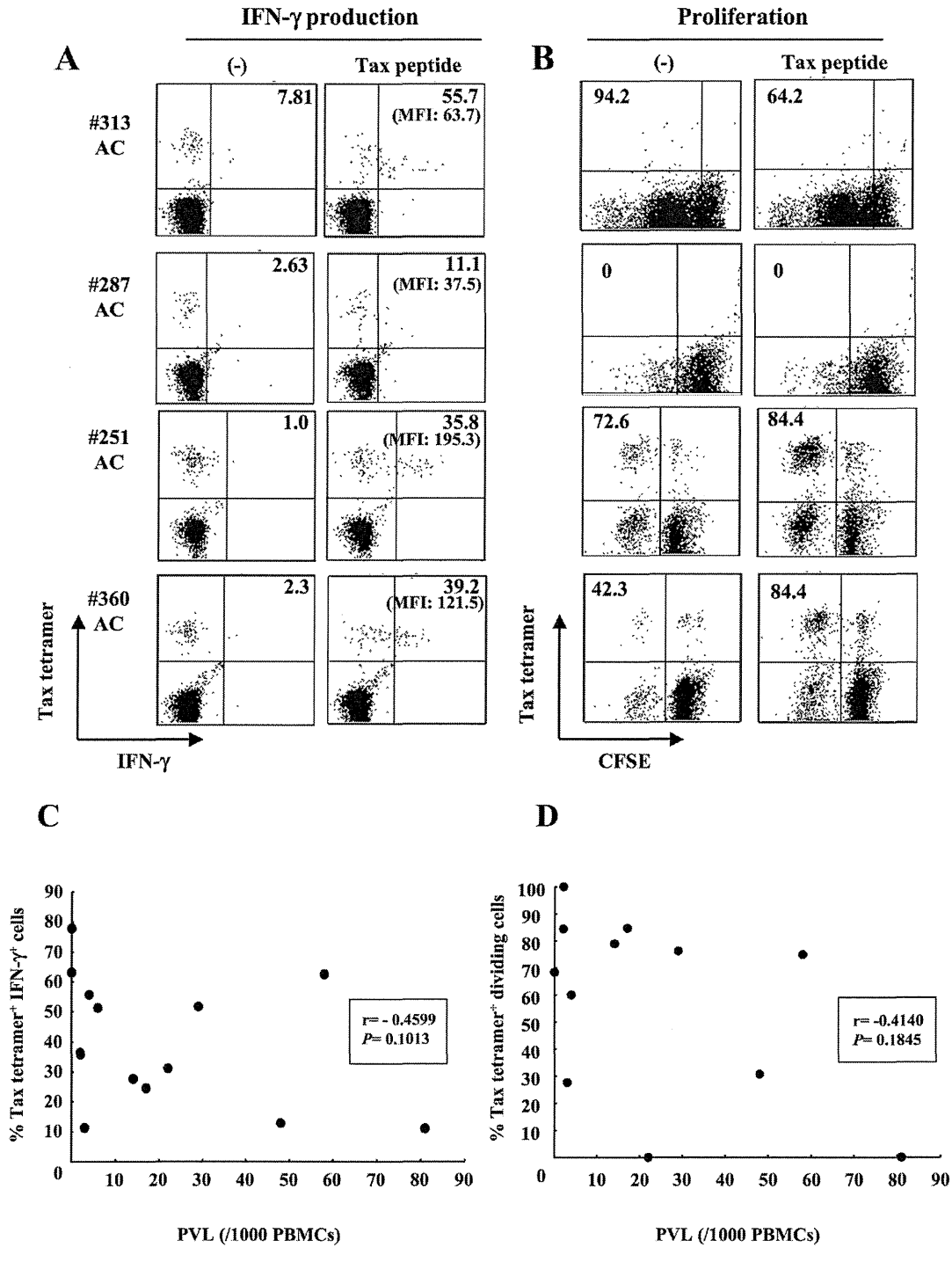

Figure 3 IFN-γ production and cell proliferation of Tax-specific CD8$^+$ T-cells in ACs. (A, B) IFN-γ production (A) and cell proliferation (B) of Tax-specific CD8$^+$ T-cells in PBMCs from 4 ACs were assessed as in Figure 2. The number given in parenthesis shows mean fluorescence intensity (MFI) of 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)

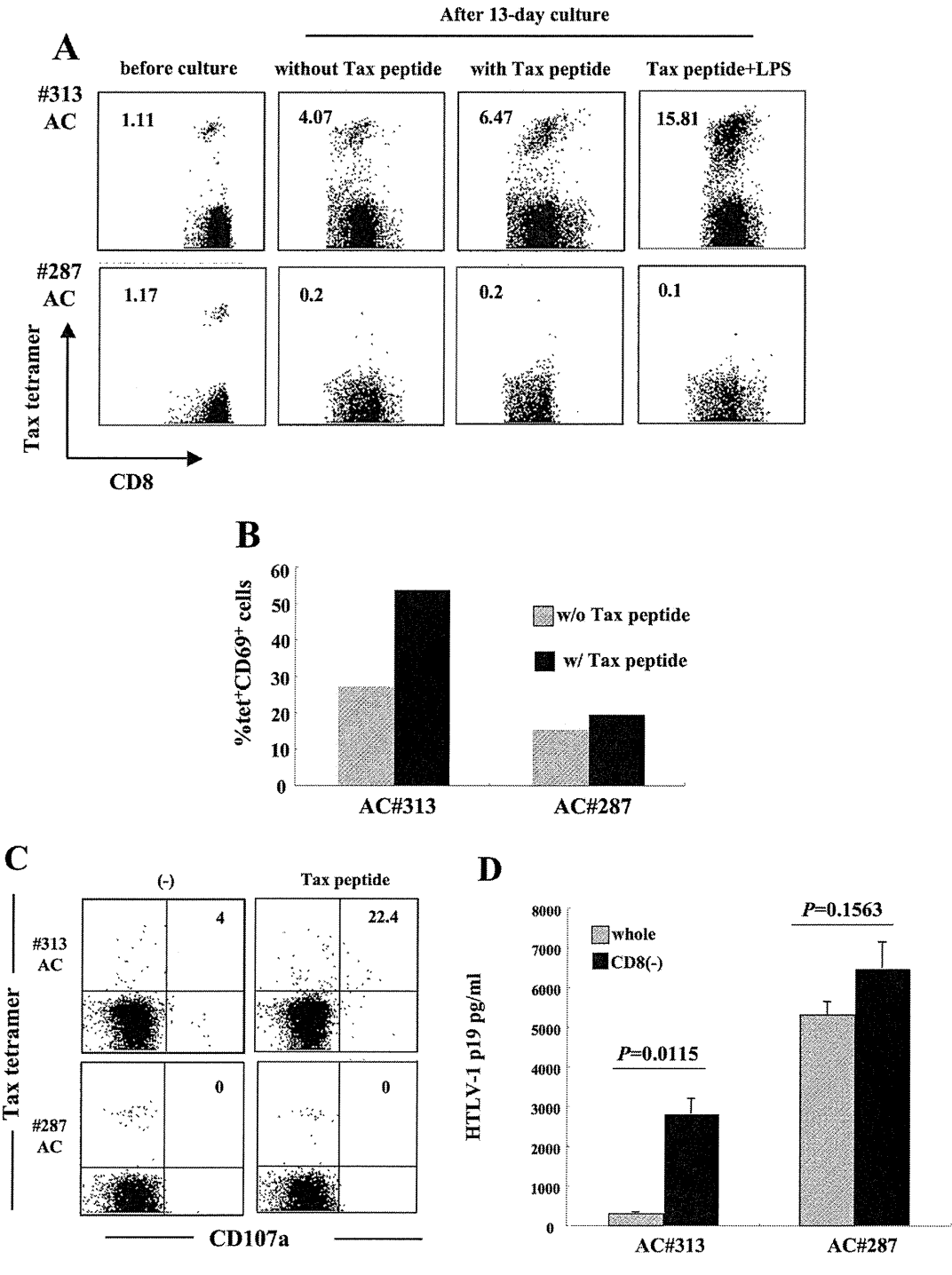

Figure 4 Dysfunction of Tax-specific CD8$^+$ T-cells and inefficient CD8$^+$ cell-mediated HTLV-1 control in AC#287. (A) For antigen-specific T-cell proliferation, PBMCs from #313 and #287 were cultured for 13 days with or without Tax peptide in the presence or absence of 0.1 μg/ml LPS. The number indicates 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.

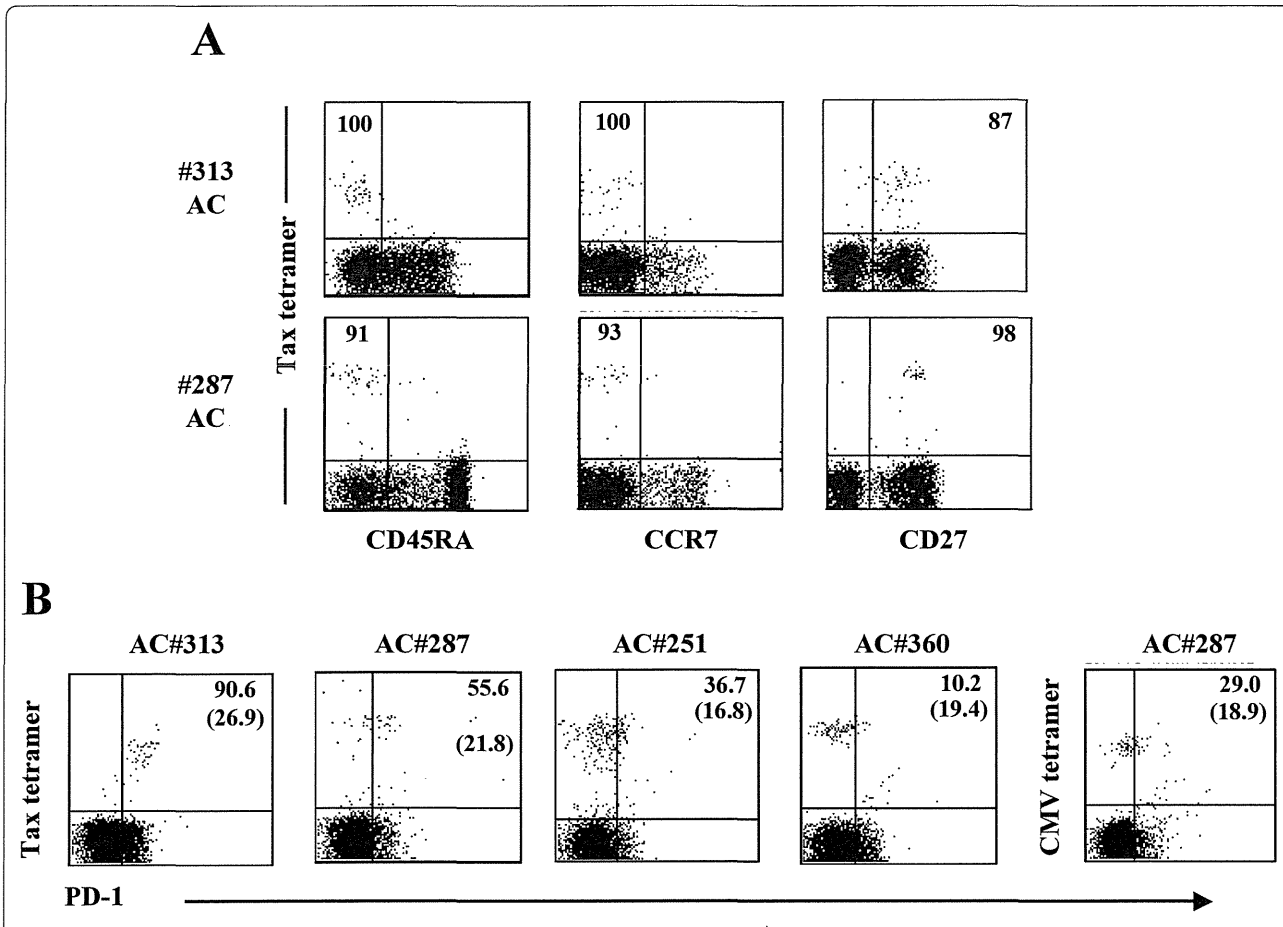

Figure 5 Phenotypic analysis of functional and dysfunctional Tax-specific CD8$^+$ T-cells. (A) Differentiation memory phenotype, based on the expression of CD45RA, CCR7, and CD27 and (B) PD-1 expression of Tax-specific CD8$^+$ T-cells from ACs were examined by flow cytometry. The number represents the 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.

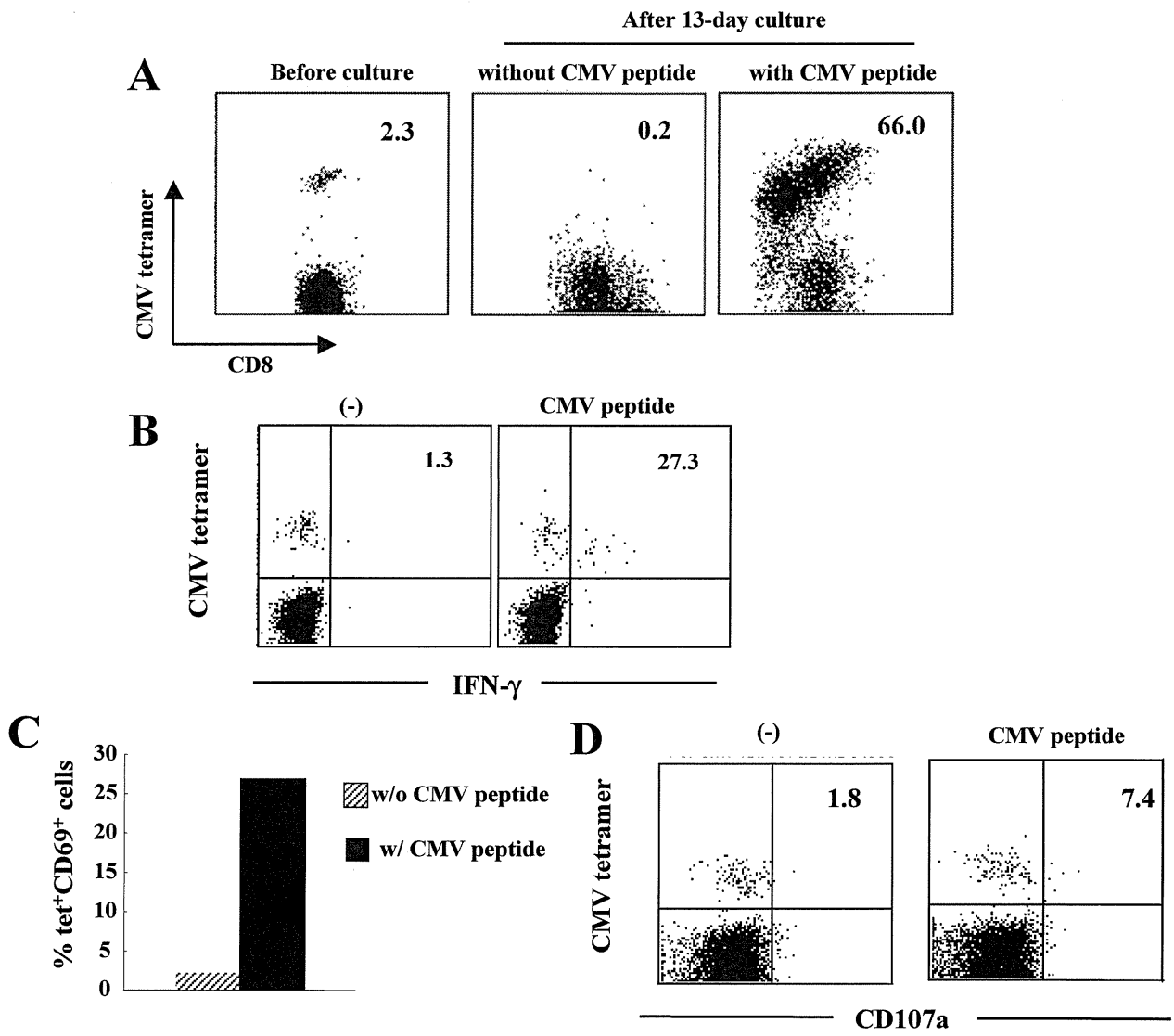

Figure 6 Conserved functions of CMV-specific CD8$^+$ T-cells in AC#287. (A) For antigen-specific T-cell proliferation, PBMCs from #287 were cultured for 13 days with or without 100 nM CMV peptide. The number indicates the percentage of CMV tetramer$^+$ cells in CD8$^+$ T-cells. (B-D) PBMCs were stimulated 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

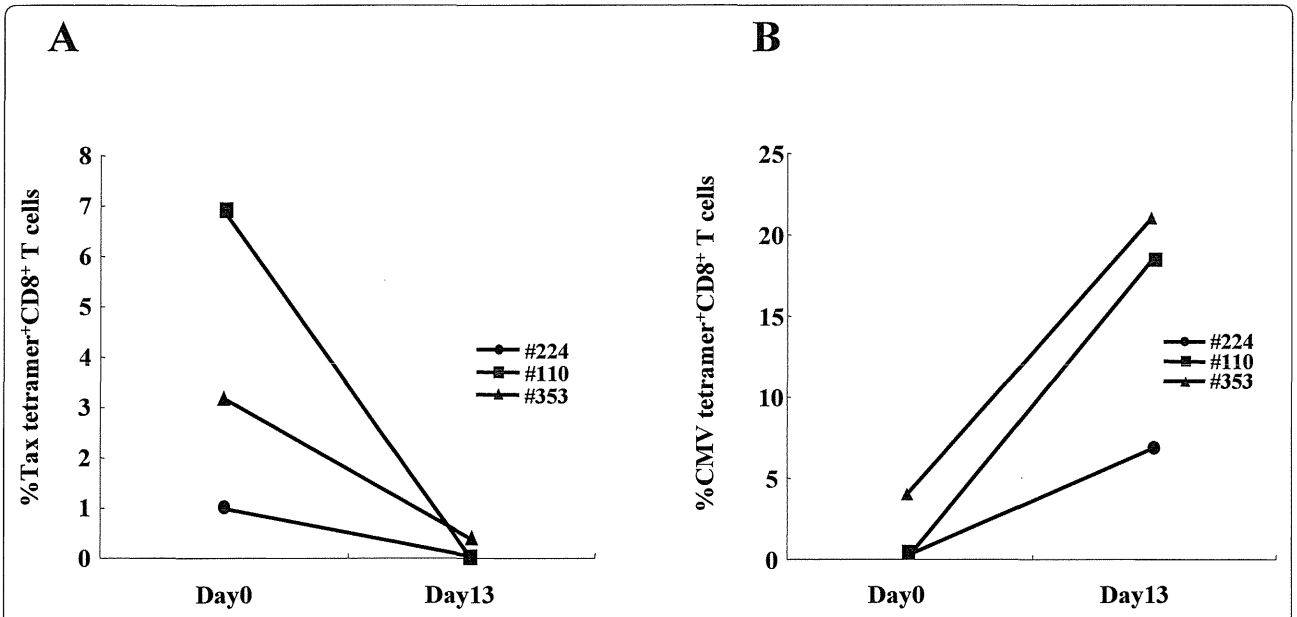

Figure 7 Impaired proliferation of Tax-specific but not CMVpp65-specific CD8$^+$ T-cells in sATL patients. For antigen-specific T-cell proliferation, PBMCs from sATL (#110; square, #353; triangle) and cATL (#224; circle) patients were cultured for 13 days with 100 nM Tax (A) or CMV (B) peptide. Each 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 Predisponsing 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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A case report of HTLV-I associated myelopathy presenting with cerebellar ataxia and nystagmus

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Abstract

HTLV-I associated myelopathy/tropical spastic paraparesis (HAM/TSP) is characterized by spastic paraparesis in the lower extremities, and urinary disturbance. HAM/TSP has also been less frequently associated with cerebellar syndromes and nystagmus. We report a case of HAM/TSP presenting with cerebellar ataxia and nystagmus. The patient was a 73-year-old woman who was born in southern Japan. At age 41, she developed pain and spasticity in the bilateral lower limbs and gradually progressive gait disturbance. At age 57, she was diagnosed with HAM/TSP based on spastic paraparesis in the lower limbs, urinary disturbance and positive anti HTLV-I antibody in serum and cerebrospinal fluid. In June 2008, she was referred to our university and hospitalized for rehabilitation. Twenty days later, she experienced rotatory vertigo sensation. Magnetic resonance imaging revealed pontocerebellar atrophy. The patient presented with cerebellar signs in the upper limbs, gaze-evoked nystagmus in the sitting position and right-beating horizontal nystagmus in the supine and head-hanging positions. Electronystagmography (ENG) showed horizontal saccadic overshoot dysmetria and horizontal saccadic pursuit. Nystagmus is rare among the literature on HAM/TSP. ENG is helpful to evaluate and confirm the cerebellar syndromes of HAM/TSP.

Keywords: HAM; Electronystagmography; Gaze-evoked nystagmus; Saccadic pursuit; Pontocerebellar atrophy

1. Introduction

Human T cell lymphotropic virus type I (HTLV-I) is an exogenous human retrovirus that has been demonstrated to be the etiological agent in adult T cell leukemia and a progressive neurological disease called HTLV-I associated myelopathy/tropical spastic paraparesis (HAM/TSP). HAM/TSP is characterized by spastic paraparesis in the lower extremities, and urinary disturbance associated with preferential damage of the thoracic spinal cord [1,2]. HAM/TSP has less frequently been associated with the following manifestations: myopathy, dysautonomia, mild cognitive dysfunction, cerebellar syndromes and peripheral neuropathy [3]. Nystagmus is also a less frequent neurological finding of HAM/TSP [3], and has been described only in a few reports [4–6]. We report a case of HAM/TSP presenting with cerebellar ataxia and nystagmus.

2. Case report

The patient was a 73-year-old woman who was born in Kagoshima, southern Japan. She had difficulty in walking since childhood. At age 41, she developed pain and spasticity in the bilateral lower limbs. Gait disturbance progressed gradually, thereafter. She suffered lumber vertebral fracture at age 48 and right femoral fracture at age 56 because of frequent falls. At age 57, she visited another prior hospital for further evaluation of gait disturbance. She presented with spastic paraparesis,

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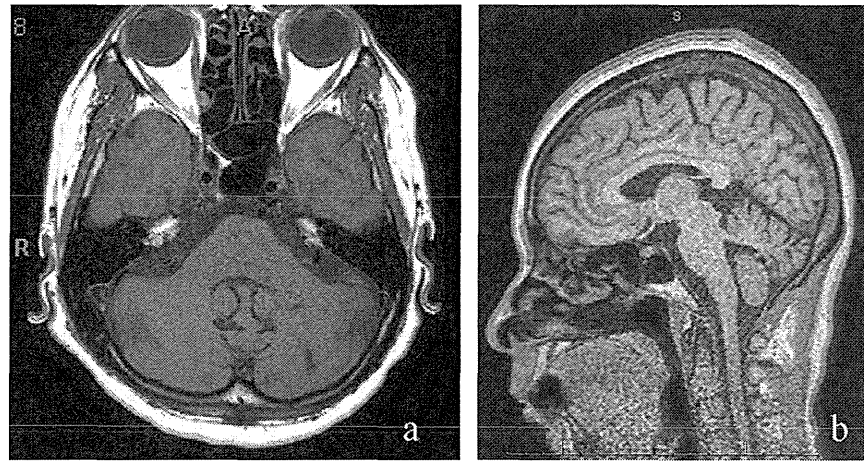

Fig. 1. Axial and sagittal T1-weighted MRI findings (a and b). Both images show moderate diffuse atrophy of the pons and cerebellum.

increased deep tendon reflexes and positive extensor planter reflex in the lower limbs, and urinary disturbance, but neither muscle weakness nor abnormal reflexes in the upper limbs. Her daughter had adult T cell leukemia and was seropositive for HTLV-I. Examinations of the serum and cerebrospinal fluid (CSF) from the patient were positive for anti HTLV-I antibody in both serum and CSF. Altogether, she was diagnosed with HAM/TSP based on the WHO criteria [3]. Steroid (predonisolone) was administered until 2001 but was not effective. Neither intravenous immunoglobulin (IVIG) nor interferon-α were effective. In June 2008, she was referred to the department of Gerontology and Neurology at our university for rehabilitation and was ultimately hospitalized. Twenty days later, she experienced rotatory vertigo sensation when she moved and was referred to the Department of Otolaryngology–Head and Neck Surgery at our university for further evaluation of vertigo. Pure tone audiometry showed that her hearing was normal. Magnetic resonance imaging (MRI) revealed diffuse pontocerebellar atrophy (Fig. 1a and b). Cerebellar examinations revealed clumsiness in the finger-to-nose test and bilateral dysdiadochokinesis. Knee-heel test was not performed because of paraparesis of the lower limbs. Nystagmus was recorded with infrared video-oculography (VOG). The patient exhibited no nystagmus in the primary position but right-beating horizontal nystagmus on right gaze and left-beating horizontal nystagmus on left gaze. Fig. 2 shows this gaze-evoked nystagmus (GEN) using the method of Ikeda et al. [7]. Eye movements recorded with VOG were converted to a nystamogram using Scion Image (Scion Corporation) and their macro (http://ds.cc.yamaguchi-u.ac.jp/(ent/gankyu3d/ikeda.html). In the supine and head-hanging positions, either in the left or right ear down positions, positional tests revealed right-beating horizontal nystagmus. Dix–Hallpike test also showed right-beating horizontal nystagmus. Electronystagmography (ENG) showed horizontal saccadic overshoot dysmetria (Fig. 3a) and horizontal saccadic pursuit (Fig. 3b). The optokinetic nystagmus pattern test was impaired bilaterally. The maximum slow phase velocity (SPV) of caloric nystagmus was 13.7°/s to the right and 15.5°/s to the left with irrigation with 5 ml of 20 °C water for 10 s. Visual suppression of the SPV of caloric nystagmus was 0% and −15.7% during the irrigation to the right and left ears, respectively. These findings were consistent with the ones of cerebellar disorder. Therefore, we diagnosed that the cerebellar signs and nystagmus of the patient were the neurological findings in clinical criteria of WHO [3].

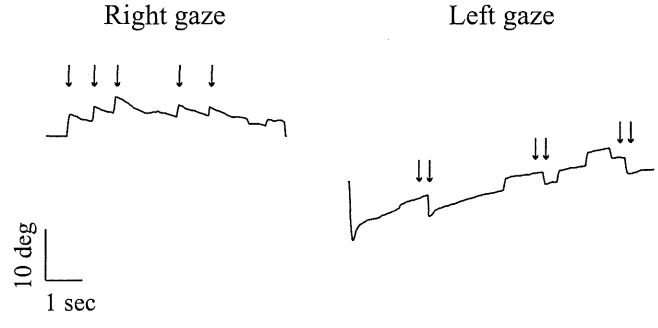

Fig. 2. The VOG recordings of horizontal gaze-evoked nystagmus. Arrows and double arrows indicate right and left gaze-evoked nystagmus, respectively.

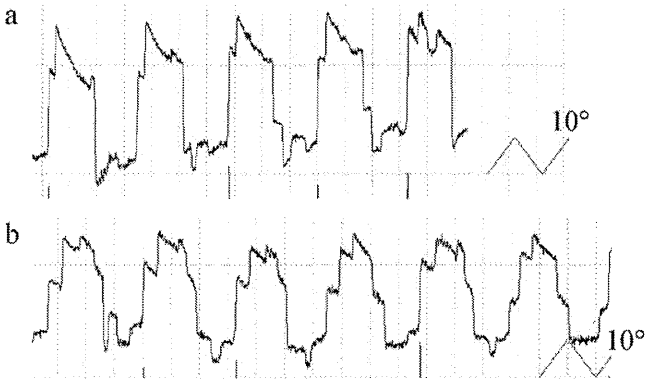

Fig. 3. Eye tracking test recordings for the patient. (a) ENG recording of horizontal saccade shows overshoot dysmetria. (b) ENG recording of horizontal sinusoidal smooth pursuit, which is superimposed on the nystagmus in the rightward and is saccadic in the leftward direction.

The vertigo sensation resolved slightly with the daily administration of 2 mg diazepam, although the nystagmus remains.

3. Discussion

HTLV-I is endemic in well-defined geographical regions: southern Japan, the Caribbean Central, South America, the Middle East, Melanesia and equatorial regions of Africa [1–3]. Our case was also born in southern Japan.

The main pathological feature of HAM/TSP is chronic inflammation of the white and grey matter of the spinal cord, mostly the thoracic cord. Mononuclear cells, mainly T cells, cause perivascular cuffing and infiltrate the parenchyma. Later in the disease, the pattern becomes less cellular and more atrophic. The striking CNS inflammatory changes demonstrated in HAM/TSP favor immune mediated mechanisms of pathogenesis.

Clinically, HAM/TSP is characterized by muscle weakness, hyperreflexia, spasticity in the lower extremities, and urinary disturbance. The symptoms usually begin during adulthood. More women than men develop HAM/TSP and the disease progresses faster in women [1,2]. The symptoms of our case were all consistent with those findings described above.

Cerebellar syndromes have been reported as a less frequent neurological finding of HAM/TSP [4,5]. Kira et al. demonstrated MRI atrophy of cerebellum and brainstem in patients with HTLV-I-associated spinocerebellar syndrome [8]. Our case presented with cerebellar signs and neuro-otological examinations showed saccadic overshoot dysmetria and saccadic pursuit. MRI revealed diffuse pontocerebellar atrophy, confirming the existence of cerebellar lesion. Arimura et al. and Uno et al. showed alterations of ocular pursuit (saccadic pursuit) in 50% (11/22) and 25% (2/8) of the patients with HAM/TSP, respectively [9,10]. These two neuro-otological studies suggest that saccadic pursuit is relatively frequently observed as a finding of cerebellar syndrome in HAM/TSP. Neuro-otological examinations using ENG can be easily performed even in patients with severe gait disturbance as with our case and are useful to evaluate cerebellar symptoms in HAM/TSP.

On the other hand, our case presented not only with cerebellar signs, but also nystagmus. Nystagmus is also one of the less frequent neurological findings of HAM/TSP [3]. Few reports have described cases of HAM/TSP with nystagmus [4–6]. Arimura et al. reported only two of twenty-two patients had ocular pursuit superimposed by jerky eye movement [9]. Uno et al. reported no cases of nystagmus [10]. These two studies suggest that the incidence of nystagmus among patients with HAM/TSP is much less than cerebellar syndromes in HAM/TSP.

The patterns of nystagmus in previous reports are varied, as follows: downbeat nystagmus (DBN), bilateral GEN, upbeat and horizontal nystagmus [4–6]. Our case presented with horizontal GEN and direction fixed right beating positional nystagmus. GEN occurs with cerebellar and brainstem lesions and reflects deficiency of the common neural integrator. The neural integrator for horizontal system is located in the vestibular nuclei/nucleus prepositus complex [11]. MRI in our case showed pontocerebellar atrophy, which is consistent with the integral disturbance of the horizontal eye movement system.

Direction fixed positional nystagmus and rotatory vertigo sensation are also observed in the patients with unilateral vestibular deficiency, e.g. vestibular neuritis in acute phase. However, in our case, the maximum SPV were bilaterally more than 10°/s, showing that the vestibular function was normal. Further, visual suppression of caloric responses were reduced or abolished. Visual suppression test is useful and reliable to detect the lesions in the vestibulocerebellum [12]. Lesions in the flocculus or nodulus of the cerebellum reduce or abolish the ability to suppress the vestibular nystagmus in the rhesus monkey [13,14]. These findings suggest that the rotatory vertigo sensation and direction fixed positional nystagmus were due to not vestibular disorder but to cerebellar one.

Castillo et al. discussed a case that presented with DBN and cerebellar vermian atrophy in MRI that was similar to alcoholic cerebellar degeneration. However, their case also presented with GEN despite the fact that there was no atrophy of the brainstem in MRI [5]. Recently, Beeravolu et al. reported a case that presented with DBN, although MRI showed no abnormal findings. MRI in our case also revealed vermian atrophy, while the nystagmus was not DBN. Taken together, in HAM/TSP, an atrophic lesion in MRI is not necessarily consistent with a lesion that is predicted from the pattern of nystagmus.

No therapy has been conclusively shown to alter long-term disability associated with HAM/TSP. Clinical improvements have been reported for a number of agents in open-label studies including corticosteroids, plasmapheresis, danazol, and pentoxifylline and interferon-α. With the exception of interferon-α, however, these drugs lack the quality of evidence required to merit a strong recommenda-

tion for their use in HAM/TSP. The role of interferon-α in HAM/TSP also remains in question, as no study has conclusively shown long-term benefit [2]. Our case was also treated with corticosteroid, interferon-α and plasmapheresis (IVIG), but none of them improved her symptoms. Further research in the treatment for long-term benefit will be needed.

In conclusion, nystagmus is rarely rather than less frequently observed in HAM/TSP. Neuro-otological examinations using ENG are helpful to evaluate and confirm cerebellar syndromes of HAM/TSP.

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MAJOR ARTICLE

Reduced Tim-3 Expression on Human T-lymphotropic Virus Type I (HTLV-I) Tax-specific Cytotoxic T Lymphocytes in HTLV-I Infection

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T cell immunoglobulin and mucin domain-containing molecule-3 (Tim-3) and programmed cell death-1 (PD-1) are T cell exhaustion molecules. We investigated the expression of Tim-3 and PD-1 in human T-lymphotropic virus type I (HTLV-I) infection. Tim-3 expression, but not PD-1 expression, was reduced on $CD4^+$ and $CD8^+$ T cells of HTLV-I–associated myelopathy/tropical spastic paraparesis (HAM/TSP) patients and HTLV-I carriers as compared with healthy controls. Tim-3 expression was also reduced in HTLV-I Tax-specific cytotoxic T lymphocytes (CTLs) as compared with cytomegalovirus-specific CTLs. Tim-3^+, but not PD-1^+, Tax-specific CTLs produced less interferon-γ and exhibited low cytolytic activity. However, we observed no difference in the expression of Tim-3 or cytolytic activity between Tax-specific CTLs of HAM/TSP patients or carriers. Moreover, HTLV-I–infected $CD4^+$ T cells showed decreased Tim-3 expression. These data suggest that Tim-3 expression is reduced in HTLV-I infection and that a high number of Tim-3^- HTLV-I–specific CTLs preserves their cytolytic activity, thereby controlling viral replication.

INTRODUCTION

Human T-lymphotropic virus type I (HTLV-I) is a retrovirus that preferentially infects $CD4^+$ lymphocytes in vivo [1]. Although HTLV-I infection is lifelong, less than 1% of infected individuals develop HTLV-I–associated myelopathy/tropical spastic paraparesis (HAM/TSP), a neurologic disease, or adult T cell leukemia (ATL), a hematologic disease [2–4]. HAM/TSP is an inflammatory disease of the spinal cord characterized by infiltration of inflammatory cells into the perivascular area [5]. Patients with HAM/TSP show spastic paraparesis and sphincter dysfunction with mild sensory disturbance [6]. HTLV-I proviral load and frequency of HTLV-I–specific $CD8^+$ cytotoxic T lymphocytes (CTLs) are higher in the peripheral blood of patients with HAM/TSP as compared with asymptomatic carriers [7–9]. Although increasing evidence supports the hypothesis that such a strong CTL response could certainly contribute to the control of viral replication and disease development, the exact pathogenic role of the CTL responses remains unclear [10].

The T-cell receptor costimulatory pathways assist in regulating T cell activation or tolerance [11, 12]. Recently, programmed cell death-1 (PD-1) signaling was shown to play an important role in T cell exhaustion during chronic viral infections [13–16]. T cell immunoglobulin and mucin domain-containing molecule-3 (Tim-3) has been similarly associated with T cell exhaustion [17]. Interaction of Tim-3 with its ligand galectin-9 regulates Th1 cell responses by promoting the

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death of interferon-γ (IFN-γ)–producing Th1 cells [18]. A recent study of human immunodeficiency virus (HIV) and hepatitis C virus (HCV) infections demonstrated that Tim-3 is upregulated in $CD4^+$ and $CD8^+$ T cells of patients with chronic viral infection. Tim-3-expressing T cells secrete less IFN-γ than do Tim-3-negative cells [19, 20]. In addition, a reduction of Tim-3 expression in T cells by using small interfering RNA or blocking antibodies increases the secretion of the antiviral cytokine IFN-γ [20, 21]. However, it is unclear whether T cells are exhausted or Tim-3 expression is upregulated in HTLV-I infection.

It remains unknown why only a small number of HTLV-I–infected individuals develop HAM/TSP, while the majority of the infected persons remain disease-free. It has been clearly demonstrated that elevated HTLV-I proviral loads increase the risk of HAM/TSP development [7, 22]. In addition, HAM/TSP patients have more HTLV-I–specific CTLs than do asymptomatic carriers [8, 23]. Recently, it has been postulated that CTLs in HAM/TSP patients have impaired function in association with degranulation of cytolytic molecules as compared with CTLs in asymptomatic carriers, which may result in an insufficient control of the virus [24]. However, it remains unclear whether CTL function is impaired in HAM/TSP patients.

In this study, we investigated Tim-3 and PD-1 expression in HTLV-I infection. In particular, we studied HTLV-I–specific CTLs and their degranulation activity in HAM/TSP patients and asymptomatic carriers as well as the role of Tim-3 and PD-1 in regulating their function.

MATERIALS AND METHODS

Patients

The study subjects consisted of 32 HAM/TSP patients, 31 asymptomatic carriers, and 11 uninfected healthy controls (Table 1). All subjects were residents of Kagoshima Prefecture, Japan. HTLV-I infection was determined using a HTLV-I antibody serological test, and HAM/TSP was diagnosed according to World Health Organization guidelines. All patients gave their written informed consent to participate in this study. Peripheral blood mononuclear cells (PBMCs) were separated from heparinized blood by Ficoll gradient centrifugation and stored in liquid nitrogen until use. To investigate HTLV-I–specific CTLs, we selected HLA-A*0201-positive or HLA-A*2402-positive cases because HTLV-I Tax11–19 and Tax301–309 are well characterized and strong immunodominant epitopes are restricted to these HLAs [25–27]. This study was reviewed and approved by the Kagoshima University Ethical Committee.

Cell Surface Staining

After thawing, 1×10^6 PBMCs were stained with a rat IgG2a anti-Tim-3 antibody (R&D Systems). The cells were washed with a staining buffer (PBS containing 5% normal goat serum and 0.1% NaN_3) and further stained with a goat anti-rat IgG–Alexa Fluor 488 secondary antibody (Invitrogen). Alternatively, the cells were stained with an anti-PD-1–fluorescein isothiocyanate (FITC) (eBioscience), anti-CD3–energy-coupled dye (ECD), anti-CD4–phycoerythrin (PE)–Cy5 (PC5), or anti-CD8–PC5 antibody (Beckman Coulter), and a PE-labeled tetramer. The HLA/antigen tetramers used were as follows: HLA-A*0201/HTLV-I Tax11–19 (LLFGYPVYV), HLA-A*0201/CMV pp65 (NLVPMVATV), HLA-A*0201/HIV Gag (SLYNTVATL), HLA-A*2402/HTLV-I Tax301–309 (SFHSLHLLF), HLA-A*2402/CMV pp65 (QYDPVAALF), and HLA-A*2402/HIV Gag (RYLKDQQLL) (Medical & Biological Laboratories). Alternatively, the cells were stained with anti-PD-L1–PE (eBioscience), anti-CD3–ECD, CD4–PC5 and CD8–FITC antibody (Beckman Coulter). Appropriate isotype antibodies were used as controls. Fluorescent signal was detected by an Epics XL flow cytometer, and Expo32 software was used for data acquisition and analysis (Beckman Coulter).

Intracellular IFN-γ Detection

PBMCs were cultured in complete medium (RPMI 1640 medium supplemented with 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 10% heat-inactivated fetal cow serum) in the absence or presence of phorbol 12-myristate 13-acetate (PMA [5 ng/mL]) and ionomycin (0.5 μg/mL) with 5 μg/mL of the secretion inhibitor brefeldin A (Sigma) for 6 hours. After harvesting, the cells were stained with a rat anti-Tim-3 antibody, followed by staining with a goat anti-rat IgG–PC5 secondary antibody (Santa Cruz Biotechnology), or with an anti-PD-1–FITC antibody. The cells were then stained with an anti-CD8–ECD antibody (Beckman Coulter) and Tax tetramer–PE. The cells were fixed with 1% paraformaldehyde, resuspended in 50 μL permeabilization buffer (0.1% saponin in staining buffer),

Table 1. Clinical Characteristics of the Study Groups

Subject	Number	Age (mean [SD])	Sex (M/F)[a]	HTLV-I proviral load[b] mean (SD)
HAM/TSP[c]	32	34–73 (57.8 [10.8])	11/21	2091.6 (3606.9)
Asymptomatic carrier	31	22–78 (55.3 [11.6])	10/21	608.9 (1159.9)
Healthy control	11	36–66 (49.4 [9.7])	1/10	N/A[d]

NOTE. [a] M/F: male/female.
[b] copies/10^4 cells.
[c] HAM/TSP: HTLV-I–associated myelopathy/tropical spastic paraparesis.
[d] N/A: not applicable.

and stained with an anti-IFN-γ–FITC antibody (Immunotech). For PD-1 detection, the cells were stained with anti-IFN-γ–biotin (eBioscience) followed by staining with a streptavidin–PC5 secondary antibody (Becton Dickinson). At least 3×10^5 $CD8^+$ cells were examined by flow cytometry.

CD107a Degranulation Assay

Cytolytic activity was assessed by flow cytometric quantification of the surface mobilization of CD107a (cluster of differentiation 107a, an integral membrane protein in cytolytic granules) [28]. PBMCs (1×10^6) from patients with HLA-A*02 were pulsed with 1 μM HTLV-I Tax11–19 or with the control influenza virus M1 peptide (GILGFVFTL) for 30 minutes; PBMCs from HLA-A*24 patients were pulsed with 1 μM HTLV-I Tax301–309 or with HIV Gag (RYLKDQQLL) peptide. Excess peptides were washed out and the cells were incubated with an anti-CD107a–PC5 antibody (Becton Dickinson [4 μL/mL]) in the presence of brefeldin A (5 μg/mL) for 4 hours. After harvesting, the cells were stained with a rat anti-Tim-3 antibody followed by an anti-rat IgG–Alexa Fluor 488 secondary antibody, or with an anti-PD-1–FITC antibody followed by staining with Tax tetramer–PE and an anti-CD8–ECD antibody. At least 1×10^5 $CD8^+$ T cells were examined by flow cytometry.

Quantitative Polymerase Chain Reaction of the HTLV-I Proviral Load

Genomic DNA was extracted from PBMCs by using the Qiagen DNA extraction kit (Qiagen). The measurements were performed as described elsewhere [7].

Intracellular HTLV-I Tax Staining

PBMCs (5×10^5) were cultured for 12 hours in complete medium in the presence of brefeldin A. After harvesting, the cells were stained with an anti-Tim-3 antibody followed by an Alexa Fluor 488-labeled secondary antibody, or with an anti-PD-1–FITC antibody and then stained with an anti-CD4–PC5 or anti-CD8–PC5 antibody. The cells were intracellularly stained with a mouse IgG3 anti-HTLV-I Tax antibody (clone Lt-4) [29] followed by a goat anti-mouse IgG3–PE antibody (Southern Biotech).

Statistical Analysis

Mann–Whitney *U* test, Wilcoxon signed-rank test, and Spearman's rank correlation test were performed using StatView software version 5.0 (SAS Institute). *P* values of less than .05 were considered significant.

RESULTS

Low Frequency of Tim-3^+ Cells Within $CD4^+$ and $CD8^+$ T cell Populations in HTLV-I Infection

Tim-3^+ cells within the lymphocyte gate were greatly reduced in asymptomatic carriers and HAM/TSP patients as compared with healthy controls (Figure 1*A*, upper row). We observed reduced frequencies of Tim-3-expressing $CD3^+CD4^+$ T cells in HTLV-I-infected individuals (mean [SD]: 2.59% [1.3%] for asymptomatic carriers and 2.62% [1.3%] for HAM/TSP patients) compared with those in healthy controls (3.72% [1.5%]) ($P = .031$ and $P = .034$, respectively [Figure 1*B*]). The same was observed on $CD3^+CD8^+$ T cells of infected individuals (7.19% [4.3%] for asymptomatic carriers and 7.54% [4.4%] for HAM/TSP patients) compared with those in healthy controls (10.6% [3.2%]) ($P = .026$ and $P = .021$, respectively [Figure 1*B*]). However, we observed increased mean fluorescent intensity (MFI) of Tim-3-expressing $CD4^+$ and $CD8^+$ T cells in asymptomatic carriers as compared with healthy controls ($P = .0031$ and $P = .046$, respectively [Figure 1*C*]). Conversely, we could not detect significant differences in Tim-3 expression (neither frequency nor MFI) on $CD4^+$ or $CD8^+$ T cells of HAM/TSP patients and asymptomatic carriers (Figures 1*B* and 1*C*). The frequency of Tim-3^+ cells within $CD4^+$ or $CD8^+$ T cells did not correlate with HTLV-I proviral loads in HAM/TSP patients, asymptomatic carriers, or when both groups were combined (data not shown).

Low Expression of Tim-3 on HTLV-I Tax-specific CTLs as compared With That on Cytomegalovirus-specific CTLs in HTLV-I Infection

Tim-3 expression on antigen-specific $CD8^+$ T cells was examined in 9 HLA-A*02 HAM/TSP patients using HLA/antigen tetramers, as shown in Figure 2*A*. We found significantly lower levels of Tim-3 on HTLV-I Tax-specific versus cytomegalovirus (CMV)–specific CTLs in HAM/TSP patients ($P = .038$ [Figure 2*B*]). The frequency of Tim-3–expressing Tax-specific CTLs was significantly lower than that in the total $CD8^+$ T-cell population ($P = .0077$ [Figure 2*B*]). The frequencies of Tax-specific CTLs in HLA-$A*02^+$ asymptomatic carriers were too low to reliably evaluate Tim-3 expression on these cells. Using PBMCs from 9 HAM/TSP patients and 10 asymptomatic carriers with HLA-A*24, we found that the frequency of Tim-3–expressing Tax-specific CTLs was also significantly lower than that in the total $CD8^+$ T cell population ($P = .0077$ and $P = .013$, respectively [Figures 2*C* and 2*D*]). We attempted to assess Tim-3 expression on CMV $tetramer^+$ cells in this HLA-A*24 group but the frequencies of CMV-specific CTLs were too small to reliably evaluate Tim-3 expression. As expected, the frequency of Tax-specific CTLs was higher in HAM/TSP patients than in asymptomatic carriers (Figure 2*E*). The frequency of Tim-3^+ cells in Tax-specific CTLs was not different between the 2 groups (Figure 2*F*). However, the MFI of Tim-3 in Tax-specific CTLs was significantly higher in asymptomatic carriers than in HAM/TSP patients ($P = .0084$ [Figure 2*G*]). In addition, we detected no correlation between the frequency of Tim-3^+ Tax-specific CTLs and HTLV-I proviral load, duration of illness, disease

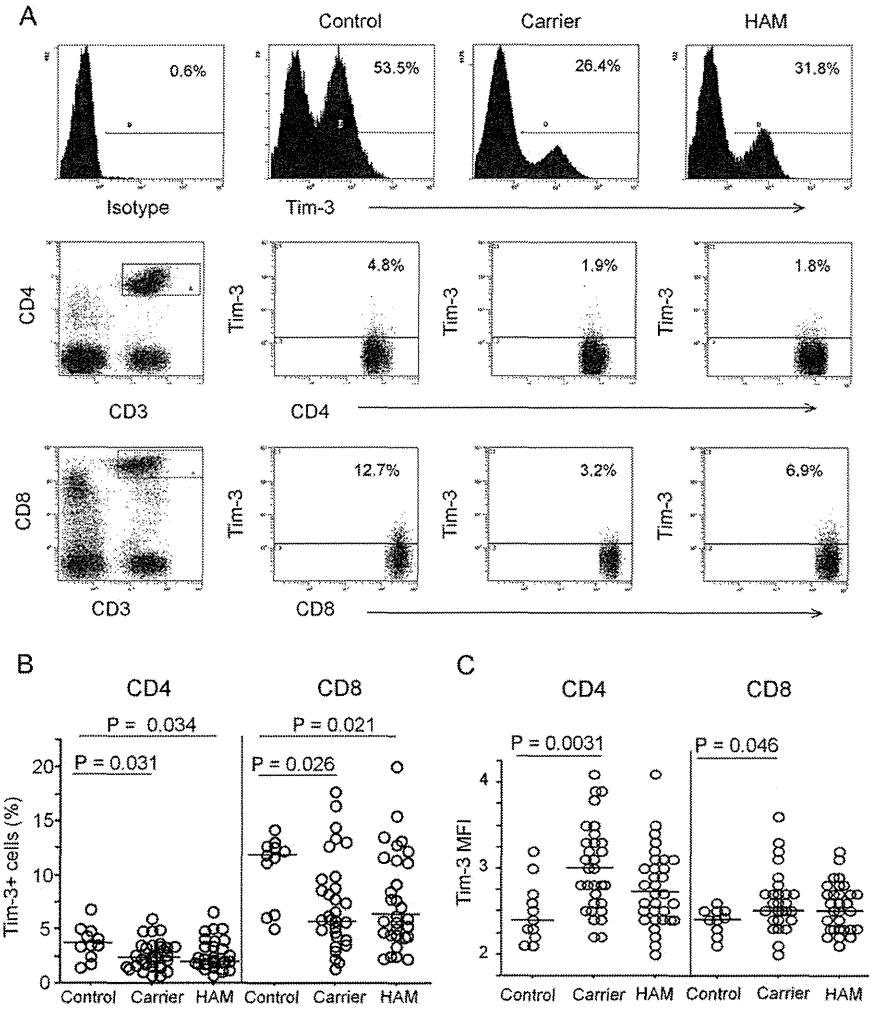

Figure 1. Low frequency of Tim-3$^+$ cells within the CD4$^+$ and CD8$^+$ T cell populations in HTLV-I infection. PBMCs from 63 HTLV-I–infected (32 HAM/TSP patients and 31 carriers) and 11 uninfected subjects were stained with antibodies against CD3, CD4, or CD8 and Tim-3. The numbers indicate the percentage of Tim-3$^+$ cells within each cell population. (*A*) Representative data from each group are shown in the last 3 columns. The upper row shows the expression levels of Tim-3 in total lymphocytes. The middle and lower rows show Tim-3 expression in CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ cells, respectively. (*B*) The combined data from all studied subjects reveal significantly lower percentages of Tim-3$^+$ cells within CD4$^+$ and CD8$^+$ T cell populations of HAM/TSP patients and carriers than those of controls. Each symbol represents an individual subject, and the horizontal bars indicate the medians. Data were analyzed by Mann–Whitney *U* test. (*C*) The combined data from all studied subjects reveal significantly higher MFI of Tim-3$^+$ cells in CD4$^+$ and CD8$^+$ T cell populations of carriers than those of controls. Data were analyzed by Mann–Whitney *U* test. Each symbol represents an individual subject, and the horizontal bars indicate the medians in each group.

NOTE: PBMCs: peripheral blood mononuclear cells; HAM/TSP: HTLV-I–associated myelopathy/tropical spastic paraparesis; Tim-3: T cell immunoglobulin and mucin domain-containing molecule-3; MFI: mean fluorescent intensity; HAM: HTLV-I–associated myelopathy/tropical spastic paraparesis.

activity, age of the patients, or serum HTLV-1 antibody titer (data not shown).

Increased PD-1 Expression on HTLV-I Tax-specific CTLs as Compared With That on CMV-specific CTLs

Since PD-1 has been also recognized as a marker for T cell exhaustion, we assessed PD-1 expression levels in 9 HAM/TSP patients, 8 asymptomatic carriers, and 10 healthy controls (Figure 3*A*). We could not detect a significant difference in PD-1 expression (neither frequency nor MFI) between HAM/TSP patients, asymptomatic carriers, and healthy controls in either CD4$^+$ or CD8$^+$ T cells (Figure 3*B*). However, we observed a significantly higher frequency of PD-1–expressing Tax-specific CTLs in asymptomatic carriers as compared with that in HAM/TSP patients (P = .043 [Figure 3*C*]). We assessed PD-L1 expression levels in all three groups. Since expression levels were relatively small (0.07–0.76%) in either CD3$^+$CD4$^+$ or CD3$^+$CD8$^+$ cells, we did not consider these results. Next, we analyzed PD-1 expression on antigen-specific cells (Figure 3*D*) and found significantly higher PD-1 expression on Tax-specific CTLs as compared with CMV-specific CTLs (P = .046

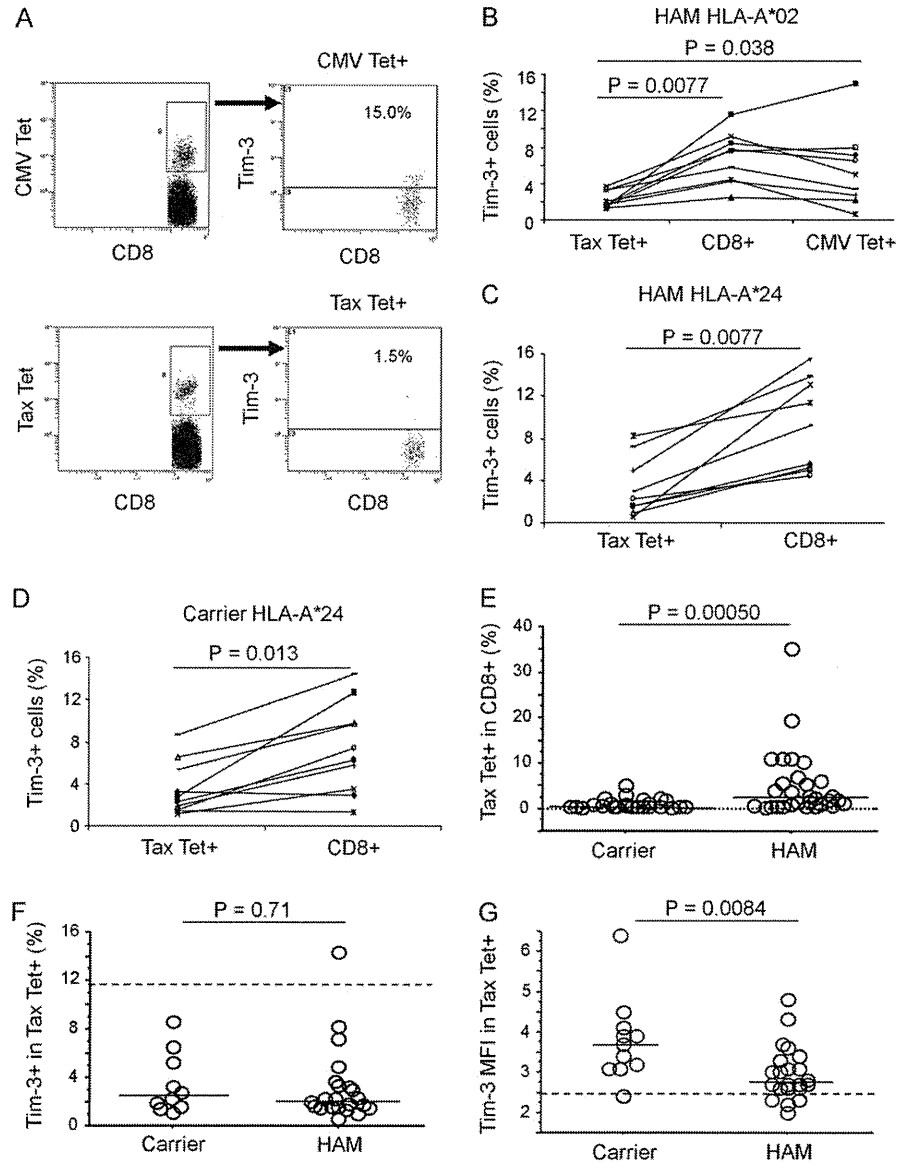

Figure 2. Low expression of Tim-3 on HTLV-I Tax-specific CTLs as compared with that on CMV-specific CTLs in HTLV-I infection. Tim-3 expression was determined in $CD8^+$, $CD8^+$Tax $tetramer^+$, and $CD8^+$CMV $tetramer^+$ cells of HAM/TSP patients and carriers. (*A*) A representative flow cytometry analysis depicts Tim-3 expression on $tetramer^+$ cells from a HAM/TSP patient. Gated $CD8^+tetramer^+$ cells were used for quantification of Tim-3^+ cells. The upper and bottom rows show Tim-3 expression in CMV-specific and HTLV-I Tax-specific CTLs. The numbers indicate the percentage of Tim-3^+ cells in each of the $tetramer^+$ cell populations. (*B*) The combined data from 9 HLA-A*02^+ HAM/TSP patients show significantly lower expression of Tim-3 in Tax-specific CTLs than in total $CD8^+$ T cells or CMV-specific CTLs, by Wilcoxon signed-rank test. (*C*, *D*) The combined data from 9 HAM/TSP patients and 10 carriers, all HLA-A*24^+, show significantly lower expression of Tim-3 in Tax-specific CTLs in comparison to total $CD8^+$ T cells, by Wilcoxon signed-rank test. (*E*) The percentage of Tax $tetramer^+$ cells within the $CD8^+$ cell population in HAM/TSP patients and carriers is depicted. Patients have significantly higher number of Tax $tetramer^+$ cells as compared with carriers, by Mann–Whitney *U* test. (*F*, *G*) Tim-3^+ cells in $CD8^+$Tax $tetramer^+$ cells of HAM/TSP patients and carriers are shown. There is no significant difference in the frequency of Tim-3^+ cells between the 2 groups. The carriers show significantly higher MFI of Tim-3 than do HAM/TSP patients. Data were analyzed by Mann–Whitney *U* test.

NOTE: In *E*–*G*, each symbol represents an individual subject and the horizontal bars indicate the medians in each group. In *F* and *G*, the dashed lines indicate the medians of Tim-3^+ cells within the $CD8^+$ cell population from healthy controls. Tim-3: T cell immunoglobulin and mucin domain-containing molecule-3; HAM/TSP: HTLV-I–associated myelopathy/tropical spastic paraparesis; CMV: cytomegalovirus; HTLV-I: human T-lymphotropic virus type I; CTLs: cytotoxic T lymphocytes; MFI: mean fluorescent intensity; HAM: HTLV-I–associated myelopathy/tropical spastic paraparesis; Tet: tetramer.

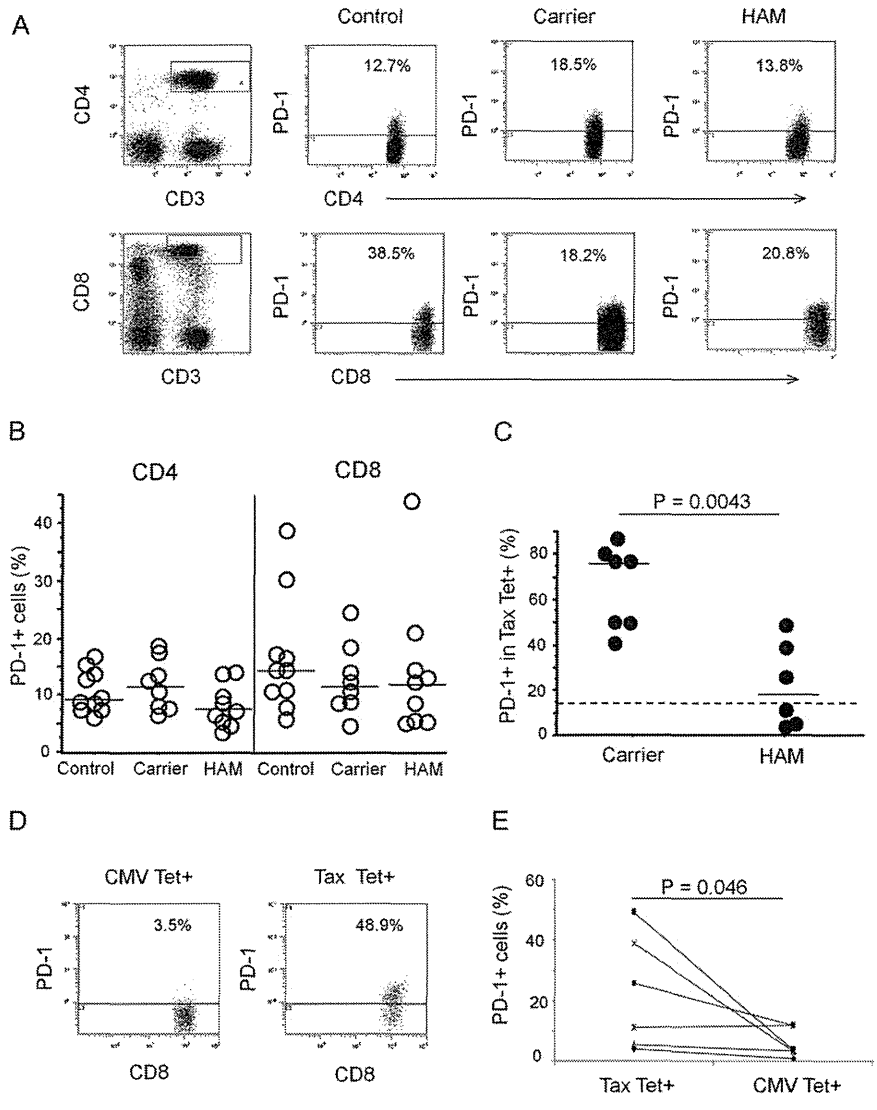

Figure 3. Increased PD-1 expression on HTLV-I Tax-specific CTLs as compared with that on CMV-specific CTLs. PD-1 expression was analyzed in PBMCs from 9 HAM/TSP patients, 8 carriers, and 10 controls after gating $CD3^+CD4^+$, $CD3^+CD8^+$, $CD8^+$Tax tetramer$^+$, or $CD8^+$CMV tetramer$^+$ cells. (*A*) The left column shows gated $CD3^+CD4^+$ and $CD3^+CD8^+$ cells. The last 3 columns show representative data of PD-1 expression in a control, a carrier, and a HAM/TSP patient after gating. (*B*) The combined data from all studied subjects show no significant difference in PD-1 expression between the 3 groups in $CD4^+$ or $CD8^+$ T cells, by Mann–Whitney *U* test. (*C*) The frequencies of PD-1^+ cells within $CD8^+$Tax tetramer$^+$ cells in HAM/TSP patients and carriers are shown. The carriers show significantly higher frequencies than HAM/TSP patients, by Mann–Whitney *U* test. The bars indicate the medians. The dashed line indicates the median value of PD-1^+ cells within the $CD8^+$ cell population from healthy controls. (*D*) The plots depict representative PD-1 expression in either $CD8^+$CMV tetramer$^+$ or $CD8^+$Tax tetramer$^+$ cells. Tax tetramer$^+$ cells show higher PD-1 expression than CMV tetramer$^+$ cells. (*E*) The combined data from 6 HAM/TSP patients show significantly higher expression of PD-1 in Tax tetramer$^+$ cells than in CMV tetramer$^+$ cells, by Wilcoxon signed-rank test.

NOTE: PD-1: programmed cell death-1; PBMCs: peripheral blood mononuclear cells; HAM/TSP: HTLV-I–associated myelopathy/tropical spastic paraparesis; CMV: cytomegalovirus; HAM: HTLV-I–associated myelopathy/tropical spastic paraparesis; Tet: tetramer.

[Figure 3*E*]). We detected no correlation between the frequency of PD-1^+ Tax-specific CTLs and HTLV-I proviral load, duration of illness, disease activity, age of the patients, or serum HTLV-1 antibody titer (data not shown). For technical reasons, we could not establish a double staining protocol for Tim-3 and PD-1.

Reduced IFN-γ Production by Tim-3^+ HTLV-I Tax-specific CTLs

We compared IFN-γ production after PMA/ionomycin stimulation between Tim-3^+ and Tim-3^- cells, or PD-1^+ and PD-1^- cells, within $CD8^+$ or Tax-specific CTL populations. As shown in Figures 4*A* and 4*D*, we determined the percentage of IFN-γ^+ cells after gating on either $CD8^+$ or $CD8^+$Tax tetramer$^+$ cells from HAM/TSP patients with a high percentage of tetramer$^+$ cells. IFN-γ was predominately produced by Tim-3^- cells, and less by Tim-3^+ cells in both groups (Figures 4*B* and 4*C*). Statistical analysis showed a significant difference in IFN-γ production (frequency and MFI) within $CD8^+$ cells (P = .043 and .043,