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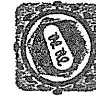

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Functional impairment of Tax-specific but not cytomegalovirus-specific CD8$^+$ T lymphocytes in a minor population of asymptomatic human T-cell leukemia virus type 1-carriers

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Abstract

Background: Human T-cell leukemia virus type 1 (HTLV-1) causes adult T-cell leukemia (ATL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) in a small percentage of infected individuals. ATL is often associated with general immune suppression and an impaired HTLV-1-specific T-cell response, an important host defense system. We previously found that a small fraction of asymptomatic HTLV-1-carriers (AC) already showed impaired T-cell responses against the major target antigen, Tax. However, it is unclear whether the impaired HTLV-1 Tax-specific T-cell response in these individuals is an HTLV-1-specific phenomenon, or merely reflects general immune suppression. In this study, in order to characterize the impaired HTLV-1-specific T-cell response, we investigated the function of Tax-specific CD8$^+$ T-cells in various clinical status of HTLV-1 infection.

Results: By using tetramers consisting of HLA-A*0201, -A*2402, or -A*1101, and corresponding Tax epitope peptides, we detected Tax-specific CD8$^+$ T-cells in the peripheral blood from 87.0% of ACs (n = 20/23) and 100% of HAM/TSP patients (n = 18/18) tested. We also detected Tax-specific CD8$^+$ T-cells in 38.1% of chronic type ATL (cATL) patients (n = 8/21), although its frequencies in peripheral blood CD8$^+$ T cells were significantly lower than those of ACs or HAM/TSP patients. Tax-specific CD8$^+$ T-cells detected in HAM/TSP patients proliferated well in culture and produced IFN-γ when stimulated with Tax peptides. However, such functions were severely impaired in the Tax-specific CD8$^+$ T-cells detected in cATL patients. In ACs, the responses of Tax-specific CD8$^+$ T-cells were retained in most cases. However, we found one AC sample whose Tax-specific CD8$^+$ T-cells hardly produced IFN-γ, and failed to proliferate and express activation (CD69) and degranulation (CD107a) markers in response to Tax peptide. Importantly, the same AC sample contained cytomegalovirus (CMV) pp65-specific CD8$^+$ T-cells that possessed functions upon CMV pp65 peptide stimulation. We further examined additional samples of two smoldering type ATL patients and found that they also showed dysfunctions of Tax-specific but not CMV-specific CD8$^+$ T-cells.

Conclusions: These findings indicated that Tax-specific CD8$^+$ T-cells were scarce and dysfunctional not only in ATL patients but also in a limited AC population, and that the dysfunction was selective for HTLV-1-specifc CD8$^+$ T-cells in early stages.

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Background

Human T-cells leukemia virus type 1 (HTLV-1) is the causative agent of a highly aggressive $CD4^+$ T-cell malignancy, adult T-cell leukemia (ATL)[1,2]. As many as 10 million individuals are thought to be infected worldwide, in southern Japan, the Caribbean basin, South America, Melanesia, and equatorial Africa[3]. Unlike human immunodeficiency virus (HIV), the majority of HTLV-1-infected individuals are clinically asymptomatic during their lifetime. However, approximately 5% develop ATL, and another 2-3% develop a variety of chronic inflammatory diseases such as HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP)[4-8].

HTLV-1-specific cytotoxic T-lymphocytes (CTLs) are thought to play a pivotal role in containing the proliferation of HTLV-1-infected T-cells[9,10]. Tax is known to be the dominant target antigen for HTLV-1-specific CTLs[10-13], and a high frequency of Tax-specific CTLs can be detected in HAM/TSP patients and some asymptomatic HTLV-1 carriers (ACs)[10-14]. However, ATL patients show general immune suppression[15], reduced frequency and dysfunction of Tax-specific CTLs[16,17]. Regulatory T cell (Treg)-like function of $FoxP3^+$ ATL cells and diminished function of dendritic cells may be involved in the immune suppression in ATL patients [18,19], but the precise mechanism is not yet clarified. We previously demonstrated that a fraction of ACs also exhibit reduced T-cell responses against Tax protein [20]. These observations suggest that the reduced HTLV-1-specific T-cell response might be an underlying risk of ATL development, but not the result of ATL. However, it is unknown how the function of HTLV-1-specific $CD8^+$ T-cells becomes impaired in a small percentage of ACs and whether its dysfunction is specific for HTLV-1 antigen or due to general immune suppression.

During chronic stage of infection with several viruses, such as HIV and hepatitis C virus (HCV), virus-specific CTLs gradually lose their cytotoxic activity, the ability to proliferate and secrete a diverse profile of cytokines, ultimately leading to exhaustion, anergy or even deletion of these cells[21-26]. Programmed death-1 (PD-1), a negative regulator in the CD28 superfamily, has recently been shown to be highly expressed on virus-specific T-cells during many chronic viral infections[27-29]. It has also been reported that the interaction of PD-1 with PD-ligand 1 (PD-L1) negatively regulates cytokine production and proliferation of T-cells[30,31]. A previous report indicates that PD-1 is up-regulated on the dominant Tax-specific CTLs in ATL patients and ACs and that immune regulation through the PD-1/PD-L1 pathway may be involved in the dysfunction of HTLV-1-specific CTLs in ATL patients[32].

Studies on memory T-cell differentiation have shown that phenotype, function, and homeostasis of memory T-cells vary for different persistent virus infections[33]. Central memory T-cells (T_{CM}; $CD45RA^-CCR7^+$) are elicited by non-persisting virus that provide transient antigen stimulation, such as in Influenza virus infection. In contrast, effector memory T-cells (T_{EM}; $CD45RA^-CCR7^-$) predominate when relatively high levels of antigen persist, such as in HIV infection. Terminally differentiated memory (T_{Diff}; $CD45RA^+CCR7^-$) can be seen when antigen persists at a low level, such as in cytomegalovirus (CMV) infection. In HTLV-1 infection, it has been reported that dominant Tax-specific CTLs in HAM/TSP patients consist of T_{EM} and T_{Diff} compartments[34].

We previously identified some major epitopes recognized by HTLV-1-specific CTLs in infected individuals carrying HLA-A2, -A11, or -A24[12,35,36]. These allowed us to monitor HTLV-1-specific CTLs and analyze their functions *ex vivo*, by using antigen/HLA tetrameric complexes. In this study, we demonstrate that IFN-γ production and proliferative capacity of tetramer-binding Tax-specific $CD8^+$ T-cells were severely impaired not only in ATL patients but also in a minor population of asymptomatic HTLV-1 carriers (ACs). Importantly, the T-cell dysfunction at the asymptomatic stage was selective for HTLV-1 but not for CMV antigen. In addition, severely impaired HTLV-1-specific but not CMV-specific $CD8^+$ T-cells responses were also observed in patients diagnosed as smoldering ATL, the clinical condition of which is close to that of AC. The dysfunction of HTLV-1-specific $CD8^+$ T-cells in an early clinical stage implies HTLV-1-specific immune suppressive mechanism might be an underlying risk for ATL.

Results

Incidence and frequency of Tax-specific $CD8^+$ T-cells in ACs, and HAM/TSP and cATL patients

In 23 ACs and 18 HAM/TSP and 21 cATL patients carrying HLA-A2, -A11 and/or -A24 alleles, we evaluated the frequencies of Tax-specific $CD8^+$ T-cells by using cognate Tax/HLA tetramers (Figure 1 and Table 1). Tax-specific $CD8^+$ T-cells were detected in 87.0% of ACs and all HAM/TSP patients tested. In contrast, only 38.1% of cATL patients have detectable frequencies of Tax-specific $CD8^+$ T-cells (Table 1). Figure 1B shows that the average frequency of Tax-specific $CD8^+$ T-cells in the $CD8^+$ T-cells of cATL patients (n = 21, 0.90% range: 0%-9.45%) was significantly lower than that in ACs (n = 23, 2.37%, range: 0%-8.23%, P = 0.0023). HAM/TSP patients had the highest average frequency of Tax-specific $CD8^+$ T-cells among the three groups (n = 18, 8.88%, range: 1.86%-29.9%, P = 0.0001; vs. AC, P < 0.0001; vs. cATL patients), which is consistent with

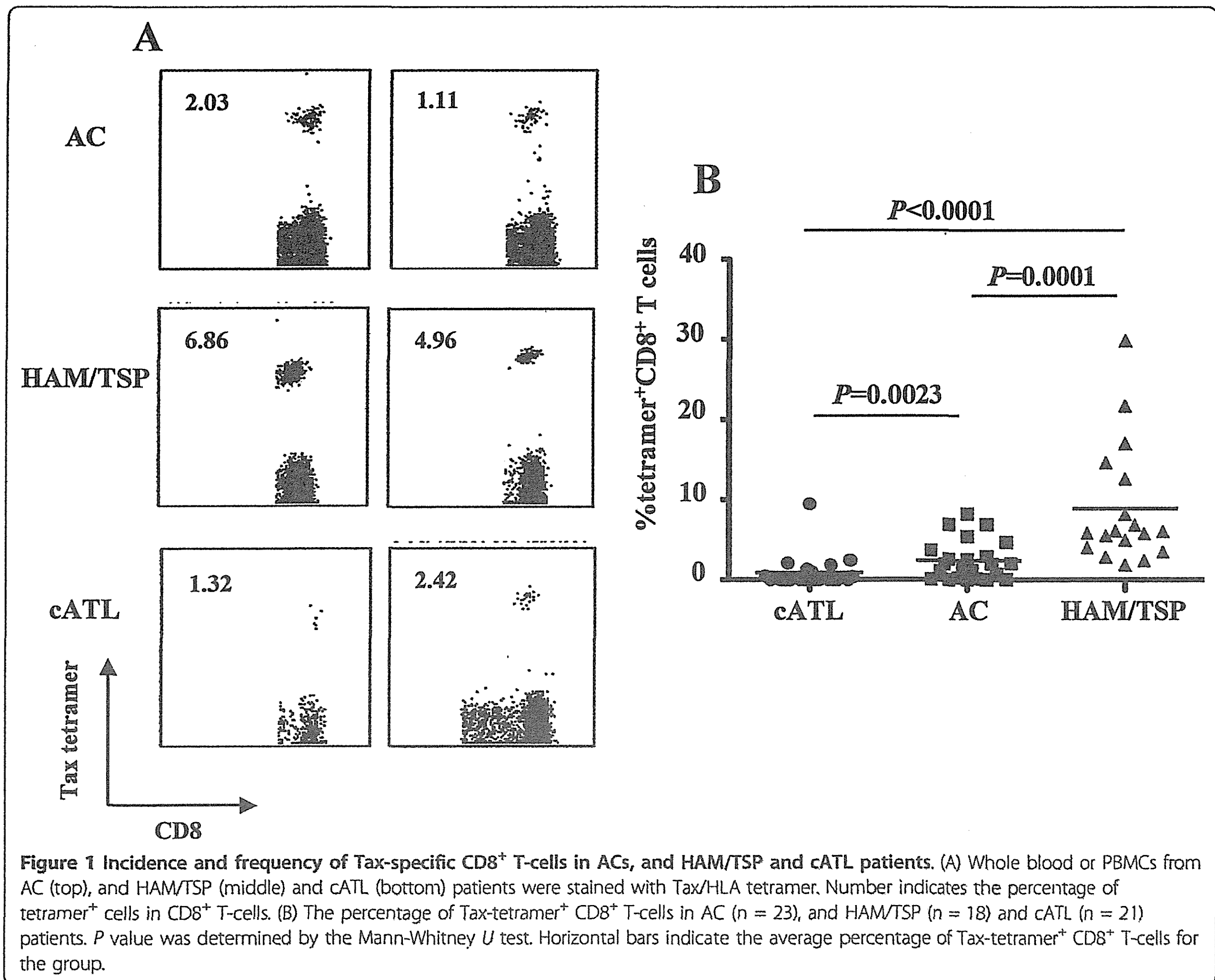

Figure 1 Incidence and frequency of Tax-specific CD8⁺ T-cells in ACs, and HAM/TSP and cATL patients. (A) Whole blood or PBMCs from AC (top), and HAM/TSP (middle) and cATL (bottom) patients were stained with Tax/HLA tetramer. Number indicates the percentage of tetramer$^+$ cells in CD8$^+$ T-cells. (B) The percentage of Tax-tetramer$^+$ CD8$^+$ T-cells in AC (n = 23), and HAM/TSP (n = 18) and cATL (n = 21) patients. *P* value was determined by the Mann-Whitney *U* test. Horizontal bars indicate the average percentage of Tax-tetramer$^+$ CD8$^+$ T-cells for the group.

previous reports [10,17,37]. It is of note that Tax-specific CD8$^+$ T-cells are detectable even in cATL patients, although the frequency is very low.

Impaired cell proliferation and IFN-γ production of Tax-specific CD8$^+$ T-cells in cATL but not HAM/TSP patients

We next examined IFN-γ production and cell proliferation of Tax-specific CD8$^+$ T-cells in HAM/TSP and cATL patients (Figure 2A). Intracellular IFN-γ staining showed that Tax-specific CD8$^+$ T-cells in all HAM/TSP patients tested produced IFN-γ when stimulated with Tax peptide (Figure 2A). Tax-specific CD8$^+$ T-cells in those HAM/TSP patients proliferated regardless of stimulation with Tax peptide (Figure 2B). In contrast to HAM/TSP patients, IFN-γ production from Tax-specific CD8$^+$ T-cells in a cATL patient was hardly detectable even when stimulated with Tax peptide (4.8%, Figure 2A). In the same donor, Tax-specific CD8$^+$ T-cells

Table 1 The number of blood samples with detectable Tax-specific CD8$^+$ T-cells in all samples tested in this study

Tax/HLA tetramers used in this study	Disease Status		
	AC	HAM/TSP	cATL
HLA-A*0201/Tax11-19	12/14[1]	7/7	2/11
HLA-A*1101/Tax88-96	4/4	4/4	3/5
HLA-A*2402/Tax301-309	13/15	13/13	5/16
No. of tetramer$^+$ samples/total no. of blood samples[2]	20/23 (87.0%)	18/18 (100%)	8/21 (38.1%)

[1] No. of samples with detectable Tax-specific CD8$^+$ T-cells/total no. of samples carrying each HLA allele. When the frequency of tetramer$^+$ cells was more than 0.04% of CD8$^+$ T-cells, the sample was regarded as detectable.

[2] In case Tax-specific CD8$^+$ T-cells was detectable by either tetramer in a sample carrying two of three HLA-A alleles above, the sample was regarded as positive.

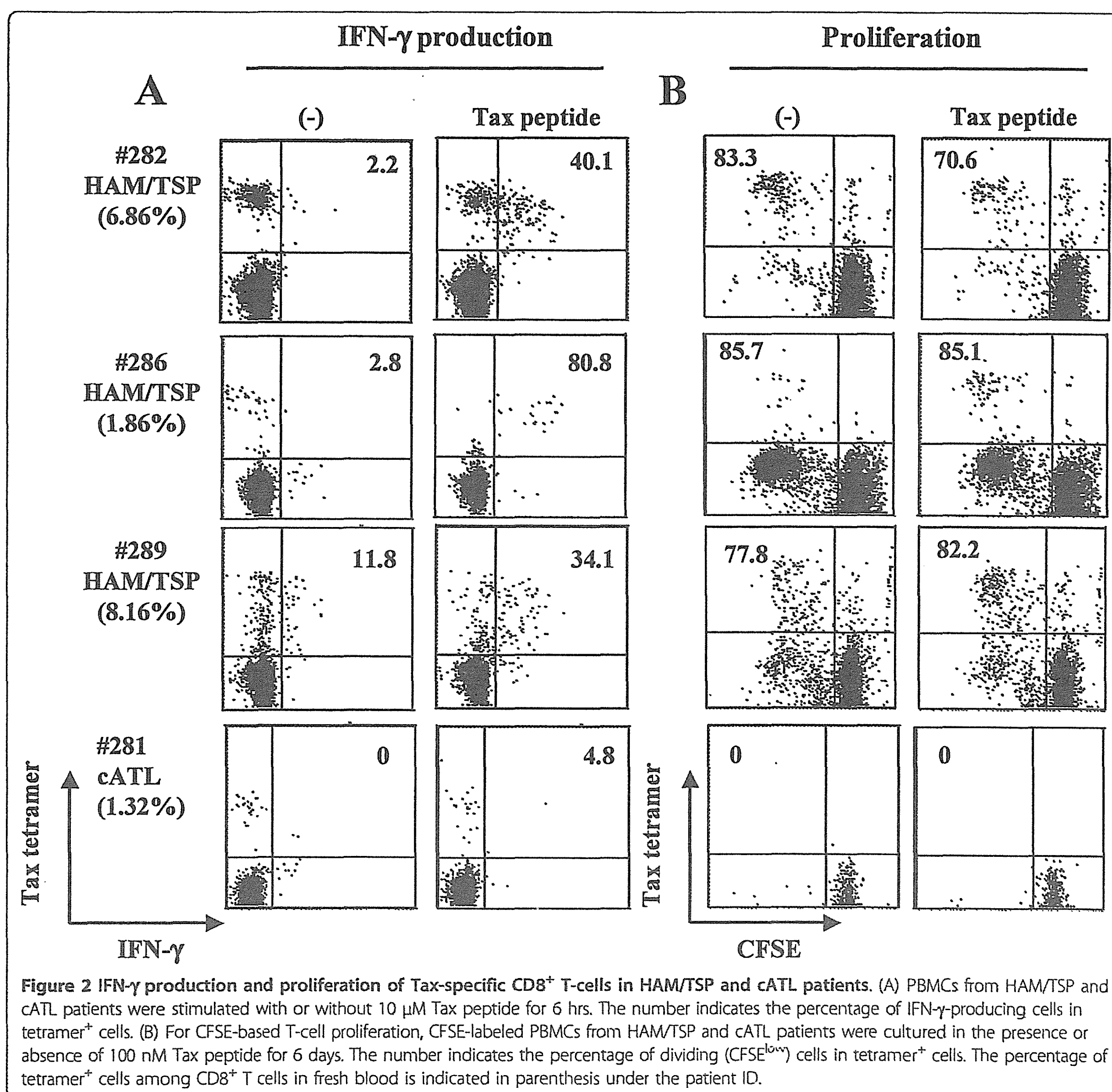

Figure 2 IFN-γ production and proliferation of Tax-specific CD8$^+$ T-cells in HAM/TSP and cATL patients. (A) PBMCs from HAM/TSP and cATL patients were stimulated with or without 10 μM Tax peptide for 6 hrs. The number indicates the percentage of IFN-γ-producing cells in tetramer$^+$ cells. (B) For CFSE-based T-cell proliferation, CFSE-labeled PBMCs from HAM/TSP and cATL patients were cultured in the presence or absence of 100 nM Tax peptide for 6 days. The number indicates the percentage of dividing (CFSE^{low}) cells in tetramer$^+$ cells. The percentage of tetramer$^+$ cells among CD8$^+$ T cells in fresh blood is indicated in parenthesis under the patient ID.

could be detected in fresh blood (1.32%) and after 6 hrs incubation as shown in Figure 2A, but not after 6 day-culture, suggesting that Tax-specific CD8$^+$ T-cells in this cATL patient had no proliferative capacity (Figure 2B). We tested PBMC from four other cATL patients who had detectable Tax-specific CD8$^+$ T-cells, but none of them showed proliferation of Tax-specific CD8$^+$ T-cells by either the CFSE-based proliferation assay or 13-day culture (Additional file 1). Collectively, these results indicate that Tax-specific CD8$^+$ T-cells from most cATL patients are impaired in their capacities to proliferate and produce IFN-γ.

Diversity in the IFN-γ production and cell proliferation of Tax-specific CD8$^+$ T-cells in ACs

Our recent studies using the GST-Tax protein-based assay demonstrated that the extent of Tax-specific T-cell responses varied widely in ACs[20]. We then evaluated proliferation and/or IFN-γ production of tetramer-binding Tax-specific CD8$^+$ T-cells in 14 ACs (Table 2). Representative data on 4 of 14 ACs are shown in Figures 3A and 3B. In 3 ACs (#251, #313, and #360), Tax-specific CD8$^+$ T-cells produced IFN-γ and proliferated in response to Tax peptide (Figures 3A and 3B). Similarly to HAM/TSP samples, a large proportion of Tax-

Table 2 Clinical information and summary for Tax-specific CD8⁺ T cells in 14ACs

ID	Age	Sex	WBC (/μl)	CD4 (%)[1]	CD8 (%)[1]	HLA	Tetramer (%)[2]	Functions and phenotype of Tax-specific CD8+ T-cells[3]			Ably (%)[7]	PVL[8]
								IFN-γ+ (%)[4]	CFSE^low (%)[5]	PD-1+ (%)[6]		
#217	70s	F	6800	ND[9]	5.72	A24	1.94	27.7	78.9	78.7	0	14
#236	30 s	F	6500	ND	11.9	A24	2.54	31.1	0	54.1	0	22
#238	60 s	F	5700	ND	12.7	A11	1.29	36.4	100	0	0	2
#243	50 s	F	4100	ND	24.6	A2/24	0.39/3.67	11.3	27.6	93.8	0	3
#245	40 s	F	5000	ND	22.6	A2	0.73	62.5	75	ND	1	58
#251	60 s	M	4800	ND	11.9	A2/11	0.70/8.23	35.8	84.4	36.7	0	2
#279	40 s	M	6200	34.1	11.6	A2/24	4.70/0.18	12.9	30.8	70.2	1	48
#287	70 s	M	4800	72.5	10.0	A2/24	1.17/0.23	11.1	0	55.6	2	81
#309	60 s	F	4600	37.5	24.8	A11/24	6.88/4.26	51.7	76.2	85.3	1.5	29
#311	60 s	F	3200	30.6	14.8	A2/24	1.02/1.94	51.3	ND	ND	0	6
#312	50 s	F	2700	27.3	36.4	A24	2.03	77.8	ND	ND	ND	UN[10]
#313	60 s	M	7300	25.4	31.0	A24	1.11	55.7	60	90.6	ND	4
#315	50 s	F	7500	26.5	7.9	A2/24	6.88/0	24.5	84.7	20	0.6	17
#360	50 s	M	6200	37.7	29.9	A2	2.6	63.1	68.4	10.2	0	UN

[1]The number indicates percentage of CD4⁺ or CD8⁺ T cells in lymphocytes.

[2]The number indicates percentages of tetramer⁺ cells in CD8⁺ T-cells. Two numbers divided by a slash represent those detected by two different tetramers corresponding to two HLA alleles shown in the HLA column.

[3]In case of a sample carrying two of three HLA-A alleles (A2, A11, or A24), Tax-specific CTLs predominantly detected by a tetramer were used. The number represents percentage of indicated cells in the tetramer-binding CD8⁺ T cells.

[4]Evaluated by intracellular IFN-γ staining following 6 hours stimulation with corresponding Tax peptide.

[5]Evaluated by CFSE intensities in labeled PBMC after 6 days incubation with corresponding Tax peptide stimulation.

[6]The number represents percentage of indicated PD-1⁺Tax-specific CD8⁺ T cells without culture.

[7]Ably; abnormal lymphocytes

[8]PVL; proviral load. The number represents copy number per 1000 PBMCs.

[9]ND; not determined

[10]UN; undetectable

specific CD8⁺ T-cells in these ACs spontaneously proliferated without stimulation with Tax peptide, probably due to viral reactivation in HTLV-1-infected cells *in vitro*[38,39]. IFN-γ production was specifically detected for peptide stimulation, and 35.8-55.7% of Tax-specific CD8⁺ T-cells produced a good amount of IFN-γ (mean fluorescence intensity, MFI: 63.7-195.3) upon stimulation in the samples of #251, #313, and #360. In contrast, Tax-specific CD8⁺ T-cells in one AC (#287) did not proliferate in response to Tax peptide and showed a very weak IFN-γ response with low amounts of IFN-γ (MFI: 37.5) in a low percentage (11.1%) of Tax-specific CD8⁺ T-cells (Figures 3A and 3B). In other ACs (#243 and #279), low frequency of IFN-γ⁺ Tax-specific CD8⁺ T-cells was observed, but the levels of IFN-γ production (MFI: #243; 58.8, #279; 77.6) and the proliferative responses were comparable to other ACs (Table 2). Tax-specific CD8⁺ T-cells in #236 failed to proliferate but showed favorable IFN-γ production (MFI: 80.1) in 31.1% of the cells.

Among AC samples tested, AC#287 carried higher proviral load (81 copies in 1000 PBMCs) than any other ACs (Table 2). Since Tax-specific CD8⁺ T-cells in #287 had severely impaired IFN-γ production and proliferative potential, we examined the relationship of the function of these T-cells with proviral loads. Both percentages of IFN-γ⁺ and dividing Tax-specific CD8⁺ T-cells among CD8⁺ T-cells were likely to be inversely correlated with proviral loads although they were not statistically significant (Figure 3C and 3D). Because of the limited availability of the samples, we focused mainly on two ACs (#287 and #313) in the studies hereafter.

Dysfunction of Tax-specific CD8⁺ T-cells and inefficient CD8⁺ cell-mediated HTLV-1 control in AC #287

To examine whether Tax-specific CD8⁺ T-cell responses were influenced by activation of antigen-presenting cells (APCs), PBMC from #313 (responder) and #287 (low responder) were stimulated with Tax peptide in the presence or absence of LPS, a potent activator of APCs such as dendritic cells (DCs) and monocytes/macrophages. In #313, the frequency of Tax-specific CD8⁺ T-cells increased from 1.11% to 6.47% or 4.07% at day 13, after stimulation with or without Tax peptide, respectively. The frequency of Tax-specific CD8⁺ T-cells in #313 further increased in the presence of Tax peptide and LPS (15.81%). In contrast to #313, the frequency of Tax-specific CD8⁺ T-cells in #287 decreased from 1.17%

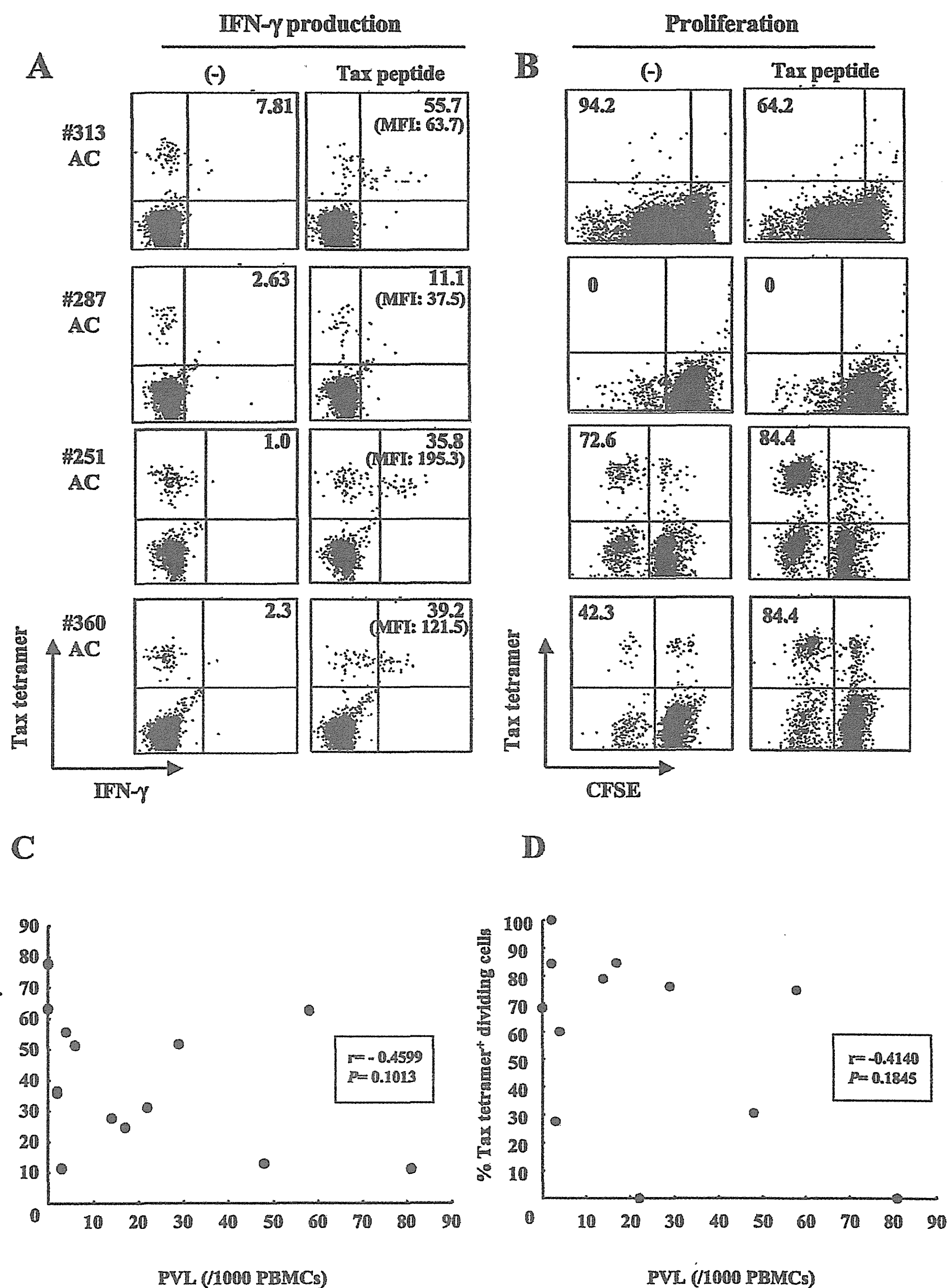

Figure 3 IFN-γ production and cell proliferation of Tax-specific CD8^{+} T-cells in ACs. (A, B) IFN-γ production (A) and cell proliferation (B) of Tax-specific CD8^{+} T-cells in PBMCs from 4 ACs were assessed as in Figure 2. The number given in parenthesis shows mean fluorescence intensity (MFI) of IFN-γ expression in the IFN-γ^{+} tetramer^{+} cells. (C, D) Relation between the percentage of IFN-γ^{+} (C) or dividing (D) Tax-specific CD8^{+} T-cells and proviral loads (PVL) in ACs. Dots represent individual ACs. The Spearman rank correlation test was used to determine correlations and *P* values.

to 0.2% after stimulation with Tax peptide, and was not recovered by LPS stimulation (Figure 4A). In addition, HTLV-1-infected cells have been reported to express C-C chemokine receptor type 4 (CCR4) and have $FoxP3^+$ Treg-like function[18,40]. However, the proliferative ability of Tax-specific $CD8^+$ T-cells in #287 was not restored even in the absence of $CCR4^+$ infected cells (data not shown).

To further examine the function of Tax-specific $CD8^+$ T-cells in #313 and #287, we observed the expression of CD69, an early activation marker transiently expressed on T lymphocytes that precedes cytokine secretion after antigenic stimulation, and CD107a, a marker of degranulation associated with cytotoxic activity in an antigen-specific manner[41]. CD69 was up-regulated on Tax-specific $CD8^+$ T-cells in #313 when stimulated with Tax peptide, but not in #287, which was in agreement with their abilities to produce IFN-γ (Figure 4B). In #313, 22.4% of Tax-specific $CD8^+$ T-cells mobilized CD107a to the surface during a 6-hr culture with Tax peptide stimulation, while CD107a surface expression was detected on 4% of Tax-specific $CD8^+$ T-cells in the culture without stimulation (Figure 4C). However, no CD107a mobilization was detected on the surface of Tax-specific $CD8^+$ T-cells in #287 with or without Tax peptide stimulation (Figure 4C). These results indicate that HTLV-1-specific $CD8^+$ T-cells in AC #287 did not properly activate upon antigen stimulation, and therefore failed to control HTLV-1-infected cells.

The Tax/HLA tetramers used in this study allow us to evaluate the functions of $CD8^+$ T-cells only against an immunodominant epitope, Tax. We therefore compared HTLV-1 Gag p19 in the culture between whole and $CD8^+$ cell-depleted PBMCs to examine the role of total HTLV-1-specific $CD8^+$ T-cells including the dominant Tax-specific $CD8^+$ T-cells, in suppression of HTLV-1 production from infected cells (Figure 4D). As expected, depletion of $CD8^+$ cells from PBMCs in #313 led to significantly higher HTLV-1 production compared to whole PBMCs ($P = 0.0115$). In contrast, HTLV-1 p19 production increased only a little in the culture of $CD8^+$ cell-depleted PBMCs in #287 ($P = 0.1563$), indicating that HTLV-1-specific $CD8^+$ T-cells other than the dominant Tax-specific $CD8^+$ T-cells might have a reduced ability to control the infected cells in this donor. It is of note that HTLV-1-infected cells from both two donors carried intact HTLV-1 proviral genomic DNA because HTLV-1 p19 could be detected after 7 day-culture.

Phenotypic analysis of functional and dysfunctional Tax-specific $CD8^+$ T-cells

We next characterized the differentiation status of memory T-cells in Tax-specific $CD8^+$ T-cells. Human CD8 T-cells may be classified as naïve T-cells ($CD45RA^+CCR7^+CD27^+$), T_{CM} ($CD45RA^-CCR7^+CD27^+$), T_{EM} ($CD45RA^-CCR7^-CD27^+$), and T_{Diff} ($CD45RA^+CCR7^-CD27^-$) cells[42-44]. As shown in Figure 5A, almost all Tax-specific $CD8^+$ T-cells in both #313 and #287 were skewed to $CD45RA^-CCR7^-CD27^+$ T_{EM} cells, and there was no essential difference between two donors.

A previous report has shown that PD-1 was highly up-regulated on Tax-specific $CD8^+$ T-cells in ATL patients and ACs[32]. We therefore examined PD-1 expression on Tax-specific $CD8^+$ T-cells in several AC samples, including #287. The frequency of $PD-1^+$ Tax-specific $CD8^+$ T-cells was very high in #309 (85.3%) and #313 (96%) (Figure 5B and Table 2) while those Tax-specific $CD8^+$ T-cells retained the proliferative and the cytokine-producing abilities (Figure 3A and Table 2). In #287, the frequency of PD-1-expressing Tax-specific $CD8^+$ T-cells (55.6%) was lower than #309 and #313, but higher than that of $PD-1^+$ CMVpp65-specific $CD8^+$ T-cells in the same donor (Figure 5B). The levels of PD-1 expression showed a similar tendency to the frequency of $PD-1^+$ T-cells. In addition, the blockade of PD-1/PD-ligand 1 (PD-L1) pathway did not restore the proliferative capacity of Tax-specific $CD8^+$ T-cells in #287 (data not shown).

Conserved functions of CMV-specific $CD8^+$ T-cells in #287

We next examined whether the impairment of proliferative capacity and effector functions observed in #287 $CD8^+$ T-cells were specific for HTLV-1 antigens or the result of general immune suppression. PBMC from #287 contained CMVpp65-specific $CD8^+$ T-cells (2.3% of $CD8^+$ T-cells), as detected by tetramer staining. The frequency of CMVpp65-specific $CD8^+$ T-cells increased from 2.3% to 66.0% following in vitro CMVpp65 peptide stimulation, but not without the peptide stimulation (Figure 6A). Antigen-specific IFN-γ and CD69 expression were clearly detected in CMVpp65-specific $CD8^+$ T-cells in #287 (Figures 6B and 6C). Furthermore, CMVpp65-specific $CD8^+$ T-cells mobilized CD107a to the surface in response to CMVpp65 peptide (Figure 6D). These results demonstrate that in #287, CMVpp65-specific $CD8^+$ T-cells, but not Tax-specific $CD8^+$ T-cells, have proliferative potential and effector functions, such as cytotoxic activity and IFN-γ release, suggesting that the impaired $CD8^+$ T-cell function in #287 was specific for HTLV-1.

Dysfunction of Tax-specific but not CMVpp65-specific $CD8^+$ T-cells also in sATL patients

Finally, we extended the study to see whether patients with early stage ATL might exhibit similar dysfunction selective for HTLV-1-specific $CD8^+$ T-cells. We found two smoldering ATL (sATL) patients (#110 and #353)

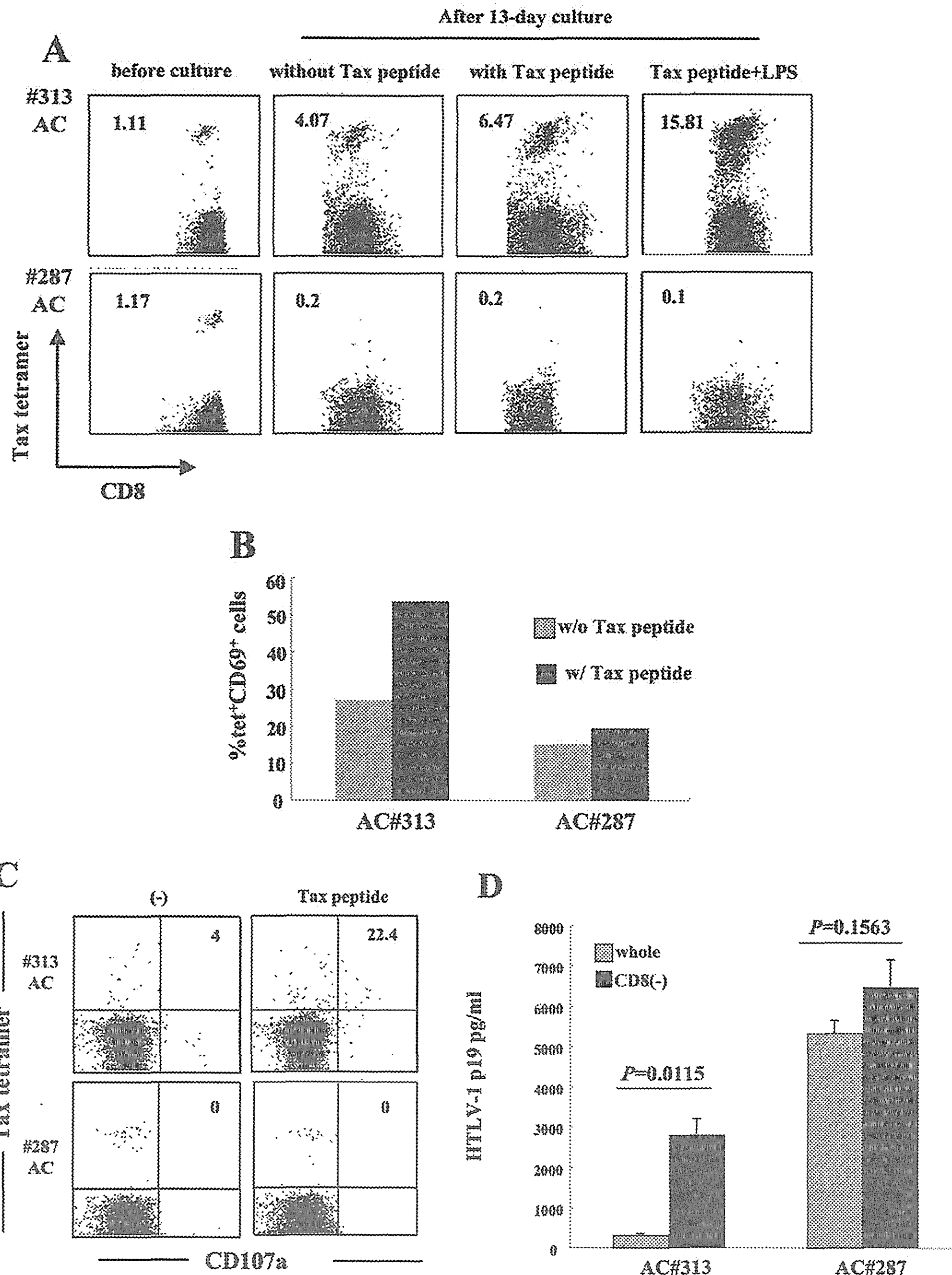

Figure 4 Dysfunction of Tax-specific CD8$^+$ T-cells and inefficient CD8$^+$ cell-mediated HTLV-1 control in AC#287. (A) For antigen-specific T-cell proliferation, PBMCs from #313 and #287 were cultured for 13 days with or without Tax peptide in the presence or absence of 0.1 μg/ml LPS. The number indicates the percentage of tetramer$^+$ cells in CD8$^+$ T-cells. (B, C) PBMCs were stimulated with or without 10 μM Tax peptide for 6 hrs. The expression of CD69 (B) and CD107a (C) in Tax-specific CD8$^+$ T-cells was analyzed by flow cytometry. (B) Bar indicates the percentage of CD69$^+$ cells in Tax-specific CD8$^+$ T-cells. (C) The number represents the percentage of CD107a$^+$ cells in Tax-specific CD8$^+$ T-cells. (D) Whole PBMCs and CD8-depleted fractions in ACs (#287 and #313) were cultured for 7 days and HTLV-1 p19 in the supernatants were measured by HTLV-1 p19 ELISA. *P* value was determined by the unpaired *t* test.

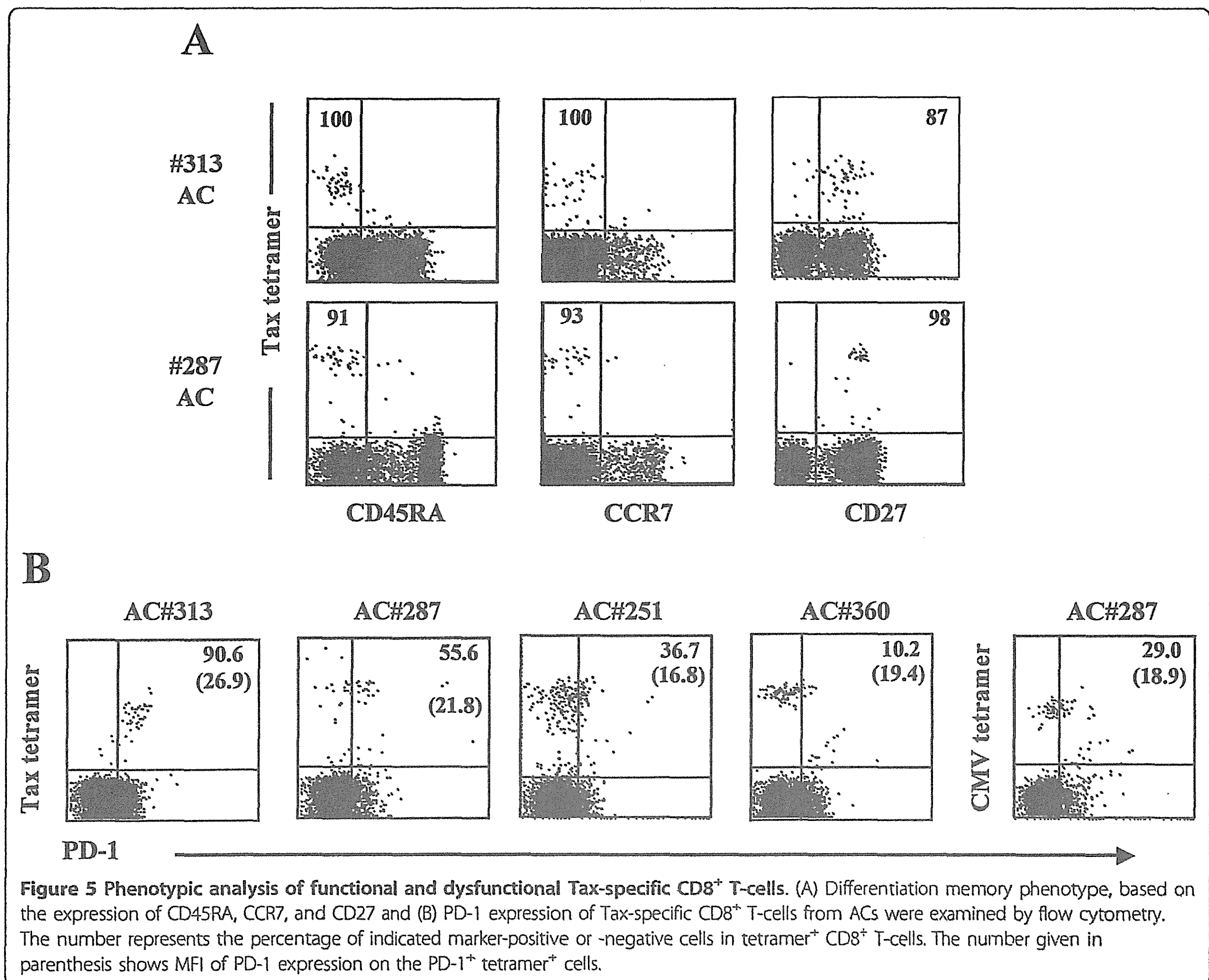

Figure 5 Phenotypic analysis of functional and dysfunctional Tax-specific CD8$^+$ T-cells. (A) Differentiation memory phenotype, based on the expression of CD45RA, CCR7, and CD27 and (B) PD-1 expression of Tax-specific CD8$^+$ T-cells from ACs were examined by flow cytometry. The number represents the percentage of indicated marker-positive or -negative cells in tetramer$^+$ CD8$^+$ T-cells. The number given in parenthesis shows MFI of PD-1 expression on the PD-1$^+$ tetramer$^+$ cells.

possessing 6.89% and 3.15% of tetramer-binding Tax-specific CD8$^+$ T-cells, respectively. The sATL patient #353 carried 5% of abnormal lymphocytes (ably) with a normal range of lymphocyte number, whose status is very close to the borderline with ACs. Patient #110 carried 4% of abnormal lymphocytes with mild lymphocytosis. Tax-specific CD8$^+$ T-cells of two sATL patients (#110 and #353) did not proliferate in response to Tax peptides as similarly observed in a cATL patient (#224) (Figure 7A) and most other cATL patients (Figure 2A and Additional file 1). In contrast, CMVpp65-specific CD8$^+$ T-cells in both sATL patients vigorously proliferated when stimulated with CMVpp65 peptides. CMVpp65-specific CD8$^+$ T-cells in a cATL (#224) also proliferated, but to a lesser degree, which might reflect general immune suppression in this patient (Figure 7).

Discussion

In this study, we detected Tax-specific CD8$^+$ T-cells in 87%, but not the rest of ACs tested, by using tetramers containing Tax major epitope-peptides presented by HLA-A*0201, A*1101, and A*2402. Tax-specific CD8$^+$ T-cells were also detected in 38% of cATL patients, but at reduced frequencies and with severely impaired functions. Further analysis of Tax-specific CD8$^+$ T-cells in 14 ACs indicated that they were functional in most of ACs tested except one (#287), whose Tax-specific CD8$^+$ T-cells poorly responded to specific peptides. However, CMVpp65-specific CD8$^+$ T-cells of this individual were fully functional. Similar T-cell dysfunction selective for HTLV-1, but not CMV, was also observed in sATL patients, one of which (#353) had no clinical symptoms but 5% abnormal lymphocytes. General immune suppression might partly account for the scarcity and/or the dysfunction of Tax-specific CD8$^+$ T-cells in ATL patients, but not those in the AC or the sATL patients as they were selective for HTLV-1. These findings suggest that HTLV-1-specific immune suppression is undergoing in a minor group of ACs and an early stage of ATL.

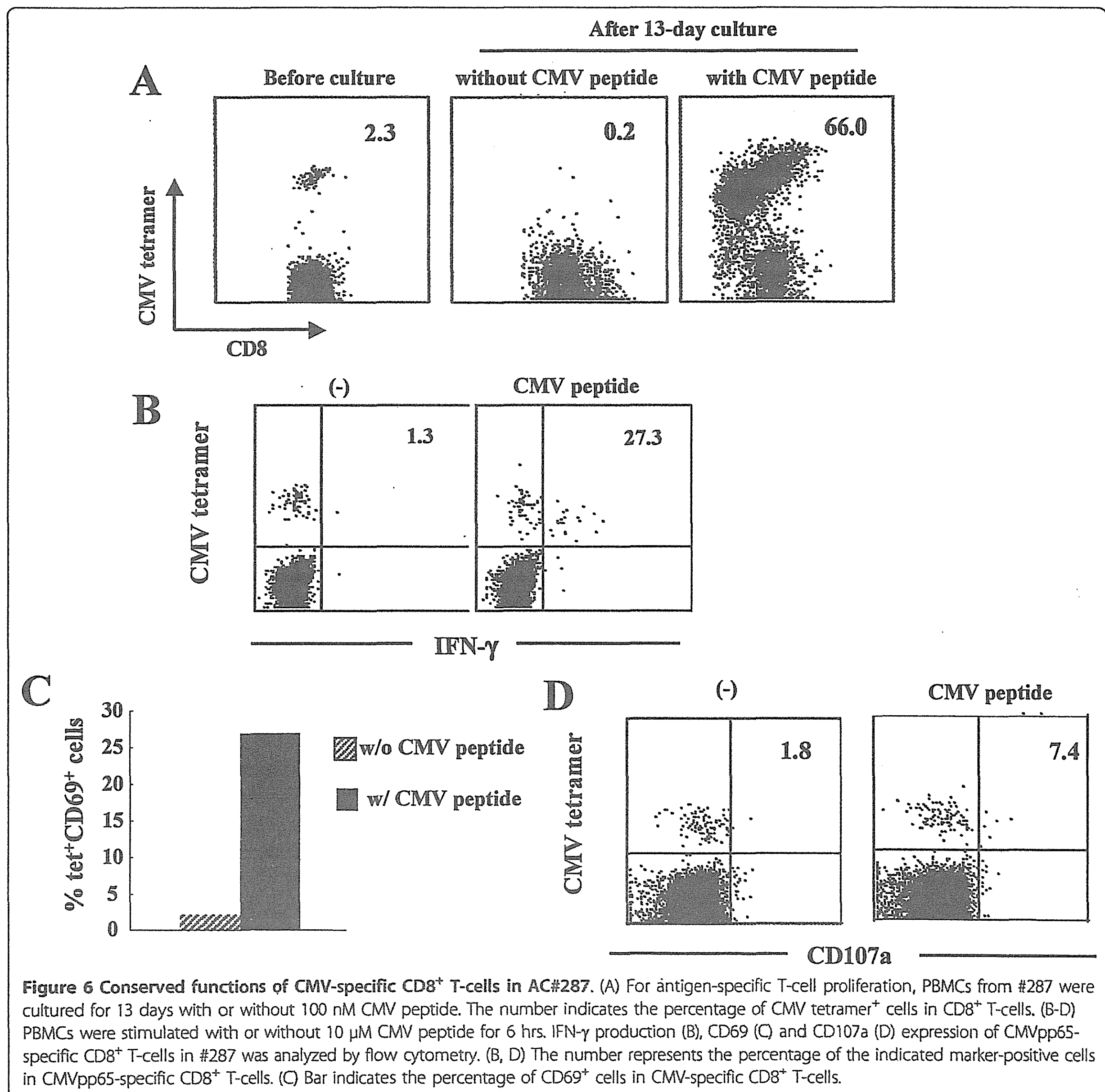

Figure 6 Conserved functions of CMV-specific CD8⁺ T-cells in AC#287. (A) For antigen-specific T-cell proliferation, PBMCs from #287 were cultured for 13 days with or without 100 nM CMV peptide. The number indicates the percentage of CMV tetramer⁺ cells in CD8⁺ T-cells. (B-D) PBMCs were stimulated with or without 10 μM CMV peptide for 6 hrs. IFN-γ production (B), CD69 (C) and CD107a (D) expression of CMVpp65-specific CD8⁺ T-cells in #287 was analyzed by flow cytometry. (B, D) The number represents the percentage of the indicated marker-positive cells in CMVpp65-specific CD8⁺ T-cells. (C) Bar indicates the percentage of CD69⁺ cells in CMV-specific CD8⁺ T-cells.

The presence of tetramer-binding Tax-specific $CD8^+$ T-cells in cATL patients, although at low frequencies, implies that they have encountered antigen during the chronic phase of ATL disease, suggesting that Tax may be expressed *in vivo*. This may be supported by a previous report showing that virus-specific $CD8^+$ T-cells fails to acquire memory T-cell property of long-term antigen-independent persistence during chronic lymphocytic choriomeningitis virus (LCMV) infection[45]. However, there is no direct evidence that infected cells produce Tax in infected individuals. HTLV-1-specific T-cell responses in cATL patients are largely different from HAM/TSP patients. In HAM/TSP patients, Tax-specific $CD8^+$ T-cells proliferated vigorously and a large population of them produced IFN-γ. In contrast, the function of Tax-specific $CD8^+$ T-cells in cATL patients was profoundly suppressed, similarly to tumor infiltrating lymphocytes (TIL)[46]. In cATL patients, Tax-specific $CD8^+$ T-cells that were detected before culture decreased in number to undetectable or very low levels after 6 days, regardless of peptide stimulation (data not shown). This is not likely to be due to TCR down-regulation, because TCRs on Tax-specific $CD8^+$ T-cells in HAM/TSP patients are down-regulated on days 1 to 4

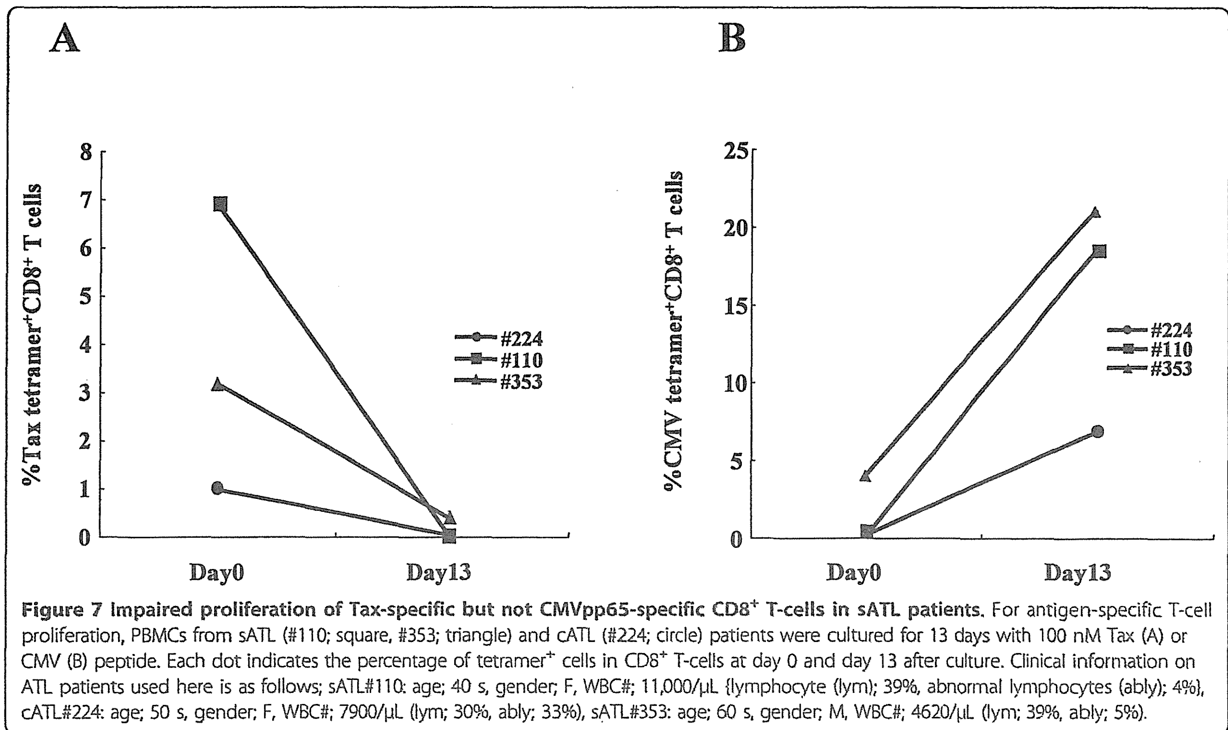

Figure 7 Impaired proliferation of Tax-specific but not CMVpp65-specific CD8⁺ T-cells in sATL patients. For antigen-specific T-cell proliferation, PBMCs from sATL (#110; square, #353; triangle) and cATL (#224; circle) patients were cultured for 13 days with 100 nM Tax (A) or CMV (B) peptide. Each dot indicates the percentage of tetramer⁺ cells in CD8⁺ T-cells at day 0 and day 13 after culture. Clinical information on ATL patients used here is as follows; sATL#110: age; 40 s, gender; F, WBC#; 11,000/μL (lymphocyte (lym); 39%, abnormal lymphocytes (ably); 4%), cATL#224: age; 50 s, gender; F, WBC#; 7900/μL (lym; 30%, ably; 33%), sATL#353: age; 60 s, gender; M, WBC#; 4620/μL (lym; 39%, ably; 5%).

and reappeared by day 6 in vitro[34]. Moreover, we could not observe any tetramer$^+$ CD8$^+$ T-cells even in the 13-day culture (data not shown), suggesting these cells might have died during the culture.

Severe dysfunction of Tax-specific CD8$^+$ T-cells was observed not only in cATL patients, but also in an AC #287. Fresh PBMCs of #287 contained 1.17% tetramer$^+$ cells in the CD8$^+$ T-cell fraction. However, none of these tetramer-positive T-cells proliferated in culture, with or without Tax peptide stimulation (Figure 3B). Although a few populations of them (11.1%) produced a small amount of IFN-γ, they lacked degranulation activity for cytotoxicity or expression of CD69, an early activation marker, upon specific stimulation (Figures 3 and 4). Importantly, CMVpp65-specific CD8$^+$ T-cells in the same donor were clearly activated, and exhibited these characteristics upon stimulation with pp65 peptides (Figure 6). These observations indicated that the impaired Tax-specific CD8$^+$ T-cells function in #287 was not attributable to general immune suppression, but to an HTLV-1-specific phenomenon. In addition, CD8-depletion study indicated that not only the dominant Tax-specific CD8$^+$ T-cell function but also other HTLV-1-specific CD8$^+$ T cell responses might be reduced in #287 (Figure 4D). Since CMV-specific CD8$^+$ T-cells responded well to the specific peptides, antigen-presenting cells in culture were not likely to be responsible for the selective suppression of Tax-specific CD8$^+$ T-cells. In addition, it has been shown that HTLV-1-infected cells generally express CCR4 and have Treg-like function[18,40]. However, depletion of CCR4$^+$ cells did not restore the proliferative ability of Tax-specific CD8$^+$ T-cells (data not shown), indicating that suppression of the infected cells were not likely to be the major reason for the impaired Tax-specific CD8$^+$ T-cell function in our culture system. These observations suggest that in #287, Tax-specific CD8$^+$ T-cells themselves might lose their functions.

Many chronic viral infections affect the phenotype, function, and maintenance of memory T-cells [24,42,47,48]. T_{EM} cells predominate in infections in which relatively high levels of antigen persist and continuous antigen stimulation are required for maintenance of T_{EM} cells. As described in HAM/TSP patients [34], Tax-specific CD8$^+$ T-cells in both ACs (#287 and #313) were primarily enriched in T_{EM} memory pool in spite of the functionality of Tax-specific CD8$^+$ T-cells (Figure 5A), which may support continuous or periodical expression of viral antigen in vivo during an asymptomatic stage.

PD-1 is known to play a major role in regulating T-cell exhaustion during chronic infection. In this study, we could not obtain any data supporting the involvement of PD-1 in the dysfunction of Tax-specific CD8$^+$ T-cells. However, we observed that Tax-specific CD8$^+$ T-cells in some ACs showed IFN-γ production, but not

proliferative capacity (Table 2). This partially lacked function of Tax-specific CD8$^+$ T-cells is similar to the features of T-cell exhaustion. Whether Tax-specific CD8$^+$ T-cells are exhausted in HTLV-1 infection, and whether other molecules associated with T-cell exhaustion are involved in the impairment of Tax-specific CD8$^+$ T-cell responses are necessary to be clarified because some inhibitory molecules such as T-cell immunoglobulin and mucin domain-containing protein-3 (TIM-3), lymphocyte activated gene-3 (LAG-3), and transcription factors including BLIMP-1 are also found to be associated with T-cell exhaustion [49].

The incidence of Tax-specific CD8$^+$ T-cell detection was high (87.0%) in ACs. Given the fact that the incidence of Tax-specific CD8$^+$ T-cells in HAM/TSP patients was 100%, a small fraction of ACs lacking detectable tetramer-binding cells might lack Tax-specific T-cell responses. Our previous study investigating GST-Tax protein-based T-cell responses supports this notion [20]. In the present study, even in ACs possessing Tax-specific CD8$^+$ T-cells, at least one individual exhibited T-cell dysfunction selectively for HTLV-1. The incidence of tetramer-positive cells was reduced in ATL patients (38.1%), and the function of these cells was impaired in all the ATL patients even with detectable tetramer-binding Tax-specific CD8$^+$ T-cells. Our findings suggest that HTLV-1-specific T-cell responses are selectively impaired in a small percentage of HTLV-1-infected individuals in the asymptomatic stages, and the proportion of individuals with such characteristics increase as the stages proceed towards ATL. Strategies to reactivate HTLV-1-specific T-cells at early stages might contribute to a reduction in the immunological risk of ATL.

Conclusions

Tax-specific CD8$^+$ T-cells were scarce and dysfunctional in a limited AC population and ATL patients, and the dysfunction of CD8$^+$ T-cells was selective for HTLV-1 in early stages. These results implied the presence of some HTLV-1-specific T-cell suppressive mechanisms even in asymptomatic stages, which are not a result of general immune suppression in ATL but could be underlying conditions toward disease progression.

Methods

Samples

Blood samples from 64 HTLV-1-seropositive individuals were used in this study: 23 asymptomatic carriers (ACs), 18 HAM/TSP patients, 2 smoldering type ATL (sATL) patients, and 21 chronic type ATL (cATL) patients. All blood samples were obtained following written informed consent, and this study was reviewed and approved by the Institutional Review Board of the Tokyo Medical and Dental University.

Peptides

Peptides used in this study were HLA-A2-restricted CTL epitopes (Tax11-19, LLFGYPVYV)[12] (Hokudo Co., Hokkaido, Japan) and (CMV495-503, NLVPMVATV)[50] (Sigma Aldrich St. Louis, MO), HLA-A11-restricted CTL epitope (Tax88-96, KVLTPPITH)[36] (Hokudo Co) and HLA-A24-restricted CTLs epitopes (Tax301-309, SFHSLHLF)[35] (Hokudo Co) and (CMV341-349, QYDPVAALF)[51] (Sigma Aldrich).

Cell Surface staining

To select samples carrying HLA-A2, -A11, or -A24, whole blood was screened with antibodies for HLA-A2, -A11, and -A24 subtypes (One Lambda, Inc., Los Angeles, CA). FITC-conjugated goat anti-mouse Ig (G+M) (Beckman Coulter Inc., Webster, TX) was used as a secondary antibody. For cell surface staining, whole blood samples were stained with the following fluorochrome-conjugated mouse anti-human mAbs; CD3-FITC, CD8-PE/Cy5, CD8-PerCP/Cy5.5 (RPA-T8, BioLegend), CD27-FITC (O323, BioLegend) CD45RA-FITC (HI 100, BD Biosciences), CD45RA-APC (HI 100, BioLegend), CD69-FITC (FN 50, BioLegend), PD-1-FITC (EH12.2H7, BioLegend), CCR7 (TG8/CCR7, Biolegend).

Tetramer staining

PE-conjugated HLA-A*0201/Tax11-19, HLA-A*1101/Tax88-96, HLA-A*2402/Tax301-309, HLA-A*0201/CMVpp65, HLA-A*2402/CMVpp65 tetramers were purchased from MBL (Nagoya, Japan). Whole blood samples or peripheral blood mononuclear cells (PBMCs) were stained with PE-conjugated Tax/HLA tetramer in conjunction with FITC-conjugated anti-CD3 (UCHT1, BioLegend San Diego, CA), and PE-Cy5-conjugated anti-CD8 monoclonal antibodies (mAbs) (HIT8a, BD Biosciences San Jose, CA). Whole blood samples were lysed and fixed in BD FACS lysing solution (BD Biosciences) before washing the cells. Samples were analyzed on a FACSCalibur (Becton Dickinson, San Jose, CA) and data analyses were performed using CellQuest software (Becton Dickinson).

Tetramer-based IFN-γ flow cytometry

Tetramer-based intracellular IFN-γ flow cytometry was performed as described previously[17], with slight modifications. In brief, PBMCs (2.0×10^5 cells) were incubated with HLA tetramer-PE and anti-CD8-PE/Cy5, washed, and stimulated with 10 μM antigenic peptide for 6 hrs at 37°C in the presence of brefeldin A (BFA, 10 μg/ml; Sigma Aldrich). The cells were stained with a

tetramer, permeabilized, and stained with anti-human IFN-γ-FITC (4S.B3, BD Biosciences).

T-cell proliferation

PBMCs (2.0-5.0 × 10^5 cells/well) labeled with carboxyfluorescein succinimidyl ester (CFSE; Sigma Aldrich) were cultured for 6 days with or without 100 nM antigenic peptide and then stained with Tax/HLA tetramer-PE and anti-CD8-PE/Cy5. In some experiments, PBMCs (2.0 × 10^5 cells) were cultured for 13 days with 100 nM antigenic peptide and 10 U/ml recombinant human IL-2 (IL-2; Shionogi, Osaka, Japan) in the presence or absence of 0.1 μg/ml Lipopolysaccharide (LPS; Sigma Aldrich). The cells were then stained with HLA tetramer-PE, anti-CD8-PE/Cy5 and anti-CD3-FITC, and analyzed by flow cytometry.

Quantification of HTLV-1 proviral load

The HTLV-1 proviral load was measured using LightCycler DNA Master SYBR Green 1 (Roche, Mannheim, Germany) with a LightCycler (Roche). Genomic DNA was extracted from PBMCs (2 × 10^6 cells) using DNeasy Blood & Tissue kits (QIAGEN, Courtaboeuf, France). The primer sets used in this study were as follows: pX2 (5'-CGGATACCCAGTCTACGTGTTTGGAGACTGT-3') and pX3 (5'-GAGCCGATAACGCGTCCATCGATGG GGTCC-3') for HTLV-1 pX, and B-globin (5'-ACACAACTGTGTTCACTAGC-3') and aB-globin (5'-CAACTTCATCCACGTTCACC-3') for β-globin. The proviral load was calculated as: [(copy number of pX)/(copy number of β-globin/2)] × 1000. HTLV-1 proviral loads in some of the PBMC samples were measured by the Group of Joint Study on Predisposing Factors of ATL Development (JSPFAD, Japan) as described previously [20].

CD107a mobilization assay

PBMCs were stained with Tax/HLA tetramers-PE and anti-CD8-PE/Cy5, washed, and stimulated with 10 μM antigenic peptide for 6 hrs at 37°C in the presence of mouse anti-human CD107a-PerCP/Cy5.5 (H4A3, Biolegend) or mouse IgG_1-PerCP/Cy5.5 (MOPC-21, Biolegend). BFA (10 μg/ml) was added 1 hr after incubation was started. The cells were then collected and stained with an HLA tetramer.

Depletion of $CD8^+$ cells and Detection of HTLV-1 p19

$CD8^+$ cells were depleted from PBMCs by negative selection using 10-fold numbers of Dynabeads M-450 CD8 (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. The PBMCs were adjust to 1 × 10^6 cells/ml before depletion, and the resulting $CD8^+$ cell-depleted fractions were resuspended in medium with the same initial volume, irrespective of the remaining cell number. PBMCs (1 × 10^6 cells/ml) and $CD8^+$ cell-depleted PBMCs were cultured for 7 days. HTLV-1 p19 in the supernatants of those PBMCs were measured by HTLV p19 antigen ELISA (RETRO tek, Buffalo, NY).

Statistics

The Mann-Whitney U-test, the unpaired t test, and the Spearman rank correlation test were performed for statistical significance by using the Graphpad Prism software (Graphpad Software). In all cases, two-tailed *P* values less than 0.05 were considered significant.

Additional material

Additional file 1: Tax-specific $CD8^+$ T-cells in cATL patients could not proliferate against Tax-peptide stimulation. (A) CFSE-labeled PBMCs were cultured with or without 100 nM Tax-peptide for 6 days. The number indicates the percentage of $tetramer^+$ cells in $CD8^+$ T cells (Day 0) or the percentage of dividing ($CFSE^{low}$) cells in Tax-specific $CD8^+$ T-cells (Day 6). In a cATL sample #54, CFSE-labeled PBMCs were cultured in the presence of mouse IgG for other experiment. (B) PBMCs (#224) and CCR4-depleted PBMCs (#280) were cultured for 13 days in the presence of 100 nM Tax-peptide. The number indicates the percentage of $tetramer^+$ cells in $CD8^+$ T-cells.

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Authors' contributions
AT carried out immunological and virological analyses, and drafted the manuscript. AH conceived of the study, participated in its design and coordination, and drafted the manuscript. AU, YM, YY, MM, IC, NU, and JO provided clinical samples. YS, YT, AS, and NZ carried out a part of the experiments. TW provided the data on proviral load of some HTLV-1-infected individuals. TM helped to draft the manuscript. MK participated in study design and helped to draft the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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High Incidence of Cytomegalovirus, Human Herpesvirus-6, and Epstein–Barr Virus Reactivation in Patients Receiving Cytotoxic Chemotherapy for Adult T Cell Leukemia

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The etiology of cytomegalovirus (CMV), human herpesvirus-6 (HHV-6), and Epstein–Barr virus (EBV) reactivation and the potential for complications following cytotoxic chemotherapy in the absence of allogeneic transplantation are not clearly understood. Patients with adult T cell leukemia (ATL) are susceptible to opportunistic infections. In this study, the incidence, kinetics and clinical significance of reactivation of CMV, HHV-6, and EBV in ATL patients were investigated. Viral DNA in a total of 468 plasma samples from 34 patients was quantified using real-time PCR. The probability of CMV, HHV-6, and EBV reactivation by 100 days after the start of chemotherapy was 50.6%, 52.3%, and 21.6%, respectively. Although most CMV reactivations were self-limited, plasma CMV DNA tended to persist or increase if the CMV DNA levels in plasma reached $\geq 10^4$ copies/ml. CMV reactivation was negatively associated with survival, but the *P*-value for this association was near the borderline of statistical significance ($P = 0.052$). One patient developed fatal interstitial pneumonia concomitant with peak CMV DNA accumulation (1.6×10^6 copies/ml plasma). Most HHV-6 and EBV reactivations were self-limited, and no disease resulting from HHV-6 or EBV was confirmed. HHV-6 and EBV reactivation were not associated with reduced survival ($P = 0.35$ and 0.11, respectively). These findings demonstrated that subclinical reactivation of CMV, HHV-6, and EBV were common in ATL patients receiving chemotherapy. There were differences in the viral reactivation patterns among the three viruses. A CMV load $\geq 10^4$ copies/ml plasma was indicative of subsequent exacerbation of CMV reactivation and developing serious clinical course. ***J. Med. Virol. 83:702–709, 2011.***

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KEY WORDS: ATL; CMV; HHV-6; EBV; real-time PCR

INTRODUCTION

Cytomegalovirus (CMV), human herpesvirus-6 (HHV-6), and Epstein–Barr virus (EBV) are ubiquitous in the human population. More that 90% of Japanese individuals over the age of 50 have antibodies against CMV or EBV [Takeda et al., 2001]. HHV-6 infects virtually all individuals during childhood [Zerr et al., 2005a]. These herpesviruses establish latency after primary infection and can reactivate under immunosuppressive condition. Reactivation of CMV, HHV-6, and/or EBV is common after solid-organ or hematopoietic stem cell transplantation and is linked to various serious clinical diseases [Boeckh et al., 1996; Shapiro et al., 1999; Yoshikawa et al., 2002; Zerr et al., 2005b; Ogata et al., 2006], and it is common practice to treat CMV or EBV reactivation in recipients of stem cell transplants [Meerbach et al., 2008; Boeckh and

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Ljungman, 2009; Omar et al., 2009]. CMV is increasingly recognized as a significant pathogen in patients receiving only chemotherapy without transplants. Han [2007] reported that a high portion of non-transplant patients with lymphoid malignancies (13.6%) were positive for CMV antigenemia. Nguyen et al. [2001] reported that the frequency of CMV pneumonia has been increasing in adults with leukemia who have not undergone transplantation.

Adult T cell leukemia (ATL) is an aggressive T cell malignancy caused by a retrovirus, human T-cell leukemia virus type I (HTLV-I), which is endemic in Japan, Melanesia/Australia, the Caribbean, parts of South America, and part of Africa [Van Brussel et al., 1999]. Prognosis for patients with ATL is extremely poor due to multidrug resistance of malignant cells and frequent complications due to opportunist infections. ATL patients are susceptible to various opportunistic infections, including pneumocyctis pneumonia, fungal infections, and herpesvirus disease due to defective cellular immunity [Uchiyama, 1997; Yasunaga et al., 2001; Chen et al., 2006]. Suzumiya et al. [1993] reported that CMV was involved in 35 of 47 (74.5%) autopsied cases of ATL and that CMV pneumonia is a significant cause of death in ATL patients. EBV-associated B cell lymphoproliferative disorder [Tobinai et al., 1991; Tanaka et al., 2008] and HHV-6 encephalitis [Idutsu et al., 2007] has also been reported in patients with ATL. Development of these herpesvirus diseases may indicate that subclinical reactivations of herpesviruses are common in ATL patients. To date, two studies evaluated CMV reactivation in ATL patients using an antigenemia assay [Fujiwara et al., 2000, 2001]. However, in both studies, antigenemia was assessed only on admission and when a patient exhibited a fever. Furthermore, less is known about incidence or significance of HHV-6 or EBV reactivation.

Understanding the dynamics of reactivation of these herpesviruses in ATL patients may facilitate the prevention of CMV-, HHV-6-, or EBV-related diseases. Presence of plasma viral DNA can be a good indicator of active CMV [Boeckh and Ljungman, 2009] or HHV-6 [Zerr, 2006b] infection. There is debate over which sample type, whole blood versus plasma or serum is most suitable for monitoring EBV reactivation; nevertheless, the detection of EBV DNA genomes in plasma that does not contain B-cells is more likely the result of a lytic EBV infection and indicates that the patients has an active EBV infection [Ljungman, 2010]. The present study used real-time polymerase chain reaction (PCR) to detect viral DNA genomes in plasma to specifically evaluate the epidemiology and kinetics of CMV, HHV-6, and EBV reactivation in ATL patients receiving chemotherapies.

MATERIALS AND METHODS

Patients

Thirty-four patients who were admitted to Oita University Hospital or Oita Prefectural Hospital and who had acute/lymphoma-type ATL that met the Japan Lymphoma Study Group criteria [Shimoyama, 1991] were enrolled in this study. The diagnosis of ATL was made based on seropositivity for HTLV-I and a histologically and/or cytologically proven peripheral T-cell malignancy. Study protocols were approved by the Ethical Committee of the Oita University Faculty of Medicine, and patients participating in this study signed an informed consent form for study protocols. Initial treatment regimens included cyclophosphamide, doxorubicin, vincristine, and prednisolone (CHOP), cyclophosphamide, pirarubicin, vincristine, and prednisolone (THP-COP), cyclophosphamide, pirarubicin, etoposide, and prednisolone (THP-CEP), or mLSG15 (sequential therapy by VCAP-AMP-VECP) [Tsukasaki et al., 2007]. Salvage treatments were selected at the discretion of the physicians for management of refractory disease. No patients were treated with prophylactic anti-viral agents. Patients who received hematopoietic stem cell transplantation were censored from this study upon beginning preconditioning for transplantation.

Real-Time PCR

Blood sampling to monitor viral DNA was started after initiation of chemotherapy and performed during the period in which patients were hospitalized in order to receive chemotherapy. Interval of sampling was at baseline, once every 7–14 days. Plasma samples were separated from EDTA-treated whole blood by centrifugation (1,750*g* for 10 min) and filtration through a 0.22-μm pores filter. The design of the PCR primers (5′-TCACCAGTGTCGTGTATGCCA-3′ and 5′-CACACAGCGCTCGTTGTAATC-3′) and a TaqMan probe (5′-[FAM]CCCATGAACGTGCTCATCGAC GTGA[TAMRA]-3′) for CMV quantitation were based on the UL97 open reading frame of CMV, and quantitation was performed as previously described [Ikewaki et al., 2005]. Primers and probe for evaluation of HHV-6 DNA load were based on sequences from U67 according to the methods originally described by Locatelli et al. [2000] and performed as previously described [Ogata et al., 2006]. For EBV, PCR primers were complementary to sequences in the BALF5 gene and performed as previously described [Kimura et al., 1999].

Analyses

Viral reactivation was defined as the detection of viral DNA in a sample of plasma. Duration of the reactivation event was defined as the period of consecutive positive results. The statistical significance of differences between the groups was assessed by Fisher's exact test or the Mann–Whitney *U* test as appropriate. The cumulative incidence of viral reactivation and survival was calculated according to the Kaplan–Meier method, and comparisons of survival were made using the log-rank test. Statistical analyses were performed using Prism 5 for Macintosh software (GraphPad Software,

San Diego, CA). Values of $P < 0.05$ were considered significant in all analyses.

RESULTS

Patients (n = 34) with acute/lymphoma-type ATL participated in this study. The characteristics of these patients are listed in Table I. One patient received two courses of pre-emptive ganciclovir treatment because positive CMV antigenemia was noticed. No other patient received any antiviral treatments. For the six patients who received hematopoietic stem cell transplantation, observation of viral reactivation for this study was stopped upon starting conditioning for transplantation. Median survival, based on Kaplan–Meier analysis, from the start of chemotherapy and 3-year overall survival rate with censoring transplantation patients were 244 days and 22.3%, respectively. A total of 22 patients died during the observation period: causes of death were ATL deterioration in 14 patients and infectious diseases in 8 patients. All patients who died of infectious diseases had concomitant, uncontrollable ATL.

Sequentially collected plasma samples were used to evaluate viral reactivation. Generally, plasma samples were collected from patients once every 7–14 days, but the interval was greater than 20 days for 6.5% of the samplings (28 of 434 intervals) because sampling was not performed during periods in which patients were not hospitalized. The median observation period for viral reactivation in individual patients was 113 days (range, 45–318 days).

Cumulative incidence of reactivation for each virus according to Kaplan–Meier analysis are shown in Figure 1, and the kinetics of plasma viral DNA load in patients who displayed positive viral DNA are shown in Figure 2. The characteristics of reactivation are shown in Table II. A Kaplan–Meier plot of the probability of survival according to viral reactivation is shown in Figure 3.

CMV Reactivation

The overall cumulative rate of a positive result for plasma CMV DNA by 100 days after the start of chemotherapy was 50.6% (Fig. 1). Univariate analysis revealed that higher white blood cell count and abnormal cell count at diagnosis were associated with CMV reactivation (Table I). As shown in Figure 2A,B, most CMV reactivations were self-limited. However, plasma CMV DNA levels had a tendency to persist or increase if and when the CMV DNA load reached a level of $\geq 10^4$ copies/ml (n = 5) (Fig. 2A). For the five patients whose plasma CMV DNA reached $\geq 10^4$ copies/ml, the median period from the detection of the first CMV-DNA-positive plasma sample to the attainment levels of $\geq 10^4$ copies/ml was 42 days (range, 21–98 days), and the period from first detection to peak levels was 98 days (range, 28–133 days). Median period from the first detection of a level of $\geq 10^4$ copies/ml CMV DNA to the death was 9 days (range, 1–152 days). One patient developed complications that were probably associated with CMV. A 72-year-old female developed fatal interstitial pneumonia at the time when the CMV DNA load peaked at 1.6×10^6 copies/ml. In this patient, the period from first detection of plasma CMV DNA to development of interstitial pneumonia was 98 days. The arrow in Figure 2A indicates the day on which the interstitial pneumonia developed. No other patient developed a clinical disease that was likely to be related to CMV reactivation. Kaplan–Meier analysis (Fig. 3) revealed median survival time from start of chemotherapy was 188 days in patients who experience CMV reactivation and 683 days in patients without CMV reactivation. CMV reactivation was negatively associated with survival, but the P-value for this association was at the borderline of statistical significance ($P = 0.052$, log-rank test).

HHV-6 Reactivations

The overall cumulative rate of a positive result for HHV-6 DNA in a plasma sample was 52.3% by 100 days after the start of chemotherapy (Fig. 1). No variables at diagnosis were identified as a risk factor associated with HHV-6 reactivation (Table I). Most HHV-6 reactivations were self-limited (Fig. 2C,D). The plasma HHV-6 DNA levels in the patient whose plasma HHV-6 DNA reached the highest value observed in this study suddenly climbed and dropped to an undetectable level three times (blue line in Fig. 2C). The duration in weeks of positive HHV-6 DNA tests in individual patients tended to be shorter compared with that of positive CMV DNA (Table II), but the difference was not statistical significance ($P = 0.11$, Mann–Whitney test). No patient developed complications, such as encephalitis or interstitial pneumonia, that were likely to be related to HHV-6 reactivation. There was no association between HHV-6 reactivation and survival (Fig. 3; $P = 0.35$, log-rank test).

EBV Reactivations

The overall cumulative rate of a positive result for EBV DNA in a plasma sample was 21.6% by 100 days of the start of chemotherapy (Fig. 1). No variables at diagnosis were identified as a risk factor associated with EBV reactivation (Table I). Most EBV reactivations were self-limited (Fig. 2E,F). Five patients had EBV DNA in the plasma sample taken within the first 7 days of treatment, and probability of incidence of EBV reactivation within the first 7 days of treatment was 14.7%. The duration, in weeks, of positive EBV-DNA tests in individual patients (Table II) was significantly shorter than that of positive CMV DNA tests ($P = 0.02$, Mann–Whitney test) but was not significantly different from that of positive HHV-6 DNA tests ($P = 0.18$). No patient developed EBV-associated lymphoproliferative disorder. There was no association between EBV reactivation and survival (Fig. 3; $P = 0.11$, log-rank test).

TABLE I. Patient Characteristics at Diagnosis and Association of These Variables With Herpesvirus Reactivation in Patients With Adult T Cell Leukemia (ATL) (n = 34)

Characteristics	Total (n = 34)	CMV reactivation			HHV-6 reactivation			EBV reactivation		
		Yes[a] (n = 22)	No[b] (n = 12)	*P*	Yes[a] (n = 20)	No[b] (n = 14)	*P*	Yes[a] (n = 11)	No[b] (n = 23)	*P*
Age in years, median (range)	65 (36–82)	69.5 (36–82)	56 (31–80)	0.03[f]	66.5 (36–82)	62 (38–80)	0.66[f]	68 (36–82)	61 (31–80)	0.85[f]
Sex										
Male	13 (38.2)	10 (45.5)	3 (25)	0.29[g]	9 (45)	4 (28.6)	0.48[g]	6 (54.5)	7 (30.4)	0.26[g]
ATL subtype										
Acute	22 (64.7)	17 (77.3)	5 (41.7)	0.06[g]	12 (60)	10 (71.4)	0.72[g]	8 (72.7)	14 (60.9)	0.70[g]
Lymphoma	12 (35.3)	5 (22.7)	7 (58.3)		8 (40)	4 (28.6)		3 (27.3)	9 (39.1)	
WBC count, /μL, median (range)	8,310 (2,500–64,550)	10,095 (3,930–64,550)	6,320 (2,500–43,800)	0.04[f]	10,375 (3,600–64,550)	7,370 (2,500–43,800)	0.16[f]	10,700 (4,200–10,700)	7,900 (2,500–64,550)	0.28[f]
Abnormal lymphocyte count, /μL, median (range)[c]	235 (0–56,804)	1,430 (0–56,804)	22.5 (0–31,098)	0.02[f]	201 (0–56,804)	387 (0–31,098)	0.98[f]	351 (0–12,527)	222 (0–56,804)	0.80[f]
LDH, more than twice the upper limit	18 (52.9)	14 (63.6)	4 (33.3)	0.15[g]	10 (50)	8 (57.1)	0.74[g]	7 (63.6)	11 (47.8)	0.48[g]
Hypercalcemia	6/33 (18.2)	5 (22.7)	1 (9.1)	0.64[g]	4 (20)	2 (6.5)	>0.99[g]	1 (9.1)	5 (22.7)	0.64[g]
sIL2R, U/L, median (range)[d]	14,200 (598–96,700)	18,750 (3,750–96,700)	8,350 (598–73,800)	0.14[f]	17,600 (879–96,700)	9,650 (598–73,800)	0.38[f]	20,750 (6,040–96,700)	11,300 (598–73,800)	0.17[f]
Initial treatment Regimen[e]										
THP-CEP	15	10	5		8	7		5	10	
THP-COP	10	6	4		5	5		3	7	
mLSG15	8	5	3		6	2		3	5	
CHOP	1	1	0		1	0		0	1	

Data represent number (%) of patients, unless otherwise indicated.
WBC, white blood cell; sIL2R, soluble interleukin-2 receptor; LDH, lactate dehydrogenase.
[a]Patients with positive DNA of each viral genome in plasma at any time after start of chemotherapy.
[b]Patients without positive DNA of each viral genome in plasma throughout the clinical course after start of chemotherapy.
[c]Data were missing for two patients.
[d]Normal range, 145–519 U/ml. Data were missing for two patients.
[e]Agents which constitutes a regimen were indicated in the Materials and Methods Section.
[f]Mann–Whitney *U* test.
[g]Fisher's exact test.