopenia.

Materials and Methods

Patients and samples

All consecutive HIV-1-infected patients who attended Kyushu Medical center between April 2005 and August 2006

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were eligible for this study. The exclusion criteria were HCV infection, active hepatitis, and treatment for antiherpes therapy at the time of sampling. Nine HIV-1-positive individuals were analyzed longitudinally before and after the initiation of antiretroviral therapy (ART). For controls, 12 HIV-1-seronegative patients with autoimmune thrombocytopenia and 17 HIV-1-seronegative healthy volunteers were sampled for baseline comparisons. Informed consent for blood sampling was obtained from all participants. The study was conducted according to the ethical guidelines of the hospital and was approved by an authorized representative of the hospital. EDTA-treated blood was taken from the subjects, and leukocytes were collected after removing red blood cells with hemolysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA). Cell pellets were stored at −20°C until use.

Real-time quantitative PCR

DNA was extracted from the cell pellets using a QIAamp Blood Mini kit (QIAGEN Inc., Tokyo, Japan). Real-time PCR was conducted with the LineGene33 (BioFlux, Tokyo, Japan) using Premix Ex Taq (TAKARA, Shiga, Japan). As an internal control measurement, to normalize for input DNA, copy numbers of $ß_2$-microglobulin ($ß_2M$) were determined in every sample tested. The primers used for amplification were as follows: HHV-8 forward, 5′-CCTCTGGTCCCCATTCATTG-3′, and reverse, 5′-CGTTTCCGTCGTGGATGAG-3′, and probe 5′-FAM-CCGGCGTCAGACATTCTCACAACC-TAMRA-3′;[11] Epstein–Barr virus (EBV) forward, 5′-CGGAAGCCCTCTGGACTTC-3′, and reverse, 5′-CCCTGTTTATCCGATGGAATG – 3′, and probe 5′-FAM-TGTACACGCACGAGAAATGCGCC-TAMRA 3′;[12] ß ₂M forward, 5′-CAGCAAGGACTGGTCTTTCTATCTCT-3′, and reverse, 5′-ACCCCACTTAACTATCTTGG-3′, and probe 5′-FAM-CACTGAAAAAGATGAGTATGCCTGCCGTGT-TAMRA-3′.[13] Standards were obtained by amplification of a control sample in a polymerase chain reaction (PCR) reaction using the same primers. The data were normalized as copies/10^6 cells by measuring copy numbers of the $ß_2M$ gene, since two $ß_2M$ copy numbers correspond to one cell. The lower limit of detection was defined as 1 copy/10^6 cells.

Statistical analyses

Frequency analysis was performed using Fisher's exact test for 2×2 tables. The viral DNA copy numbers of HHV-8, EBV, and HIV-1 were $\log_{10}$ transformed and compared between different groups by means of the Mann–Whitney *U* test. The associations between them were determined using an analysis of covariance (ANCOVA), with EBV-DNA and HIV-RNA as covariates, where HHV-8 DNA was adjusted on the bases of EBV-DNA and HIV-RNA. Spearman's rank correlation coefficient was used to compare changes in platelet counts and changes in HHV-8 DNA loads. A partial correlation was used to assess this relationship, while controlling for any changes in HIV-RNA and EBV-DNA.

Results

Subjects characteristics

A total of 125 patients fulfilled the inclusion criteria. Demographic and clinical characteristics of the subjects included in this study are shown in Table 1. There were 8 women and 117 men, 112 of whom were men who have sex with men; the others were heterosexual. These patients were ranged from 20 to 69 years of age (mean age, 37.8 years). Of these, 58 received ART and 67 remained untreated. The duration of ART was 3 months to 9.5 years (mean 3.25 years). Nine HIV-1-positive individuals were analyzed longitudinally before and after the initiation of ART. Among 125 HIV-1-positive subjects included in our study, we evaluated 97 subjects for the prevalence of splenomegaly by means of an abdominal ultrasound examination; 23 subjects were thus found to have splenomegaly.

HHV-8 qualitative and quantitative DNA analysis

The presence and load of HHV-8 DNA were investigated in 125 HIV-1-positive subjects and 17 HIV-1-negative healthy controls (Fig. 1). HHV-8 DNA was detected in 37 of 125 (29.6%) leukocyte samples from HIV-1-positive subjects, with values ranging from 2 to 91,171 copies/10^6 leukocytes. HHV-8 DNA was not detected in any healthy controls.

Relationship between HHV-8 DNA and KS

HHV-8 DNA was detected in three of five (60%) HIV-infected subjects with KS, whereas they were found in 34 of 120 (28.3%) subjects without KS ($p = 0.15$). Figure 2 shows that the HHV-8 DNA load of subjects with KS to be higher than that of the subjects without KS, but not significantly so.

The correlation between HHV-8 DNA and HIV-1-related immunovirological parameters

The HIV-RNA load in the serum of HHV-8 DNA-positive subjects was significantly higher than that of HHV-8 DNA-negative subjects (Fig. 3a). In addition, the correlation between HHV-8 DNA and hypergammaglobulinemia (the percentage of gammaglobulin is more than normal range) was examined, which is often observed in HIV-1-positive subjects. The HHV-8 DNA load in leukocytes in subjects with

TABLE 1. DEMOGRAPHIC AND CLINICAL CHARACTERISTICS OF 125 HIV-POSITIVE SUBJECTS[a]

	Total	*Pretreatment of ART*	*Treatment of ART*	p
N (male/female)	125 (117/8)	67 (63/4)	58 (54/4)	
Age (years)	37.8 ± 10.4	34.3 ± 8.2	42.1 ± 11.2	<0.001
CD4 (cells/ml)	396 ± 218	387 ± 226	407 ± 211	0.616
Viral load (copies/ml) ($\log_{10}$)		4.36 ± 0.86	Under detection limit	
Duration of ART (years)		0	3.25 ± 2.68	

[a]The mean values ± SE are shown. Pretreatment of ART versus treatment of ART.

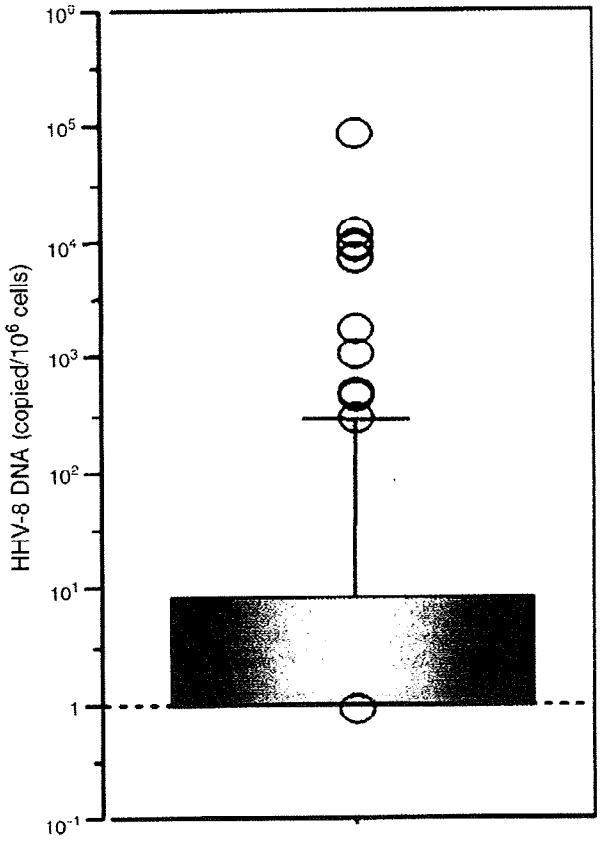

FIG. 1. Quantification of HHV-8 DNA by real-time PCR. Log_{10}-transformed HHV-8 copy numbers per 10^6 leukocytes are shown. The bar shows the mean and standard deviations. The dotted line shows the detection limit of the assay.

hypergammaglobulinemia was significantly higher than in subjects without hypergammaglobulinemia (Fig. 3b). HHV-8 DNA was detected in 24 of 44 (54.5%) HIV-infected subjects with hypergammaglobulinemia, whereas it was found in 13 of 81 (16.0%) subjects without hypergammaglobulinemia ($p < 0.0001$). We could not find any significant correlation between HHV-8 DNA loads and CD4-positive T cell counts.

The effect of ART on HHV-8 DNA

HHV-8 DNA load in leukocytes in subjects with ART was significantly lower than in subjects without ART (Fig. 4a). HHV-8 DNA was detected in 12 of 58 (20.7%) HIV-infected subjects with ART, whereas it was found in 25 of 67 (37.3%) subjects without ART ($p = 0.042$). There was no difference in the duration of ART between HHV-8 DNA-negative and HHV-8 DNA-positive subjects with ART (3.161 ± 2.764 vs. 3.575 ± 2.439 years, $p = 0.624$). HHV-8 DNA loads (log_{10}) per 10^6 leukocytes in subjects with a short duration of ART (<6 months) were higher than in subjects with a long duration of ART (≥6 months), but the difference was not statistically significant (0.758 ± 0.447 vs. 0.323 ± 0.111, $p = 0.309$).

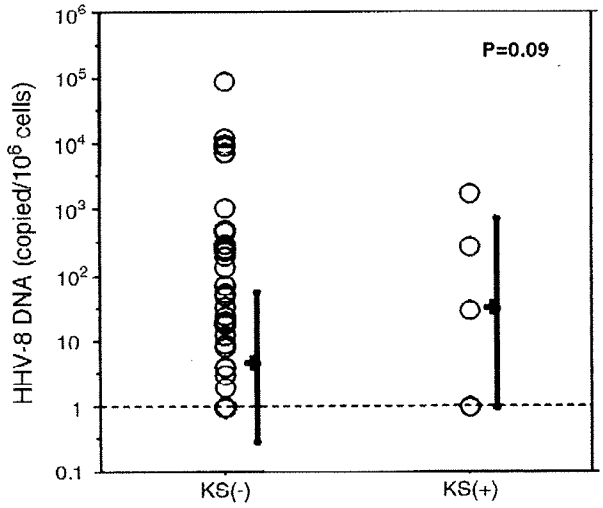

FIG. 2. HHV-8 DNA loads among the HIV-1-positive subjects with or without KS. Log_{10}-transformed HHV-8 copy numbers per 10^6 leukocytes are shown. The bar shows the mean and standard deviations. p-values were estimated by the Mann–Whitney U test. The dotted line shows the detection limit of the assay.

Longitudinal analysis of HHV-8 DNA loads after initiation of ART

The longitudinal profiles of HHV-8 DNA loads were examined in nine subjects after initiation of ART (Fig. 4b). Within 1 month after starting ART, HHV-8 DNA transiently increased in three patients (Fig. 4b, black straight lines); in two of the three patients, KS had progressed transiently. These findings show the reactivation of HHV-8 by initiation of ART. Three months after the initiation of ART, all nine patients had achieved and maintained the decreased levels of HHV-8 DNA.

The influence of EBV on HHV-8 infection

Like HHV-8, EBV is lymphotropic and is a member of the gammaherpesvirinae; it is reactivated by HIV-1 infection. Therefore, the influence of EBV on HHV-8 infection was investigated. EBV-DNA loads (log_{10}) per 10^6 leukocytes were significantly higher in HHV-8 DNA-positive subjects ($0.69 + 0.11$ vs. $1.46 - 0.21$, $p = 0.0007$) and EBV DNA was detected in 32 of 88 (36.4%) HHV-8-negative subjects, whereas they were found in 25 of 37 (67.5%) HHV-8-positive subjects ($p = 0.002$).

Relationship between HHV-8 DNA and thrombocytopenia

Figure 5 shows a case of Castleman's disease in which the platelet counts changed in inverse association with HHV-8 DNA in the leukocytes. Therefore, the association between platelet counts and HHV-8 DNA in leukocytes was examined. First, the changes in platelet counts and HHV-8 DNA were analyzed in the nine subjects before and after the initiation of ART (Fig. 6). Both the decrease in HHV-8 DNA and the

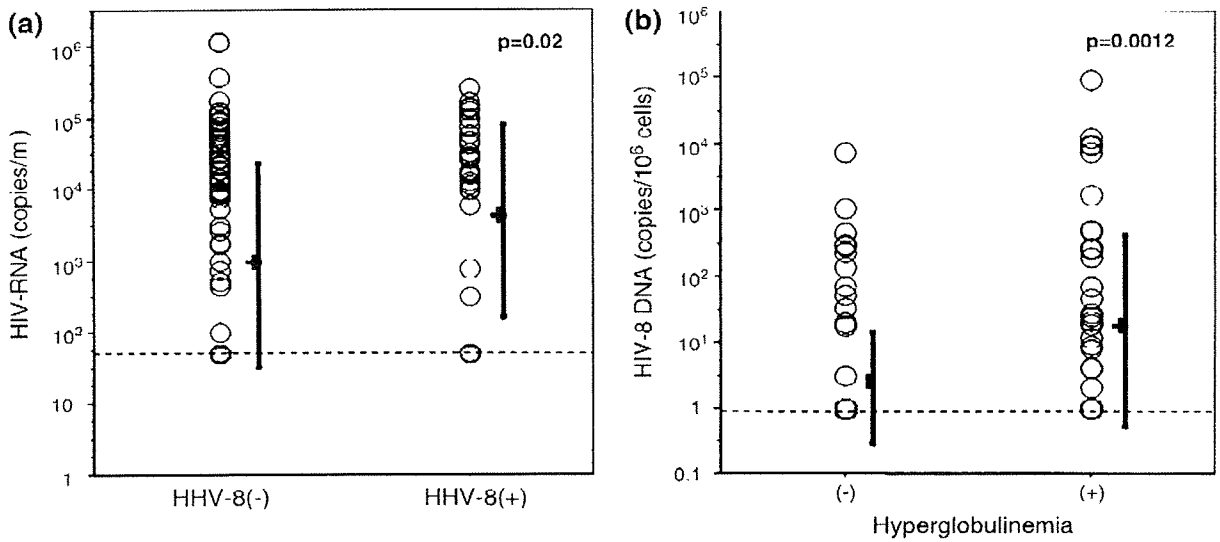

FIG. 3. The correlation between HHV-8 DNA and HIV-1-related immunovirological parameters. (a) HIV-RNA loads among the HIV-1-positive subjects with or without HHV-8 DNA. Log_{10}-transformed HIV-1 copy numbers/ml of serum are shown. The bar shows the mean and standard deviation. p-values were estimated by the Mann–Whitney U test. (b) HHV-8 DNA loads among the HIV-1-positive subjects with or without hypergammaglobulinemia. HHV-8 copy numbers per 10^6 leukocytes are shown. The bar shows the mean and standard deviations. p-values were estimated by the Mann–Whitney U test. The dotted line shows the detection limit of the assay.

increase in platelet counts were significant. Furthermore, the correlation between changes in platelet counts and changes in HHV-8 DNA was obtained (Spearman; $r = -0.783$, $p = 0.0267$). Patients with HIV infection sometimes develop thrombocytopenia, and EBV is also reported to cause mild thrombocytopenia. Therefore, partial correlations were also used to assess this relationship while controlling for EBV-DNA and HIV-RNA, and the correlation between the change in HHV-8 DNA and platelet counts was obtained (partial correlation coefficient; $r = -0.733$, $p = 0.0219$).

Next, all 125 subjects with HIV infection were analyzed. There was no significant difference between the group with

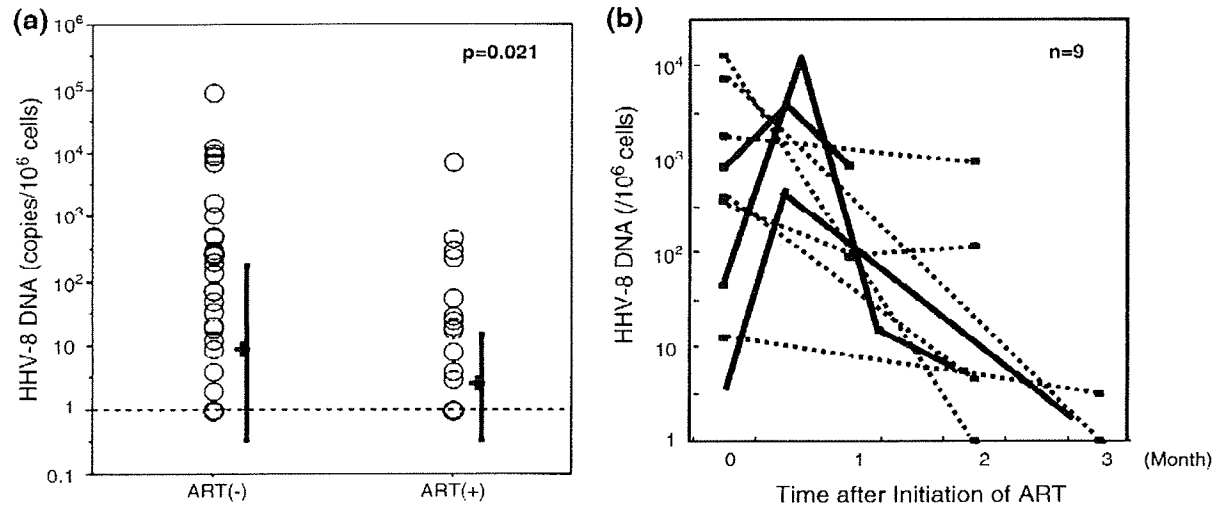

FIG. 4. The effect of ART on HHV-8 DNA. (a) HHV-8 DNA load in PBMCs in subjects with or without ART. HHV-8 copy numbers per 10^6 leukocytes are shown. The bar shows the mean and standard deviation. p-values were estimated by the Mann–Whitney U test. (b) HHV-8 DNA load after initiation of ART. HHV-8 DNA loads of the nine subjects were measured at the indicated time points. The dotted line shows the detection limit of the assay.

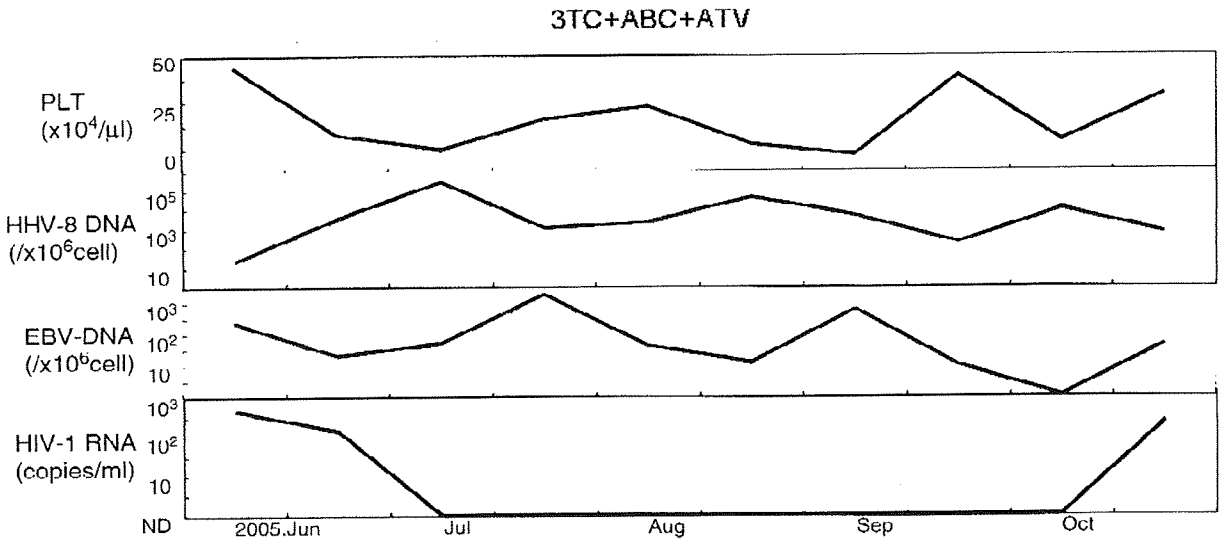

FIG. 5. Time course of the case of Castleman's disease with HIV-1 infection. Platelet counts, $\log_{10}$-transformed HHV-8 and EBV copy numbers per 10^6 leukocytes, and $\log_{10}$-transformed HIV-1 copy numbers/ml of serum are shown. The data were excerpted from Minami and Yamamoto.[10] PLT, platelet; HHV-8, human herpes virus 8; EBV, Epstein–Barr virus, 3TC, lamivudine; ABC, abacavir; ATV, atazanavir.

thrombocytopenia and the group without thrombocytopenia in terms of the number of subjects with ART, the duration of ART, and the prevalence of subjects with splenomegaly (with thrombocytopenia vs. without thrombocytopenia; 30.8% vs. 22.6%, $p = 0.520$). Similarly, no significant difference was observed between the group with HHV-8-DNA and the group without HHV-8-DNA in terms of the duration of ART and the prevalence of subjects with splenomegaly (with HHV-8 vs. without HHV-8; 26.7% vs. 22.4%, $p = 0.647$). The effects of HHV-8 DNA in leukocytes on platelet counts were examined using ANCOVA, with EBV-DNA as a covariate. ANCOVA revealed a significant interaction between EBV-DNA and HHV-8 DNA but no significant interaction between thrombocytopenia and EBV-DNA. Post hoc tests revealed that HHV-8 DNA was significantly higher in subjects with platelet counts <150,000 platelets/μl than in subjects with platelet counts >150,000 platelets/μl (Fig. 7a). HHV-8 DNA was detected in 11 of 14 (78.6%) subjects with low platelet counts,

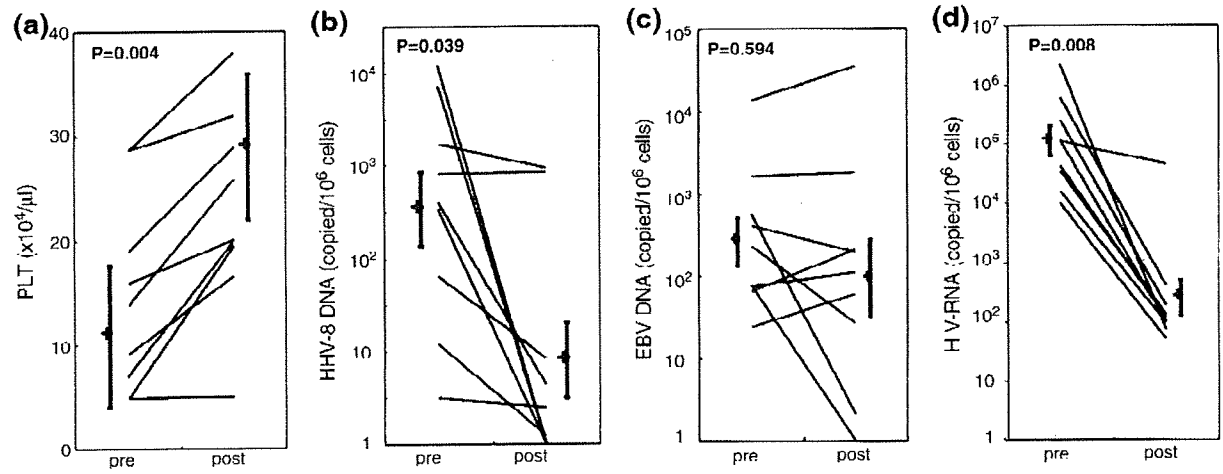

FIG. 6. Changes in the platelet counts, HHV-8 DNA loads, EBV-DNA loads, and HIV-RNA of pre- and post-ART. Platelet counts (a), HHV-8-DNA (b), EBV-DNA (c), and HIV-RNA (d) of the nine subjects were analyzed before (pre) and after 2 or 3 months of initiation of ART (post). The viral loads were $\log_{10}$ transformed. Bar shows the mean and standard deviation. p-values for the difference between the two time points were estimated by the Wilcoxon signed rank test.

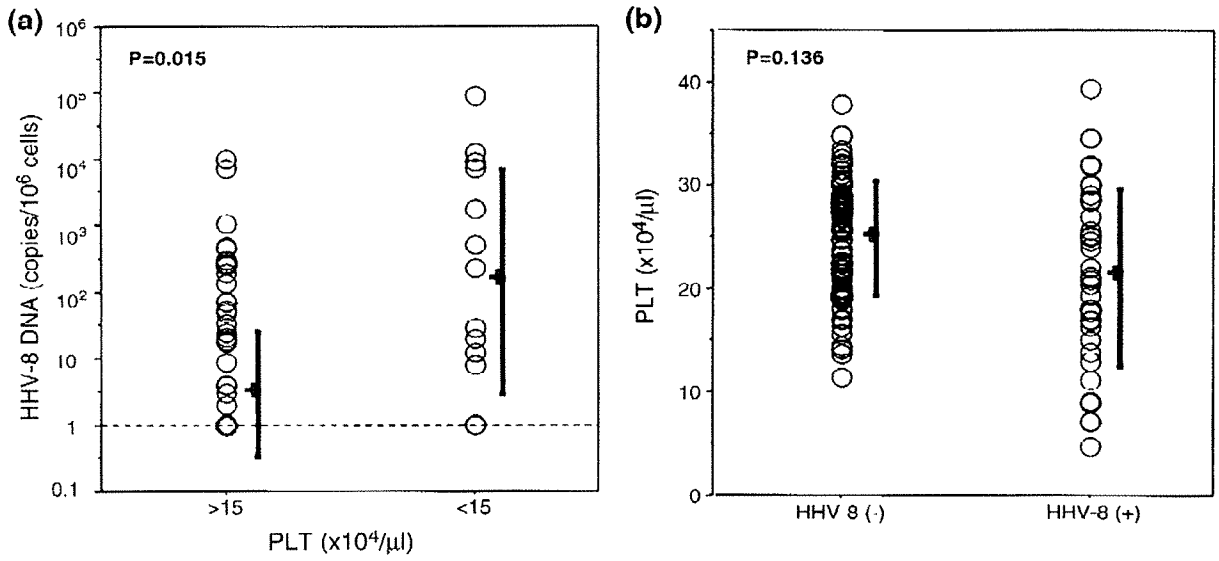

FIG. 7. Relationship between HHV-8 DNA and mild thrombocytopenia.(a) HHV-8 DNA load in leukocytes in subjects with platelet counts of <150,000 platelets/mm^3 or with platelet counts of ≥150,000 platelets/mm^3. HHV-8 copy numbers per 10^6 leukocytes are shown. The bar shows the mean and standard deviation. The *p*-values were estimated by a post hoc test after ANCOVA with EBV DNA as a covariate. (b) Platelet counts among the HIV-1-positive subjects with or without HHV-8 DNA. The bar shows the mean and standard deviation. The *p*-values were estimated by a post hoc test after ANCOVA with EBV-DNA as a covariate. The dotted line shows the detection limit of the assay.

whereas it was found in 26 of 111 (23.4%) of subjects with normal platelet counts ($p = 0.0059$: estimated by post hoc test after ANCOVA with the prevalence of EBV-DNA as a covariate) The platelet counts were lower in HHV-8 DNA-positive subjects than in HHV-8 DNA-negative subjects, *but not significanlyt* (Fig. 7b). In subjects without ART, HIV-RNA might be a confounding factor, and thus ANCOVA was conducted with HIV-RNA and EBV-DNA as covariates.

In subjects without ART, HHV-8 DNA was also significantly higher in subjects with mild thrombocytopenia with an adjustment for EBV-DNA and HIV-RNA ($p = 0.013$, data not shown). HHV-8 DNA was not detected in the 12 subjects with autoimmune thrombocytopenia. We also examined the correlation between HHV 8 DNA loads and platelet counts in subjects without KS, because the subject with KS might have occult Castleman's disease, which can cause thrombocytopenia. HHV-8 DNA was significantly higher in subjects with platelet counts of <150,000 platelets/μl than in subjects with platelet counts of ≥150,000 platelets/μl [$\log_{10}$ (HHV-8) = 2.103 ± 1.797 vs. 0.438 ± 0.924; $p = 0.0285$]. HHV-8 DNA was detected in 9 of 12 (75.0%) subjects with low platelet counts, whereas it was found in 25 of 108 (23.1%) subjects with normal platelet counts ($p = 0.0002$). The platelet counts were significantly lower in HHV-8 DNA-positive subjects than in HHV-8 DNA-negative subjects ($21.5 + 8.61/\mu$l vs. $25.0 + 5.56/\mu$l; $p = 0.1319$).

Discussion

Some previous studies have examined the prevalence of HHV-8 infection in HIV-1-infected subjects. A unique aspect of this study is that the effect of HHV-8 DNA in the peripheral blood leukocytes on HIV-1 infection was examined; a significant association between HHV-8 DNA and the clinical appearance of HIV-1 infection, especially thrombocytopenia complicated with HIV-1, was found.

Some reports have shown that the presence of KS was associated with the prevalence of HHV-8 DNA in leukocytes[14] and the severity was associated with HHV-8 DNA loads in leukocytes.[15] The current data also showed a certain association with the presence of KS and HHV-8 DNA load, but in this study there were only five subjects with KS, because the prevalence of KS in HIV-1-infected patients in Japan is low. As a result, it was impossible to determine whether the HHV-8 DNA load predicted the onset and severity of KS. To draw any conclusions concerning the clinical and prognostic utility on KS, more subjects with KS need to be analyzed.

HHV-8 encodes homologues of cytokine and cytokine response genes, such as viral interleukin-6 (vIL-6)[16] and viral interferon regulatory factor (vIRF). vIL-6 can bind the gp130 receptor to activate IL-6 response genes and promote B cell activation, which is the mechanism of hypergammaglobulinemia induced by HHV-8 infection. It was also found that HHV-8 plays an important role in hypergammaglobulinemia, which is often found in subjects with HIV-1 infection.

The current data showed that HIV-RNA load in the serum of HHV-8 DNA-positive subjects was significantly higher than that found in the serum of HHV-8 DNA-negative subjects. HHV-8 replication is activated by HIV-Tat,[17] and it has been reported that HHV-8 reactivation is associated with HIV-1 acute infection.[18] Furthermore, an open reading frame (ORF), the major transactivator of the HHV-8 lytic cycle, can also induce increased levels of HIV replication. In conclusion, these findings show that HHV-8 coinfection is associated with

immunological characteristics and disease severity of HIV infection.

ART reduced HHV-8 DNA loads as well as HIV-RNA (Fig. 4). The inhibition of HIV-1 replication with ART probably leads to the reconstitution of the immune system and regenerates effective immune responses against HHV-8. Moreover, protease inhibitors block the production of inflammatory cytokines,[19] which in turn may result in the downregulation of HHV-8 replication. In this longitudinal study during ART, HHV-8 DNA loads transiently increased in three subjects soon after the initiation of ART. This might be due to the disorder of cytokine balance, which occurs transiently in the process of the reconstitution of the immune system, and this might be one of the explanations for the immune reconstitution syndrome of KS. Some inflammatory cytokines are induced in immune reconstitution syndromes. It has recently been reported that some cytokines, which are associated with the development of KS, were elevated in other reconstitution syndromes.[20] It is possible that reactivation of HHV-8 by ART might activate the inflammation of the other reconstitution syndromes by the increase in vIL-6 or other cytokines.

The difference between HHV-8 DNA in the subjects with platelet counts of <150,000 platelets/μl and in the subjects with platelet counts of ≥150,000 platelets/μl was significant, but the difference between the platelet counts in the HHV-8 DNA-positive subjects and the platelet counts in the HHV-8 DNA-negative subjects was not significant. This is because HHV-8 DNA correlates with "mild" thrombocytopenia.

This is the first report that analyzed the association between HHV-8 DNA and thrombocytopenia in HIV-1-infected subjects, though there are a few studies that showed the relationship between HHV-8 infection and hematological disorders[21] or posttransplantation bone marrow failure in HIV-1-negative subjects.[22] Some case reports have described thrombocytopenia complicated with HHV-8-induced Castleman's disease, hemophagocytic syndrome, or other inflammatory syndromes. The HIV-1-positive subjects sometimes develop thrombocytopenia, which has been thought to be caused by an autoimmune mechanism. Recently, antibody cross-reactivity between a known epitope region of HIV-1 proteins and platelet CPIIIa19-66 has been reported to be responsible for HIV-1-associated thrombocytopenia.[23] However, the present study showed that HHV-8 also contributes independently to the thrombocytopenia complicated in HIV-1 infection.

The molecular mechanism of thrombocytopenia induced by HHV-8 remains to be established, but it is possible that some cytokines or chemokines induced by HHV-8 might be involved in thrombocytopenia. For example, interleukin-8 (IL-8), which is induced by HHV-8 infection,[24] binds the receptors on megakaryocytes and platelets and inhibits megakaryocytopoiesis.[25] Furthermore, HHV-8 is reported to infect CD34-positive hematopoietic progenitor cells[26,27]; HHV-8 harboring CD34-positive cells might also contribute to hematological disorders including thrombocytopenia.[28] Another possible explanation for thrombocytopenia is an autoimmune mechanism such as immune thrombocytopenia (ITP). Chronic stimulation of the B cell clones induced by HHV-8 could favor the production of autoantibodies, some of which might react against platelet. In fact, Evan's syndrome complicating multicentric Castleman's disease[29] and ITP complicating KS have been reported.[30]

In Italy, the area of subendemicity of HHV-8, the presence of HHV-8 DNA in HIV-1-negative elderly people has been reported to be associated with mild thrombocytopenia.[31] In Japan, HHV-8 infection in HIV-1-negative subjects is rare; as a result, when the 12 subjects with autoimmune thrombocytopenia were examined, the association between HHV-8 and autoimmune thrombocytopenia in HIV-1-negative subjects was not distinct. However, the fact that HHV-8 DNA was not detected in the HIV-1-negative subjects with autoimmune thrombocytopenia suggests that HHV-8 is not a main causative agent of autoimmune thrombocytopenia.

In conclusion, a significant association was observed between HHV-8 infection and the clinical appearance of HIV-1 infection, especially in regard to mild thrombocytopenia, hypergammaglobulinemia, and disease severity. It is possible that some of these effects of HHV-8 infection are related to other independent etiological cofactor or some other viruses. However, it may be useful to monitor HHV-8 DNA in leukocytes of HIV-1-positive subjects to assess and predict disease severity and to select the optimal treatment modalities.

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Disclosure Statement

No competing financial interests exist.

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HAARTによる脂質代謝異常と高分子アディポネクチンの関連

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国立病院機構九州医療センター 免疫感染症科 ほか

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ATVへの変更はHMW-Ad濃度を回復させる

メタボリックシンドロームにおける病態の一部は，成熟脂肪細胞から分泌されるアディポサイトカインによって説明できることが明らかになってきた。特に活性体である高分子量アディポネクチン(HMW-Ad)は，インスリン抵抗性改善作用，抗動脈硬化作用を有するため重要視されている。そこで，抗HIV薬の副作用として起こってくる代謝障害において，HMW-Adがどのように関与しているかを検討した。

当院通院中のHIV感染者57例を，HAARTのキードラッグ別にエファビレンツ(EFV)群，アタザナビル(ATV)以外のプロテアーゼ阻害薬(PI)群，ATV群，他剤からATVへの変更群に分け，HAART開始前，開始後もしくは薬剤変更後1年以内に血清脂質とHMW-Adを測定した。

EFV群とPI群では中性脂肪(TG)と低比重リポ蛋白コレステロール(LDL C)が増加し，HMW Ad濃度が低下したが，ATV群では有意な影響を認めなかった。また，他剤からATVへの変更群ではLDL-Cが低下，HMW-Ad濃度が上昇し，その差は有意であった(**表1**，**図1**)。リポジストロフィーを有する患者では，HMW-Adの変化率が大きい傾向にあり，両者の関連性が認められた(**図2**)。

抗HIV薬による脂肪細胞減少がHMW-Ad低下の一因

*In vitro*において，脂肪前駆細胞(3T3-L1)にイソブチルメチルキサンチン，デキサメタゾン，インスリンを添加して分化誘導し，分化前・分化後に抗HIV薬

表1　BMI，脂質の変化

	BMI 前	BMI 後	TG 前	TG 後	LDL-C 前	LDL-C 後	HDL-C 前	HDL-C 後
EFV	21.4±3.3	21.2±3.6	144.4±36.8	233.5±125.4**	108.9±35.4	122.0±46.2**	42.8±16.1	53.5±11.5**
PI	21.6±2.9	21.7±3.2	151.1±60.0	231.9±105.6*	95.7±41.3	128.5±40.3*	37.6±11.5	46.9±7.4**
ATV	22.3±3.0	22.8±3.4	165.1±104.8	174.4±85.4	88.5±16.1	104.3±23.2*	38.7±11.3	41.0±8.0
他剤→ATV	21.1±2.9	20.7±2.5	188.4±92.0	189.1±111.3	120.7±61.5	101.5±52.0**	54.6±28.9	55.1±52.0

*$p < 0.001$ **$p < 0.005$ (paired t 検定)

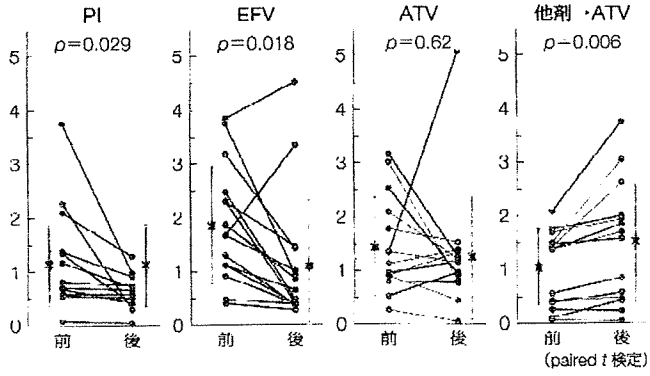

図1　HMW-Adの変化

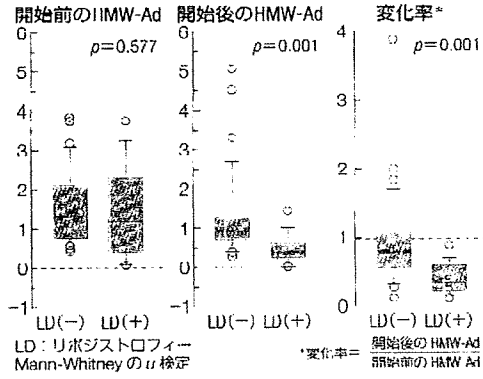

図2　リポジストロフィーとの関連