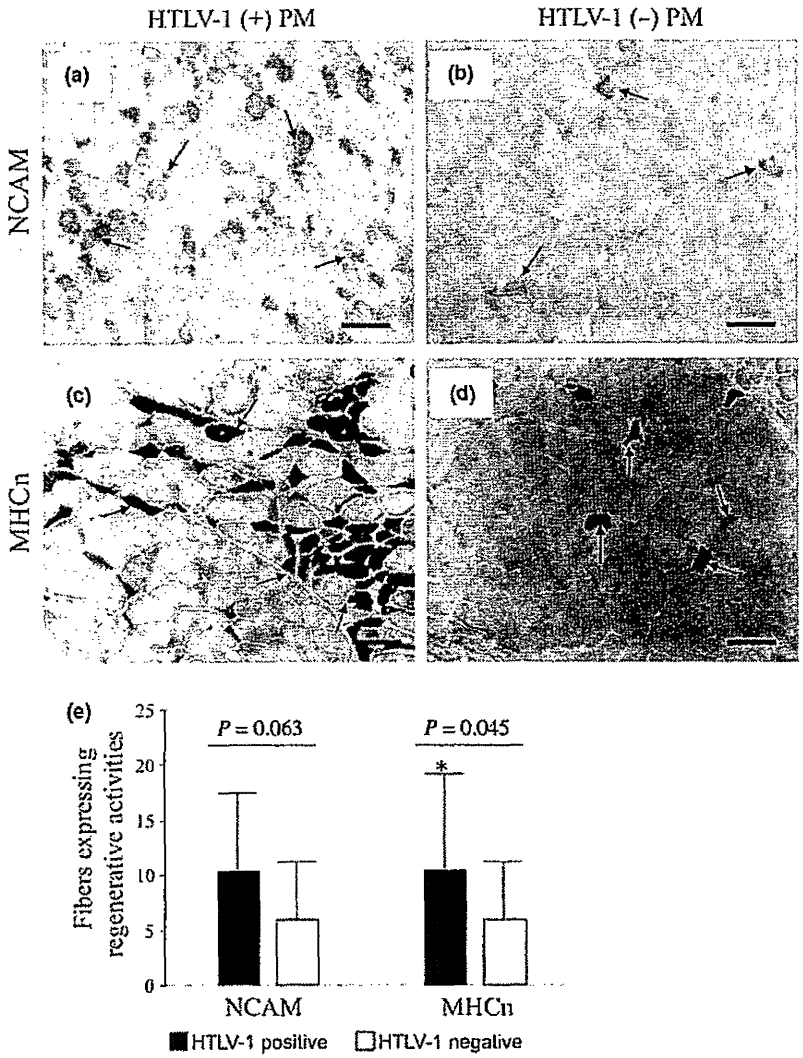

Figure 5 (a,b) Immunohistochemistry for neural cell adhesion molecule (NCAM) and (c,d) neonatal myosin heavy chain (MHCn). Myofibers show increased and decreased regenerative activity with NCAM (arrows) in (a) human T-lymphotropic virus type 1 (HTLV-1)-positive and (b) HTLV-1-negative polymyositis (PM), respectively. (c) Regenerative activity is more pronounced in HTLV-1-positive PM on MHCn staining (arrows). (d) Myofibers show decreased regenerative activity in HTLV-1-negative PM on MHCn staining (arrows). (e) Combined data for all study patients. An increase in the numbers of fibers showing regenerative activity in HTLV-1-positive PM with NCAM staining ($P = 0.063$, Mann–Whitney *U*-test) and significantly greater numbers of fibers showing regenerative activity with MHCn staining ($P = 0.045$; Mann–Whitney *U*-test) in HTLV-1-positive PM compared with HTLV-1-negative PM. Bars (a)–(d) 100 μm.

contrast, such abnormal mitochondria were not observed in normal controls or all five examined HTLV-1-negative patients (Fig. 7a,b). The percentage of mitochondria with morphological abnormalities was correlated with the number of partially reduced or completely CCO negative-stained fibers ($P = 0.0304$; Spearman's rank correlation; Fig. 7f).

Detailed histological, histochemical and electron microscopy findings in the skeletal muscle of all 21 PM patients are shown in Table 2.

Discussion

The present retrospective study included all patients with inflammatory myopathy who had undergone biopsy over the past 10 years at Kagoshima University Hospital, South Kyushu, Japan. From these patients, we selected our cohort after excluding patients with myositis associated with any other diseases or factors other than HTLV-1 that might affect our results.

On retrospective re-evaluation of all 21 selected patients using the modified criteria introduced by Dalakas and Hohlfeld,[7] six patients (29%) were diagnosed with definite PM; in the remaining 15 patients, probable myositis was diagnosed because of the absence of the CD8/MHC-1 complex, possibly a result of a minimal inflammatory reaction or patchy MHC-1 expression.[12–14]

In the present study, the incidence of PM was 31% (21/68), which is higher than that of other types of inflammatory myopathies. This might be

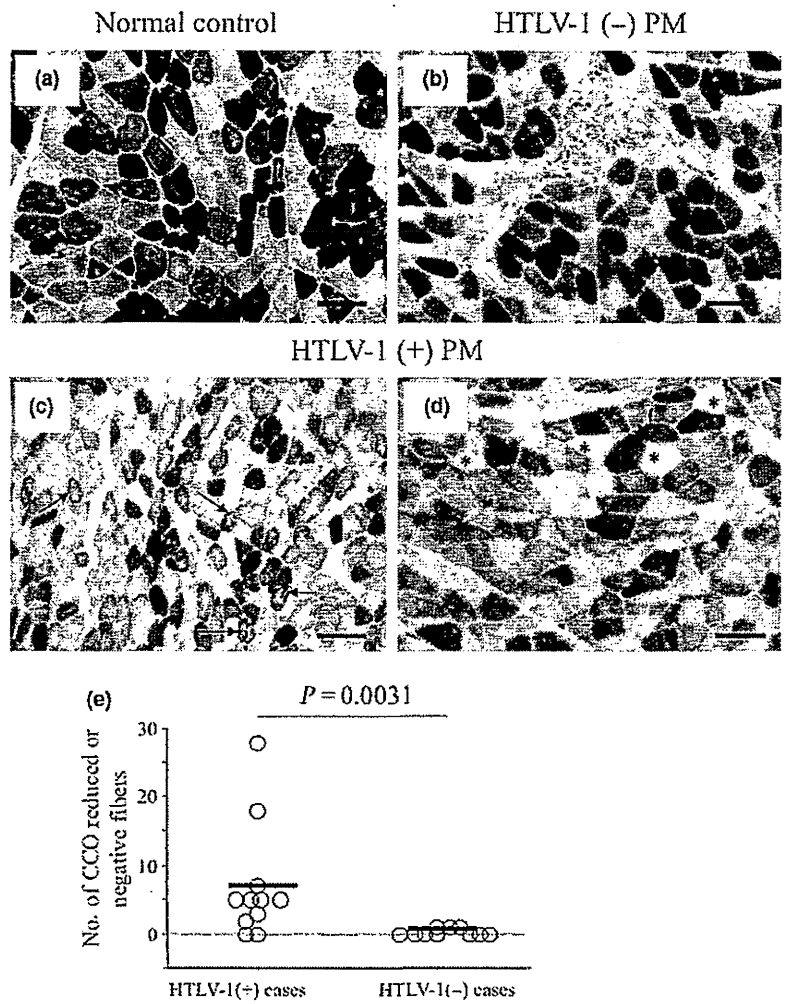

Figure 6 Enzyme histochemistry for cytochrome c oxidase (CCO). (a) Normal CCO staining in normal control showing two types of muscle fibers: type I (dark) and type II (light). (b) Normal CCO staining adjacent to inflammatory cells in human T-lymphotropic virus type 1 (HTLV-1)-negative polymyositis (PM). (c) Frequently observed partially reduced CCO-stained fibers with a mosaic appearance (arrows) in HTLV-1-positive PM. (d) Many completely CCO-negative fibers in HTLV-1-positive PM (stars). (e) Combined data for all study patients. Significantly greater numbers of partially reduced or completely CCO-negative fibers are seen in HTLV-1-positive than in HTLV-1-negative PM ($P = 0.0031$, Mann–Whitney *U*-test). Bars (a)–(d) 100 μm.

because the present study was carried out on patients with myositis in Kagoshima and Okinawa prefectures, where HTLV-1 is endemic and usually shows the PM phenotype. Therefore, the association of PM with HTLV-1 might not be coincidental. Using these 21 cases of PM, we assessed whether HTLV-1 affects the clinical course and histopathological features of myositis.[12,13]

To confirm HTLV-1 positivity, we carried out a highly sensitive nested PCR using DNA extracted from fresh frozen muscle biopsy specimens; we found that all HTLV-1-positive patients were concomitant on retrospective serological examination without any disparities. This was carried out as a confirmatory procedure to clarify the difference between HTLV-1-positive and HTLV-1-negative cases. Based on these results, we determined that there were 11 HTLV-1-seropositive and PCR positive PM cases; and 10 HTLV-1-seronegative and PCR negative PM cases.

Regarding the clinical course of the patients, we found that HTLV-1-positive PM patients show a more protracted course, because they have a significantly longer duration of illness from the onset of symptoms until the time of biopsy. The longer duration before biopsy might reflect that the disease requires more time to progress to such a degree that obligates patients to seek medical advice; at the time of biopsy, all patients had sufficient clinical and histological features for a diagnosis of myositis. Although further follow-up studies of the patients in this cohort are necessary to conclude the protracted clinical course of HTLV-1-positive PM, the results of the present study are consistent with those of previous studies carried out in Kyushu and Jamaica – both areas of endemic HTLV-1 infection[15] – that report HTLV-1-seropositive

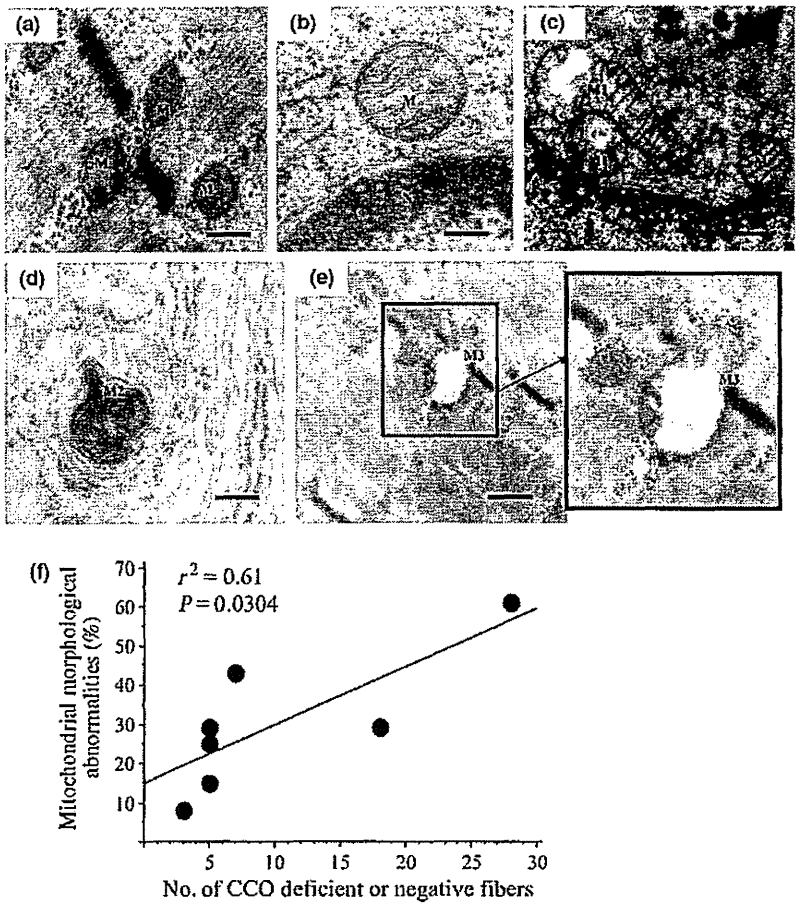

Figure 7 Ultrastructural examination of mitochondria. (a) Normal mitochondria near Z-discs in human T-lymphotropic virus type 1 (HTLV-1)-negative polymyositis (PM). (b) Normal mitochondria near the nucleus in HTLV-1-negative PM. (c) Mitochondria showing variable degrees of morphological abnormalities near the nucleus (M1) and other apparently normal mitochondria (M) in HTLV-1-positive PM. (d) Mitochondrion showing myelin-like formations in HTLV-1-positive PM (M2). (e) Well-fixed area showing many mitochondria; one of them shows abnormal high-amplitude swelling, cristae fragmentation, membrane interruption and loss of matrix in HTLV-1-positive PM (M3). (f) The percentages of mitochondria with morphological abnormalities is correlated with the counts of CCO-reduced or CCO-negative stained fibers ($P = 0.0304$; Spearman's rank correlation). Bars (a), (e) 0.3 μm; bars (b)–(d) 0.2 μm.

PM patients having more frequent hospital admissions and significantly longer durations of symptoms before presentation.[16–18]

At the histopathological level, although both groups showed the classical features of PM, we found the following significant features of the HTLV-1-positive PM group compared with the HTLV-1-negative PM group: (i) endomysial inflammatory infiltrates are more prominent; (ii) necrotic fibers are observed less frequently; (iii) regenerative activities are more prominent; and (iv) partial or complete CCO reduction is more frequent. The present results show significantly higher endomysial infiltration of $CD8^+$ and $CD4^+$ T-cells in HTLV-1-positive PM; some of the infiltrating $CD4^+$ cells in muscle lesions are considered to be the HTLV-1 genome-harboring cells, as we have reported in previous studies.[19,20]

We also observed the accumulation of $CD8^+$ T-cells around aberrant normal myofibers. This suggests that myositis in patients with HTLV-1 infections is not caused by direct viral infection of muscle fibers, but by an immune reaction between HTLV-1-infected $CD4^+$ cells and HTLV-1-specific $CD8^+$ cytotoxic T-cells damaging muscle fibers.[21–23] This characteristic inflammatory process associated with HTLV-1 is also detected in other tissues, such as tissues of the central nervous system in patients with HAM/TSP[24] and HTLV-1 uveitis in the eye.[25]

Regarding necrosis and regeneration, the results obtained from the present study were contrary to our expectations and were paradoxical to the general rule that regeneration follows necrosis; therefore, we preferred to designate them as regenerative processes (i.e., processes for mending or maintaining partially damaged muscle fibers) rather than regeneration. HTLV-1 was recently linked to regenerative activities via $p12^I$, which is an accessory hydrophilic protein of HTLV-1 that might contribute to and amplify the physiological stimulation of cells for proliferation.[26,27]

We speculate that such upregulation of regenerative processes contributes to the protracted course of

Table 2 Detailed histological, histochemical and electron microscopy findings in skeletal muscle of all 21 polymyositis patients

Case	Fiber diameter (μm)	Necrotic fibers	CCO negative fibers	SDH staining profile	Rimmed vacuoles	RRF	Fiber type atrophy	Neurogenic involvement	Glycogen and lipid content	Abnormal MT (%)
Po 1	10–60	+	++	N	–	–	Both types atrophy	–	N	43
Po 2	8–50	+	–	N	–	–	Type II atrophy	–	N	NA
Po 3	10–70	+	+++	+	–	–	Type II atrophy	–	N	61
Po 4	10–70	–	+	N	–	–	Type II atrophy	–	N	NA
Po 5	10–50	+	+	N	–	–	Both types atrophy	–	N	29
Po 6	10–80	–	–	N	–	–	Both types atrophy	–	N	NA
Po 7	5–60	–	+	N	–	–	Both types atrophy	–	N	25
Po 8	8–60	+	+	N	–	–	Type II atrophy	–	N	8
Po 9	10–60	–	++	N	–	–	Both types atrophy	–	N	29
Po 10	10–60	–	+	N	–	–	Both types atrophy	–	N	15
Po 11	5–60	–	+	N	–	–	Type II atrophy	–	N	NA
Neg 1	10–50	+	+	N	–	–	Type II atrophy	–	N	–
Neg 2	8–50	+	–	N	–	–	Type II atrophy	–	N	NA
Neg 3	10–60	++	–	N	–	–	Both types atrophy	–	N	–
Neg 4	10–70	+++	–	N	–	–	Both types atrophy	–	N	NA
Neg 5	10–70	+	+	N	–	–	Both types atrophy	–	N	–
Neg 6	8–70	+	–	N	–	–	Type II atrophy	–	N	NA
Neg 7	5–60	+	–	N	–	–	Both types atrophy	–	N	–
Neg 8	10–60	++	–	+	–	–	Both types atrophy	–	N	NA
Neg 9	5–70	+	+	N	–	–	Both types atrophy	–	N	–
Neg 10	10–70	+	–	N	–	–	Both types atrophy	–	N	NA

–, Absent; +, 1–2/200 fibers, mild; ++, 3–5/200 fibers, moderate; +++, more than 5/200 fibers, frequent. Abnormal MT % is the percentage of abnormal mitochondria. CCO, cytochrome c oxidase; MT, mitochondria; N, normal; NA, not assessed; RRF, ragged red fibers; SDH, succinate dehydrogenase.

HTLV-1-positive PM. These regenerative processes might be continuous but not complete, as fibers showing such processes appear slightly smaller than others in addition to upregulation of neonatal proteins.

We observed a reduction in CCO activity in HTLV-1-positive PM with intact normal staining profiles of SDH. CCO is an enzyme that is localized in the inner membrane and cristae of mitochondria, which are multifunctional and highly complex organelles.[28] This finding attracted our attention; therefore, we examined the mitochondrial ultrastructure using electron microscopy. Mitochondrial morphological abnormalities were more frequently observed in HTLV-1-positive than in HTLV-1-negative patients or normal control subjects. Therefore, these mitochondrial morphological abnormalities are more likely to be related to HTLV-1 infection. In particular, we found a significant correlation between these morphological abnormalities and the numbers of CCO-deficient or CCO-negative fibers. Such a link between HTLV-1 and similar mitochondrial morphological abnormalities was previously observed *in vitro* and is reported to be caused by $p13^{II}$, an accessory protein of HTLV-1.[23] In addition, $p13^{II}$ accumulates in the inner mitochondrial membranes and causes mitochondrial morphological abnormalities by altering membrane permeability.[29]

It would be interesting if future studies could detect such viral proteins and confirm their relationship to the present findings *in vivo*.

In summary, we observed the clinical and morphological differences of muscle biopsy specimens between cases of HTLV-1-positive and HTLV-1-negative PM. Although these differences were significant, they were not specific to HTLV-1-positive PM, because they were also observed (although less frequently) in HTLV-1-negative PM. These results suggest that HTLV-1 is responsible for modifying the clinical course and the histopathological features of myofibers observed in the present study.

Acknowledgements

The present study was supported by a Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Science and research grants from the Health Sciences Research Grants from the Ministry

of Health, Labor, and Welfare in Japan. We thank Ms N. Hirata and Ms T. Inoue for their excellent technical assistance.

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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. $Tim\text{-}3^+$, but not $PD\text{-}1^+$, 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 $Tim\text{-}3^-$ 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

Received 13 July 2010; accepted 15 November 2010.
Potential conflicts of interest: none reported.
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The Journal of Infectious Diseases 2011;203:948–59

1537-6613/2011/2037-0001$15.00
DOI: 10.1093/infdis/jiq153

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

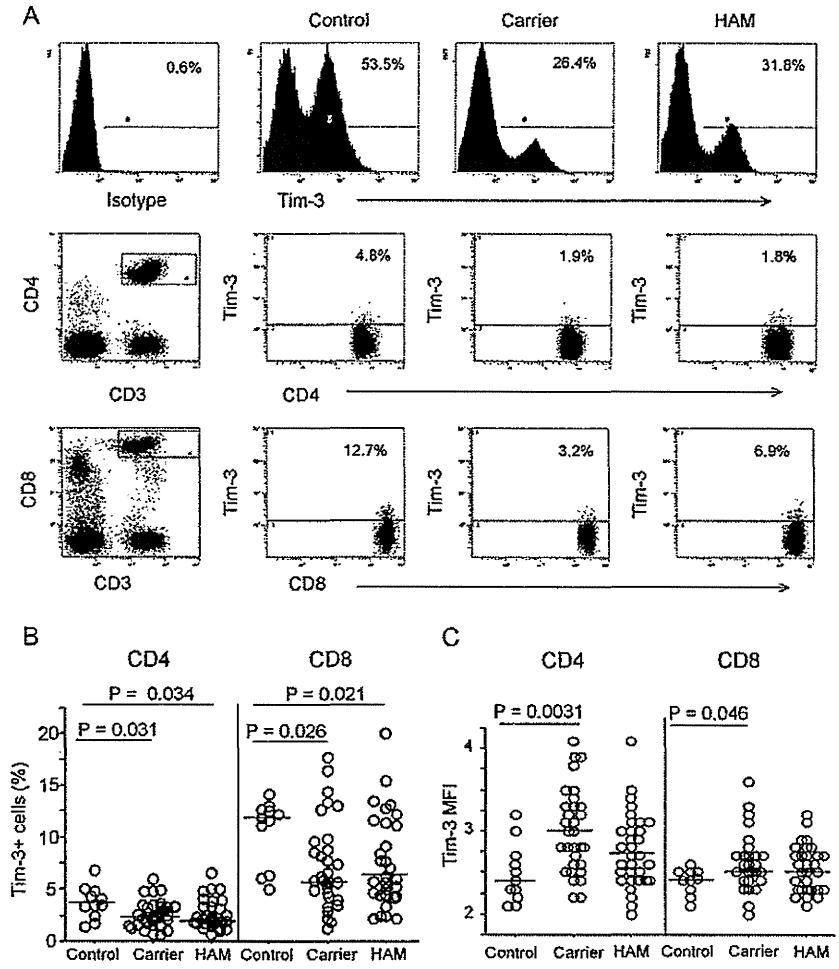

Figure 1. Low frequency of Tim-3$^+$ cells within the CD4$^+$ and CD8$^+$ T cell populations in HTLV-I infection. PBMCs from 63 HTLV-I–infected (32 HAM/TSP patients and 31 carriers) and 11 uninfected subjects were stained with antibodies against CD3, CD4, or CD8 and Tim-3. The numbers indicate the percentage 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

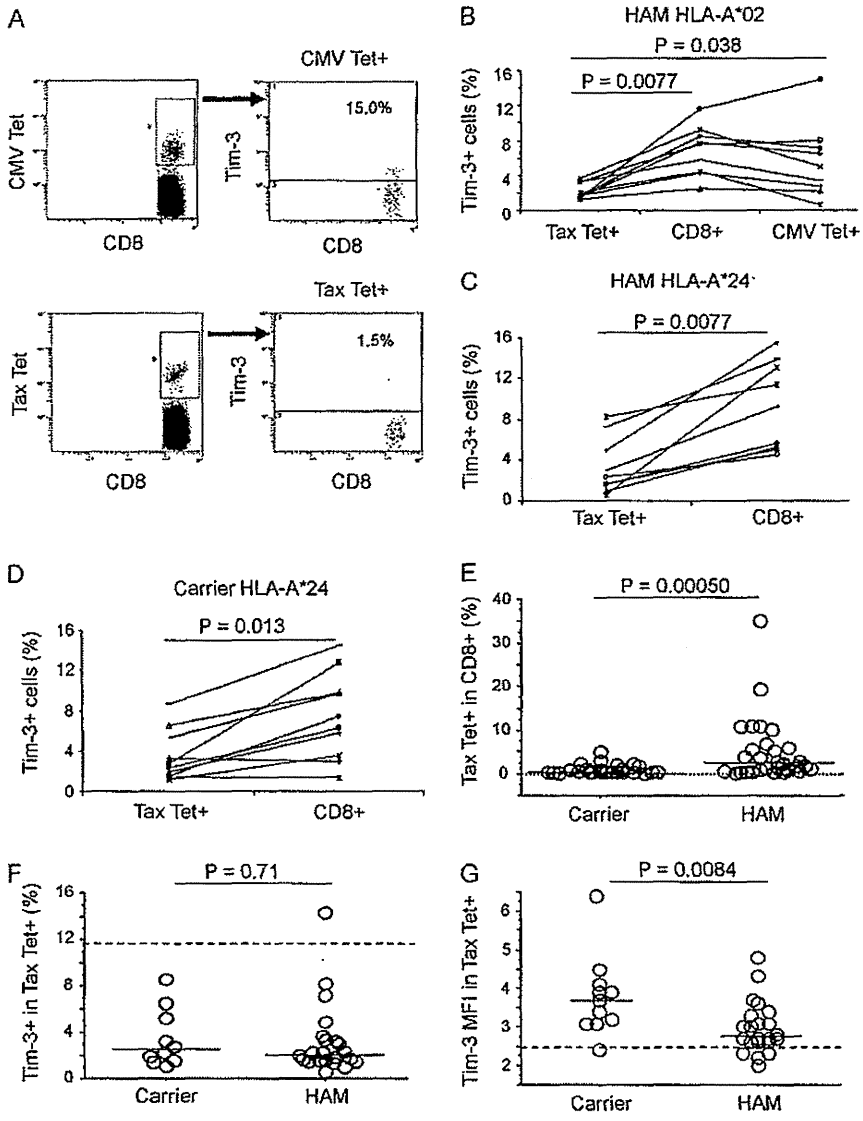

Figure 2. Low expression of Tim-3 on HTLV-I Tax-specific CTLs as compared with that on CMV-specific CTLs in HTLV-I infection. Tim-3 expression was determined in $CD8^+$, $CD8^+$Tax tetramer$^+$, and $CD8^+$CMV tetramer$^+$ cells of HAM/TSP patients and carriers. (*A*) A representative flow cytometry 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.

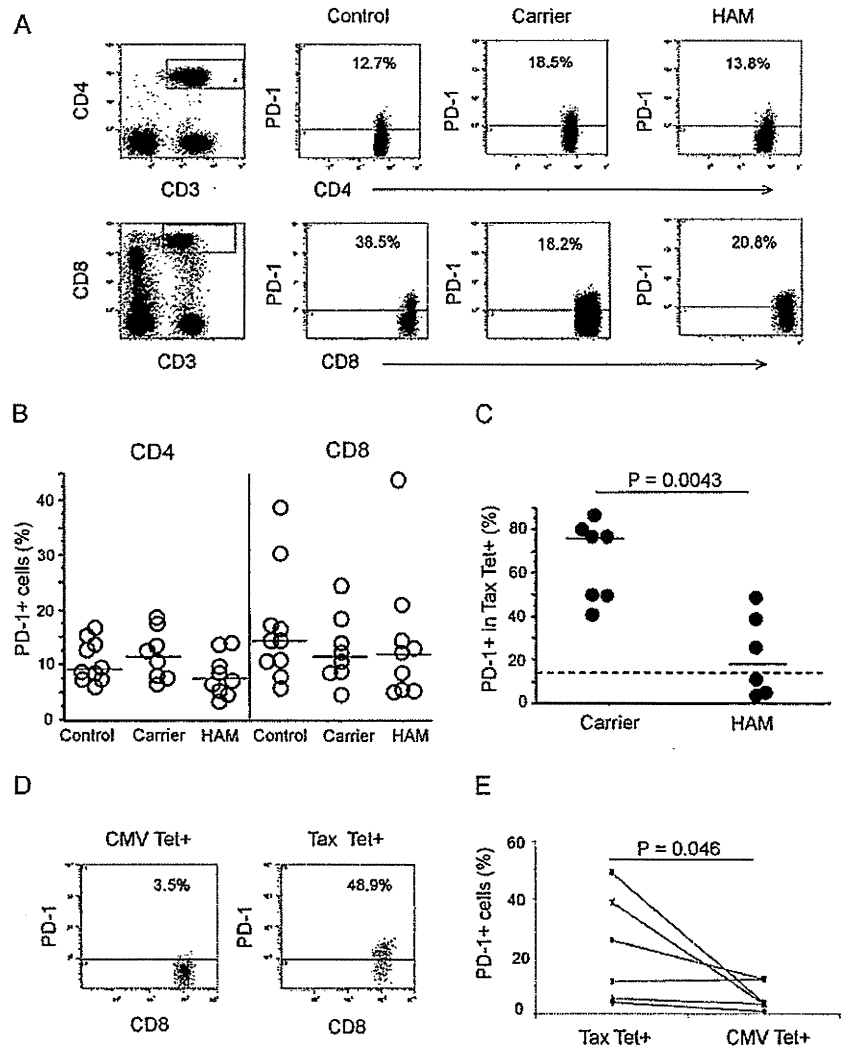

Figure 3. Increased PD-1 expression on HTLV-I Tax-specific CTLs as compared with that on CMV-specific CTLs. PD-1 expression was analyzed in PBMCs from 9 HAM/TSP patients, 8 carriers, and 10 controls after gating $CD3^+CD4^+$, $CD3^+CD8^+$, $CD8^+$Tax tetramer$^+$, or $CD8^+$CMV tetramer$^+$ cells. (*A*) 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,

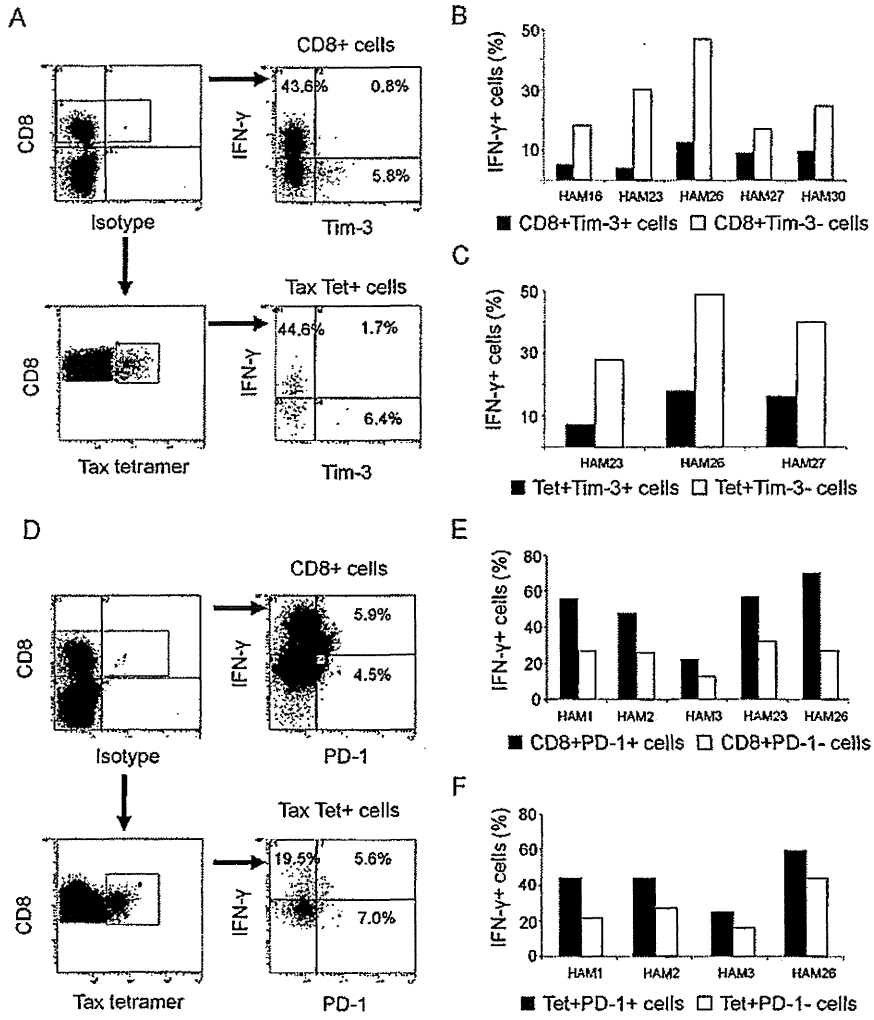

Figure 4. Reduced IFN-γ production by Tim-3$^+$ HTLV-I Tax-specific CTLs. PBMCs from 5 HAM/TSP patients were stimulated with PMA and ionomycin, and cultured for 6 hours in the presence of brefeldin A. IFN-γ production was determined by flow cytometry in CD8$^+$ and CD8$^+$Tax tetramer$^+$ cells with or without Tim-3 or PD-1 expression. (*A, D*) Representative data from a HAM/TSP patient are shown. The upper and lower rows show the percentage of IFN-γ$^+$ cells in gated CD8$^+$ and CD8$^+$Tax tetramer$^+$ cell populations, respectively. In *A*, Tim-3$^+$ cells within CD8$^+$ and Tax tetramer$^+$ cell populations have a lower percentage of INF-γ$^+$ cells than do Tim-3$^-$ cells. In *D*, PD-1$^+$ cells within CD8$^+$ and Tax tetramer$^+$ cell populations have a higher percentage of INF-γ$^+$ cells than do PD-1$^-$ cells. (*B*) Summary data from 5 HAM/TSP patients show a significantly lower percentage of INF-γ$^+$ cells within the CD8$^+$Tim-3$^+$ cell population than within the CD8$^+$Tim-3$^-$ one, after background subtraction ($P = .043$ by Wilcoxon signed-rank test). (*C*) Summary data from 3 HAM/TSP patients with high percentage of CTLs show a lower percentage of INF-γ$^+$ cells within the Tax tetramer$^+$Tim-3$^+$ cell population than within the Tax tetramer$^+$Tim-3$^-$ one, after background subtraction. (*E*) Summary data from 5 HAM/TSP patients show a significantly higher percentage of INF-γ$^+$ cells within the CD8$^+$PD-1$^+$ cell population than within the CD8$^+$PD-1$^-$ one ($P = .043$ by Wilcoxon signed-rank test). (*F*) Summary data from 4 HAM/TSP patients with high percentage of CTLs show a higher percentage of INF-γ$^+$ cells within the Tax tetramer$^+$PD-1$^+$ cell population than within the Tax tetramer$^+$ PD-1$^-$ one.

NOTE: PBMCs: peripheral blood mononuclear cells; HAM/TSP: HTLV-I–associated myelopathy/tropical spastic paraparesis; PMA: phorbol 12-myristate 13-acetate; INF-γ: interferon-γ; Tim-3: T cell immunoglobulin and mucin domain-containing molecule-3; PD-1: programmed cell death-1; CTLs: cytotoxic T lymphocytes; Tet: tetramer.

respectively). Conversely, IFN-γ was predominately produced by PD-1$^+$ cells and less by PD-1$^-$ cells in both groups (Figures 4*E* and 4*F*). Statistical analysis showed a significant difference in IFN-γ production within CD8$^+$ cells, as measured by frequency ($P = .043$). However, no difference was observed in the MFI.

Reduced CD107a Expression on Tim-3$^+$ HTLV-I Tax-specific CTLs

To assess the cytolytic activity of HTLV-I Tax-specific CTLs with or without Tim-3 or PD-1 expression, we measured CD107a expression after specific peptide stimulation of Tax-specific

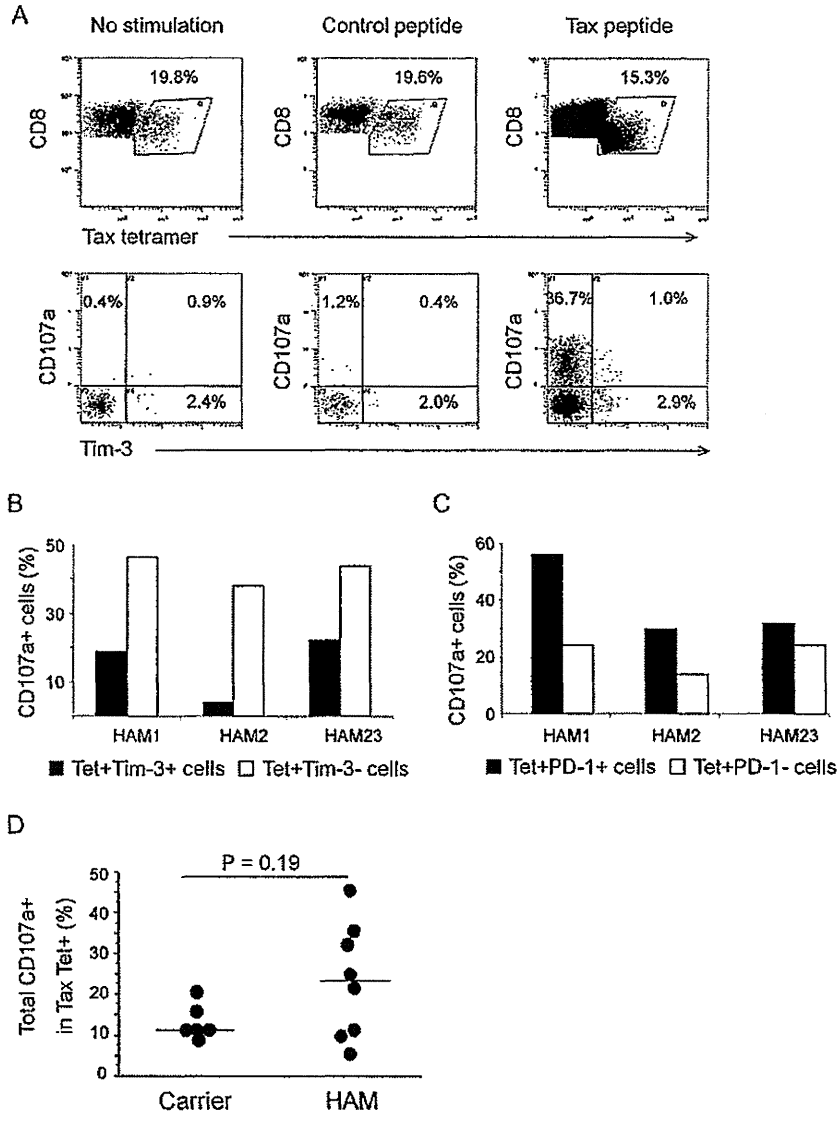

Figure 5. Reduced CD107a expression on Tim-3$^+$ HTLV-I Tax-specific CTLs. PBMCs from 8 HAM/TSP patients and 6 carriers were stimulated with HTLV-I Tax peptide or a control peptide, and cultured in the presence of an anti-CD107a antibody and brefeldin A for 4 hours. The expression of CD107a on CD8$^+$Tax tetramer$^+$ cells was analyzed. (*A*) Representative data from a HAM/TSP patient are shown. In the upper row, Tax peptide-stimulated Tax tetramer$^+$ cells show a parallel decrease in fluorescence intensity for CD8 and Tax tetramer. The same was not observed with the control peptide. The percentage of tetramer$^+$ cells is reduced after Tax peptide stimulation. In the lower row, the frequency of CD107a-expressing cells is analyzed in tetramer$^+$Tim-3$^+$ and tetramer$^+$Tim-3$^-$ cells. Tetramer$^+$Tim-3$^+$ cells show a lower percentage of CD107a$^+$ cells than tetramer$^+$Tim-3$^-$ cells. (*B, C*) Three HAM/TSP patients from whom more than 10^4 Tax-tetramer$^+$ cells could be collected were chosen for a precise evaluation. (*B*) The summary data show low CD107a expression in tetramer$^+$Tim-3$^+$ cells in comparison with tetramer$^+$Tim-3$^-$ cells, after background subtraction. (*C*) The summary data show high CD107a expression in tetramer$^+$ PD-1$^+$ cells in comparison to tetramer$^+$ PD-1$^-$ cells. (*D*) The percentage of CD107a$^+$ cells within Tax tetramer$^+$ cells from 8 HAM/TSP patients and 6 carriers is shown. No significant difference was observed in the percentage of CD107a$^+$ cells between the 2 groups ($P = .19$ by Mann–Whitney *U* test). The bars indicate the medians.

NOTE: PBMCs: peripheral blood mononuclear cells; HAM/TSP: HTLV-I–associated myelopathy/tropical spastic paraparesis; HTLV-I: human T-lymphotropic virus type I; CD107a: cluster of differentiation 107a; HAM: HTLV-I–associated myelopathy/tropical spastic paraparesis; Tet: tetramer.

CTLs from 8 HAM/TSP patients and 6 asymptomatic carriers. Representative data from a HAM/TSP patient are shown in Figure 5*A*. Specific antigen-induced CD107a expression was higher in tetramer$^+$Tim-3$^-$ cells than in tetramer$^+$Tim-3$^+$ cells. At the same time, CD107a expression was higher in tetramer$^+$PD-1$^+$ cells than in tetramer$^+$PD-1$^-$ cells from 3 HAM/TSP patients from whom we could collect more than 10^4 tetramer$^+$ cells for a more precise evaluation (Figures 5*B* and 5*C*).

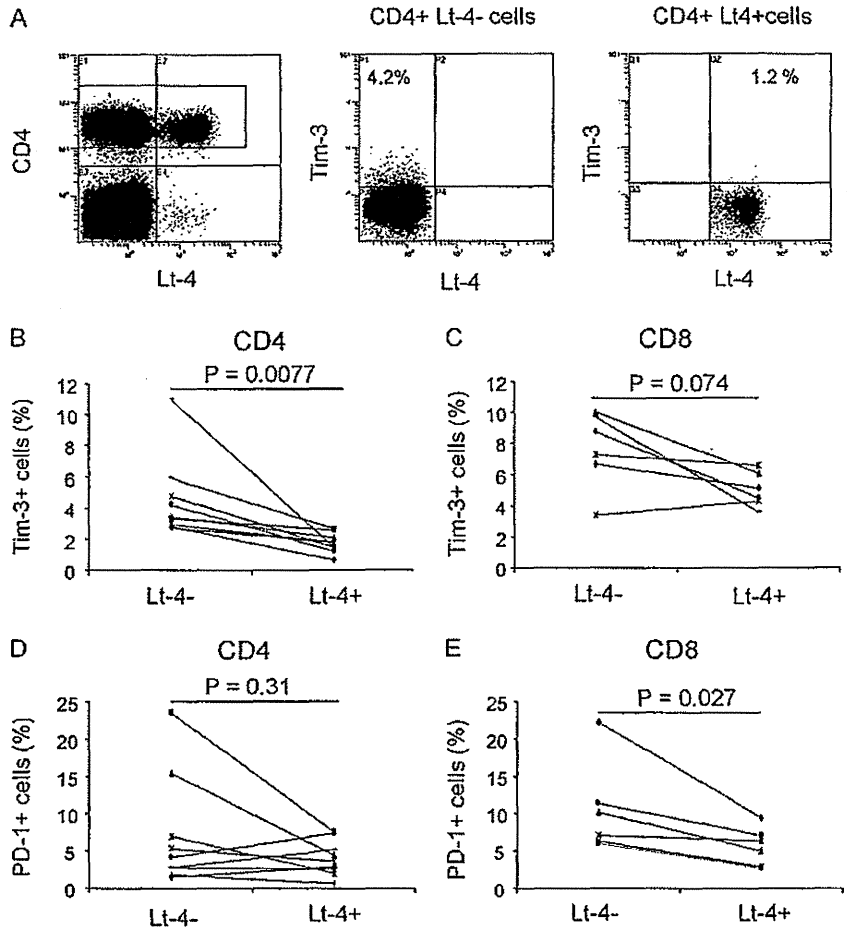

Figure 6. Low expression of Tim-3 on HTLV-I–infected cells. PBMCs from 9 HAM/TSP patients were cultured for 12 hours. Cells were double stained for the intracellular HTLV-I Tax protein, using the Lt-4 antibody, and the cell surface Tim-3 or PD-1. (*A*) After gating on $CD4^+$ cells, expression of Tim-3 was analyzed in either $CD4^+Lt\text{-}4^+$ or $CD4^+Lt\text{-}4^-$ cells. Representative data from a HAM/TSP patient show low percentage of $Tim\text{-}3^+$ cells in $CD4^+Lt\text{-}4^+$ cells in comparison to $CD4^+Lt\text{-}4^-$ cells. (*B, D*) Combined data from 9 HAM/TSP patients show significantly lower Tim-3 expression in $CD4^+Lt\text{-}4^+$ cells than in $CD4^+Lt\text{-}4^-$ cells. No significant difference in PD-1 expression between both groups was found by Wilcoxon signed-rank test. (*C, E*) Combined data from 6 HAM/TSP patients show that Tim-3 expression tended to be lower in $CD8^+Lt\text{-}4^+$ cells than in $CD8^+Lt\text{-}4^-$ cells and that PD-1 expression was significantly lower in $CD8^+Lt\text{-}4^+$ cells than in $CD8^+Lt\text{-}4^-$ cells, by Wilcoxon signed-rank test.

NOTE: PBMCs: peripheral blood mononuclear cells; HAM/TSP: HTLV-I–associated myelopathy/tropical spastic paraparesis; HTLV-I: human T-lymphotropic virus type I; Tim-3: T cell immunoglobulin and mucin domain-containing molecule-3; PD-1: programmed cell death-1.

Furthermore, when we reanalyzed the frequency or MFI of $CD107a^+$ cells within the Tax $tetramer^+$ cell population, we could not detect a significant difference between HAM/TSP patients and asymptomatic carriers (Figure 5*D*). Also, we could not detect a significant correlation between the frequency of $CD107a^+$ cells and HTLV-I proviral load (data not shown).

Low Expression of Tim-3 on $CD4^+$ HTLV-I–infected Cells

To assess Tim-3 expression on HTLV-I–infected cells, we cultured PBMCs from 9 HAM/TSP patients for 12 hours in order to induce the expression of the HTLV-I Tax protein [30]. After harvesting, Tax protein was simultaneously detected with Tim-3 or PD-1 (Figure 6*A*). We observed that Tim-3 expression was significantly lower in Tax^+ $CD4^+$ cells ($Lt\text{-}4^+$ cells) than in Tax^-CD4^+ cells ($P = .0077$ [Figure 6*B*]). On the contrary, we observed no significant differences in PD-1 expression between Tax^+CD4^+ and Tax^-CD4^+ cells ($P = .31$ [Figure 6*D*]). In addition, we assessed the expression of Tim-3 or PD-1 in infected $CD8^+$ cells from 6 cases that showed a reasonable percentage of infected $CD8^+$ cells. We found that Tim-3 expression tended to be lower in Tax^+CD8^+ cells than in Tax^-CD8^+ cells ($P = .074$ [Figure 6*C*]), whereas PD-1 expression was significantly lower in Tax^+CD8^+ cells than in Tax^-CD8^+ cells ($P = .027$ [Figure 6*E*]). No significant correlations were observed between the MFI of Lt-4–positive cells and the frequency or

MFI of Tim-3 or PD-1 within $CD4^+$ or $CD8^+$ cells (data not shown). The percentage of infected $CD4^+$ and $CD8^+$ cells in asymptomatic carriers or $tetramer^+CD8^+$ cells in HAM/TSP patients was too small to assess Tim-3 or PD-1 expression.

CONCLUSIONS

We found that the proportion of $Tim\text{-}3^+$ cells within $CD4^+$ and $CD8^+$ T cell populations of HTLV-I–infected individuals (both HAM/TSP patients and asymptomatic carriers) is significantly lower than in healthy controls (Figure 1). This reduction was much clearer in Tax-specific CTLs because the frequency of Tim-3–expressing cells in CTLs was lower than in the total $CD8^+$ population of infected individuals (Figures 2*B–D*). In addition, $Tim\text{-}3^+$ cell frequency in HTLV-I Tax-specific CTLs was significantly lower than in CMV-specific CTLs from HAM/TSP patients (Figure 2*B*). Interaction of Tim-3 with its ligand, galectin-9, regulates Th1 cell responses by promoting the death of IFN-γ–producing Th1 cells, suggesting that Tim-3 may play a role in suppressing Th1-mediated immune responses [18]. Our results showing that the frequency of $Tim\text{-}3^+$ cells is reduced within $CD4^+$ and $CD8^+$ T cells in HTLV-I infection strongly suggest that the Th1/Tc1 immune response is not negatively regulated by Tim-3 in HTLV-I infection. Rather, immune cells such as HTLV-I–specific CTLs may be resistant to cell death through the Tim-3/galectin-9 pathway [18]. In this sense, the increased number of $Tim\text{-}3^-$ HTLV-I Tax-specific CTLs may contribute to the control of viral replication. In the present study, we found that IFN-γ production was decreased in $CD8^+$ cells and HTLV-I Tax-specific CTLs that expressed Tim-3 as compared with their $Tim\text{-}3^-$ counterparts in HAM/TSP patients (Figure 4). In addition, CD107a expression was lower in $Tim\text{-}3^+$ HTLV-I Tax-specific CTLs from HAM/TSP patients (Figure 5). These results indicate that Tim-3 identifies a subset of CTLs with impaired production of cytokines and cytolytic activity. The decreased expression of Tim-3 in HTLV-I infection is in marked contrast to other chronic viral infections such as HIV and HCV infections, where Tim-3 expression is increased in T cells, including the virus-specific CTLs [19, 20]. It would be of interest to determine whether Tim-3 expression is also reduced in other chronic viral infections and to clarify the mechanisms underlying Tim-3 down-regulation in HTLV-I infection.

Interestingly, our data demonstrated that Tim-3 and CD107a expression in HTLV-I Tax-specific CTLs was not significantly different between HAM/TSP patients and asymptomatic carriers (Figures 2*F* and 5*D*); however, Tim-3 MFI was higher in asymptomatic carriers than in HAM/TSP patients. Our data suggest that the killing activity of the CTLs is not different between the 2 groups. Controversially, others have reported that CD107a expression is lower in HTLV-I Tax-specific CTLs from HAM/TSP patients than from asymptomatic carriers, and that CTL function is impaired in HAM/TSP patients as compared with asymptomatic carriers [24]. This controversy may result from differences in sample type and procedures, including the gating for $tetramer^+$ cells after antigen stimulation. To address this issue, more detailed analyses of HTLV-I–specific CTL function in HAM/TSP patients and asymptomatic carriers would be necessary to ascertain whether differences could define the clinical condition.

In this study, we found that PD-1 expression levels on T cells of HAM/TSP patients and asymptomatic carriers were not different from those of healthy controls. However, we observed that PD-1 expression was significantly higher in HTLV-I Tax-specific CTLs than in CMV-specific CTLs (Figure 3*E*) and significantly higher in HTLV-I Tax-specific CTLs from asymptomatic carriers than from HAM/TSP patients. This result is in partial agreement with a previous study on HTLV-I infection, in which a marked increase of PD-1 expression was found in HTLV-I Tax-specific CTLs from both asymptomatic carriers and ATL patients as compared with CMV- and EBV-specific CTLs [31]. We found that IFN-γ production was higher in $CD8^+$ cells and HTLV-I Tax-specific CTLs that expressed PD-1 as compared with their $PD\text{-}1^-$ counterparts in HAM/TSP patients (Figures 4*E* and 4*F*). In addition, CD107a expression was higher in $PD\text{-}1^+$ HTLV-I Tax-specific CTLs of HAM/TSP patients (Figure 5*C*). These results indicate that $PD\text{-}1^+$ HTLV-I Tax-specific CTLs are capable of producing proinflammatory cytokines and have high cytolytic activity during HTLV-I infection. An increase in IFN-γ production by $PD\text{-}1^+$ T cells has been recently shown in simian immunodeficiency virus (SIV) infection and in an animal model of autoimmune nephritis [32, 33]. Interestingly, $PD\text{-}1^+$ cells were predominantly detected within $CD107a^+$ antigen-specific T cells in SIV infection [34]. In this context, it is proposed that the primary mechanism by which PD-1 affects $CD8^+$ T cell function involves regulation of cell proliferation and survival [32, 35]. Our results suggest that HTLV-I Tax-specific CTLs exhibit an increased expression of PD-1, albeit a reduced expression of Tim-3. This is in marked contrast to other chronic viral infections such as HIV and HCV infections, in which both PD-1 and Tim-3 are expressed at high levels in the virus-specific CTLs [19, 20]. Double staining for Tim-3 and PD-1 revealed that these are expressed by distinct populations of $CD8^+$ T cells in HIV infection and that the predominance of either $Tim\text{-}3^+PD\text{-}1^-$ or $Tim\text{-}3^-PD\text{-}1^+$ cells in HIV-specific CTLs differs among individuals [19]. At the same time, CMV- and HCV-specific CTLs are predominantly $Tim\text{-}3^-PD\text{-}1^+$ and $Tim\text{-}3^+PD\text{-}1^+$, respectively [20]. In our study, the average percentage of $PD\text{-}1^+$ cells in HTLV-I Tax-specific CTLs was 65.9% and 22.3% in carriers and HAM/TSP patients, respectively (Figure 3*C*); the average percentage of $Tim\text{-}3^+$ cells was 3.5% and 3.2%, respectively (Figure 2*F*), suggesting that the majority of the CTLs expressing T cell exhaustion molecules has a $Tim\text{-}3^-PD\text{-}1^+$ phenotype. Taken together, these results suggest that PD-1 and Tim-3 may have a distinct function in regulating immune responses in HTLV-1 infection.

We observed that HTLV-I Tax-expressing cells show a significant reduction in Tim-3 expression as compared with Tax^-CD4^+ cells and that Tim-3 expression in Tax^+CD8^+ cells tends to be lower than in Tax^-CD8^+ cells. Tax^+CD8^+ cells also showed significantly lower PD-1 expression (Figure 6). This suggests that HTLV-I–infected cells may be resistant to cell death through the Tim-3/galectin-9 pathway. HTLV-I Tax combines a positive effect on cell cycle with a negative effect on apoptosis through transactivation of several host genes [36]. It would be of interest to further investigate whether Tax might regulate Tim-3 expression.

In summary, we demonstrated that the expression of the negative immune regulator Tim-3, but not of PD-1, is reduced on HTLV-I Tax-specific CTLs, $CD4^+$, and $CD8^+$ T cells in both HAM/TSP patients and asymptomatic carriers. Moreover, we showed that Tim-3^+, but not PD-1^+, cells produce less IFN-γ and exhibit low cytolytic activity within the CTL population. Tim-3 expression and CTL cytolytic activity were not different between HAM/TSP patients and asymptomatic carriers. In addition, $CD4^+$ HTLV-I Tax-expressing cells showed a significant reduction in Tim-3 expression as compared with Tax^-CD4^+ cells. These results suggest that HTLV-I Tax-specific CTLs preserve their cytolytic activity, thereby controlling viral replication.

Funding

Grant-in-Aid for Research on Brain Science from the Ministry of Health, Labor and Welfare, Japan, and Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Acknowledgments

We thank Ms. Takako Inoue for the excellent technical assistance.

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Journal of Medical Virology 84:1120–1127 (2012)

Human T-Lymphotropic Virus Type I (HTLV-I)-Specific CD8+ Cells Accumulate in the Lungs of Patients Infected With HTLV-I With Pulmonary Involvement

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Pulmonary involvement has been identified in human T-lymphotropic virus type I (HTLV-I) carriers and patients with HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP). However, the relationship between HTLV-I infection and lung disease is poorly understood. The occurrence of HTLV-I-specific immune responses in the lungs of patients infected with HTLV-I with pulmonary involvement was investigated. The frequency of HTLV-I-specific CD8+ cells and the amount of HTLV-I proviral DNA were determined in bronchoalveolar lavage fluid cells and peripheral blood mononuclear cells (PBMCs) from five patients with HAM/TSP and one HTLV-I carrier who had pulmonary involvement. HTLV-I-specific CD8+ cells were detected by flow cytometry using human leukocyte antigen/antigen complex multimers. The analysis of bronchoalveolar lavage fluid revealed lymphocytosis in five of six patients. HTLV-I provirus was detected in the bronchoalveolar lavage fluid cells of all patients, and the proviral load in these cells was comparable to that in PBMCs. The frequency of HTLV-I-specific CD8+ cells in the bronchoalveolar lavage fluid cells was 5.1 times higher than that in PBMCs. Immunohistochemically, clusters formed by HTLV-I-specific CD8+ cells were detected in lung tissue by in situ tetramer staining. No samples were available from patients infected with HTLV-I without lung disorders. Whether accumulation of CD8+ cells is specific to patients with pulmonary involvement remains unclear. These results indicate that HTLV-I-specific CD8+ cells accumulate and HTLV-I-infected cells exist in the lungs of patients infected with HTLV-I with pulmonary involvement. ***J. Med. Virol. 84:1120–1127, 2012.*** © 2012 Wiley Periodicals, Inc.

KEY WORDS: HTLV-I; lung; bronchoalveolar lavage fluid; CD8+ cells; proviral load

INTRODUCTION

Human T-lymphotropic virus type I (HTLV-I), a human retrovirus, is the etiological agent of adult T-cell leukemia, a hematological malignancy of CD4+ T lymphocytes [Uchiyama et al., 1977]. HTLV-I is also responsible for several inflammatory disorders, including HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP), HTLV-I-associated uveitis and HTLV-I-associated arthropathy [Gessain et al., 1985; Osame et al., 1986; Nishioka et al., 1989; Mochizuki et al., 1992]. Pulmonary involvement has also been reported in patients infected with HTLV-I [Kimura et al., 1986; Maruyama et al., 1988]. A radiological study revealed that the incidence of

Grant sponsor: Japanese Ministry of Education, Culture, Sports, Science and Technology.

Conflicts of interest: None.

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Accepted 20 March 2012

DOI 10.1002/jmv.23307

Published online in Wiley Online Library (wileyonlinelibrary.com).

pulmonary involvement is higher in HTLV-I carriers than in non-infected individuals [Okada et al., 2006]. In HTLV-I carriers, HTLV-I infection increases the risk for pulmonary cryptococcosis, tuberculosis, and community-acquired pneumonia [Kohno et al., 1992; Marinho et al., 2005; Atsumi et al., 2009]. In patients with adult T-cell leukemia, pulmonary involvement is mainly caused by opportunistic infections or pulmonary infiltration of leukemic cells [Yoshioka et al., 1985]. Other pulmonary involvement has been observed in HTLV-I carriers and patients with HAM/TSP. Importantly, analysis of bronchoalveolar lavage fluid obtained from these patients revealed marked lymphocytosis [Sugimoto et al., 1987]. These findings may indicate that HTLV-I infection can contribute to inflammatory lung diseases.

HTLV-I-associated lung disease has various pulmonary manifestations, including bronchiolitis, alveolitis, diffuse panbronchiolitis, and interstitial pneumonia [Setoguchi et al., 1991; Kikuchi et al., 1996; Sugimoto et al., 1998]. Several studies have identified pulmonary involvement specific to HTLV-I-associated lung disease. HTLV-I proviral load in peripheral blood mononuclear cells (PBMCs) correlates with the degree of bronchoalveolar lymphocytosis [Mori et al., 2005], and mRNA of the HTLV-I gene is upregulated in bronchoalveolar lavage fluid cells than in PBMCs [Higashiyama et al., 1994; Seki et al., 2000b]. Patients with HTLV-I-associated lung disease have elevated levels of soluble adhesion molecules and soluble interleukin-2 receptor α in bronchoalveolar lavage fluid and increased mRNA levels of cytokines and chemokines in bronchoalveolar lavage fluid cells [Sugimoto et al., 1989; Seki et al., 1999; Yamazato et al., 2003]. These data suggest that HTLV-I infection induces pulmonary inflammation. However, the immunological parameters measured in the above studies are not specific for HTLV-I, and whether HTLV-I-induced inflammation may occur in the lungs remains unclear. The objective of this study was to investigate whether HTLV-I-specific immune responses occur in the lungs of patients infected with HTLV-I with pulmonary involvement using peptide/human leukocyte antigen (HLA) complex multimers.

MATERIALS AND METHODS

Subjects

The study subjects evaluated at Kagoshima University Hospital from 1994 to 2008 included five patients with HAM/TSP and one HTLV-I carrier who had pulmonary involvement (Table I). The selection criteria for inclusion in the study were as follows: (1) both PBMCs and bronchoalveolar lavage fluid cells had to be obtained and (2) the patients had to have specific HLAs (HLA-A*0201 or -A*2402) because HTLV-I Tax11–19 and Tax301–309 epitopes are well characterized and represent strong immunodominant epitopes restricted to these HLAs [Yashiki et al., 2001; Kozako et al., 2006]. HLA was determined by polymerase chain reaction (PCR) with sequence-specific primers as described previously [Bunce et al., 1995]. The serum titre of anti-HTLV-I antibody was measured by the particle agglutination method (Serodia-HTLV-I; Fujirebio, Tokyo, Japan), and HAM/TSP was diagnosed according to WHO criteria. HTLV-I status and clinical condition of the lungs are shown in Tables I and II, respectively. After obtaining informed consent, fiberoptic bronchoscopy and bronchoalveolar lavage were performed in these patients. Bronchoalveolar lavage fluid cells and PBMCs were obtained under written informed consent and stored in liquid nitrogen until use. This study was reviewed and approved by the Kagoshima University Ethical Committee and was conducted in accordance with the Declaration of Helsinki.

Quantitative PCR for HTLV-I Provirus

Genomic DNA was extracted from PBMCs and bronchoalveolar lavage fluid cells using a commercial kit (Qiagen, Tokyo, Japan). A quantitative PCR assay was performed as described previously [Nagai et al., 1998].

Flow Cytometry

HLA-A*0201/HTLV-I Tax11–19 and HLA-A*2402/HTLV-I Tax301–309 pentamers labeled with phycoerythrin (PE) were purchased from Proimmune (Oxford,

TABLE I. Clinical Characteristics of the Patients

No.	Age	Sex	HTLV-I status	Duration (years)[a]	HTLV-I Ab[b]	HLA[c]
1	63	F[d]	HAM[e]/SjS[f]	7	×65536	A*24
2	81	F	carrier	N/A[g]	×32768	A*24
3	55	F	HAM	3	×4096	A*02
4	65	F	HAM	7	×4096	A*24
5	54	F	HAM/SjS	2	×65536	A*24
6	36	F	HAM	3	×64	A*24

[a]Duration of HAM/TSP in years.
[b]Serum antibody titre as determined by the agglutination method.
[c]Human leukocyte antigen.
[d]Female.
[e]HAM/TSP.
[f]Sjögren syndrome.
[g]Not applicable.

TABLE II. Clinical Condition of Patient Lungs

No.	Chest computed tomography findings	Smoking	Bacterial feature
1	Partial bronchiectasis and centrilobular nodules	N[a]	Not examined
2	Lobular centrilobular nodules and bronchiectasis	N	*M. intracellulare*
3	Lobular centrilobular nodules and bronchiectasis	N	*Micrococcus sp*
4	Diffuse centrilobular nodules and bronchiectasis	C[b]	*H. influenzae*
5	A few centrilobular nodules	N	*Staphylococcus*
6	Hyperinflation	N	*H. parainfluenzae*

[a]Never smokers.
[b]Current smokers.

UK) [Ogg and McMichael, 1998]. Bronchoalveolar lavage fluid cells or PBMCs were stained with HTLV-I Tax pentamer or HIV Gag pentamer (control) followed by PE–Cy5-conjugated anti-CD8 antibody (clone T8; Beckman Coulter, Tokyo, Japan). The cells were analysed using an Epics XL flow cytometer (Beckman Coulter, Tokyo, Japan) with Expo32 software. Lymphocytes, determined on the basis of forward and side scatter, were gated for CD8 high cells, and the frequency of HTLV-I-specific CD8+ cells stained with the pentamers was determined in this gate.

Immunohistological Analysis

A biopsy of the lower lung was obtained by video-assisted thoracoscopic surgery from a 65-year-old woman with HTLV-I infection. She also had HAM/TSP, bronchiolitis obliterans, and HLA-A*2402. In situ detection of antigen-specific T cells using tetramers was performed according to previously published methods with some modifications [Skinner et al., 2000]. In brief, an 8-μm-thick tissue section was incubated with HLA-A*2402/HTLV-I Tax301–309 tetramer labeled with PE (MBL, Nagoya, Japan) and mouse anti-CD8 antibody (clone DK25, mouse IgG1; Dako, Tokyo, Japan) at 4°C overnight. After fixation with 4% paraformaldehyde, the section was incubated with rabbit anti-PE antibody (BioGenesis, Poole, UK) followed by a combination of Alexa Fluor 488-conjugated goat anti-rabbit antibody (Invitrogen, Tokyo, Japan) and Alexa Fluor 594-conjugated goat anti-mouse IgG1 antibody. The section was finally stained with 4′, 6-diamidino-2-phenylindole. The fluorescence signal was detected using a confocal laser scanning microscope (FV500; Olympus, Tokyo, Japan). For in situ detection of HTLV-I-infected cells, a section was fixed with 4% paraformaldehyde and incubated with a combination of anti-HTLV-I Tax antibody (Lt-4, mouse IgG3) [Lee et al., 1989] and anti-CD4 antibody (4B12, mouse IgG1; Dako) or with a combination of anti-HTLV-I Gag antibody (TP-7, mouse IgG1; Abcam, Cambridge, UK) and rat anti-CD4 antibody (YNB46.1.8; Abcam), followed by appropriate secondary antibodies labeled with fluorochrome.

Statistical Analysis

Wilcoxon signed-rank test and Spearman's rank correlation test were used for statistical analysis with StatView version J5.0. A P-value of <0.05 was considered to be significant.

RESULTS

Lymphocytosis in Bronchoalveolar Lavage Fluid From Patients Infected With HTLV-I

The cell count in bronchoalveolar lavage fluid was increased in the HTLV-I carrier (patient 2) and two of five patients with HAM/TSP (patients 5 and 6; Table III). Patient 4 was a smoker whose cell count

TABLE III. Cellular Composition of Bronchoalveolar Lavage Fluid in the Patients

No.	Cell count ($\times 10^5$/ml)	Macrophages (%)	Lymphocytes (%)	Neutrophils (%)	Eosinophils (%)
1	0.68	49.0	47.7	0.7	1.0
2	1.76	52.3	22.0	24.7	0.7
3	0.57	91.6	6.2	2.0	0.2
4	1.52	67.6	19.2	13.1	0.1
5	1.87	40.0	50.0	3.0	0.2
6	1.23	62.0	37.2	0.8	0.0
NS[a]	0.61 ± 0.36[b]	88.0 ± 9.9	11.0 ± 9.3	0.7 ± 1.6	0.3 ± 0.6
CS[c]	2.38 ± 1.58	95.7 ± 3.8	3.6 ± 3.1	0.5 ± 1.6	0.2 ± 0.5

[a]Never smokers.
[b]Mean ± SD. Values are from data published by the BAL Cooperative Group Steering Committee [Anonymous, 1990].
[c]Current smokers.