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'-GAGCCGATAACGCGTCCATCGATGGGGTCC-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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Low-Dose Intravenous Recombinant Tissue-Type Plasminogen Activator Therapy for Patients With Stroke Outside European Indications

Stroke Acute Management with Urgent Risk-factor Assessment and Improvement (SAMURAI) rtPA Registry

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Background and Purpose—The purpose of this study was to determine the safety and efficacy of intravenous recombinant tissue-type plasminogen activator (0.6 mg/kg alteplase) within 3 hours of stroke onset in Japanese patients outside the indications in the European license.

Methods—Of the 600 patients who were treated with recombinant tissue-type plasminogen activator, 422 met the inclusion criteria of the European license (IN group) and 178 did not (OUT group).

Results—The OUT group was inversely associated with any intracerebral hemorrhage (adjusted OR, 0.50; 95% CI, 0.29–0.84), positively associated with an unfavorable outcome (2.48; 1.55–3.94) and mortality (2.04; 1.02–4.04), and not associated with symptomatic intracerebral hemorrhage (0.53; 0.11–1.79) or complete independency (0.65; 0.40–1.03) after multivariate adjustment.

Conclusions—Functional and vital outcomes 3 months after low-dose recombinant tissue-type plasminogen activator in patients outside the European indications were less favorable compared with those included in the indications; however, the risk of intracerebral hemorrhage was not. **(*Stroke*. 2012;43:253-255.)**

Key Words: acute stroke ■ diabetes mellitus ■ elderly patients ■ intracerebral hemorrhage ■ outcomes ■ thrombolysis

Patients with severe stroke as indicated by a baseline National Institutes of Health Stroke Scale (NIHSS) score of ≥25, those >80 years old, and those with any history of prior stroke and concomitant diabetes were excluded from a European postmarketing monitoring study for intravenous recombinant tissue-type plasminogen activator (rtPA) therapy (the Safe Implementation of Thrombolysis in Stroke-MOnitoring STudy [SITS-MOST] registry) without sufficient rationale.[1] European regulatory agencies do not advocate rtPA therapy for patients having such exclusion items. Using our multicenter registry,[2] this study documented the safety and efficacy of low-dose intravenous rtPA (0.6 mg/kg) in patients with stroke outside the European indications as compared with those who fulfilled the SITS-MOST criteria.

Patients and Methods

Patients were derived from the Stroke Acute Management with Urgent Risk-factor Assessment and Improvement (SAMURAI) rtPA Registry.[2] Patient eligibility for alteplase was determined based on the Japanese guideline[3] stating that patients ≥75 years old, those with NIHSS score ≥23, those with a history of prior stroke, and those with poorly controlled diabetes are to be carefully considered but not excluded. Other exclusion criteria are almost identical between the European and Japanese indications. Each patient received alteplase (0.6 mg/kg) intravenously with 10% given as a bolus within 3 hours of stroke onset and the remainder delivered through continuous intravenous infusion over 1 hour. Patients not meeting the inclusion criteria of the European license were categorized into the OUT group and those who did were categorized into the IN group.

Outcomes included: any and symptomatic intracerebral hemorrhage (ICH) within the initial 36 hours, complete independence

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Table. Safety and Efficacy Outcomes

			Age		NIHSS		Prior Stroke Plus Diabetes	
	IN Group (N=422)	OUT Group (N=178)	≤80 Y (N=471)	>80 Y (N=129)	<25 (N=560)	≥25 (N=40)	Absent (N=575)	Present (N=25)
Any ICH within 36 h, no. (%)	93 (22.0)	26 (14.6)	96 (20.4)	23 (17.8)	113 (20.2)	6 (15.0)	116 (20.2)	2 (8.0)
Multivariate OR (95% CI)	1	0.50 (0.29–0.84)*	1	0.83 (0.46–1.46)	1	0.62 (0.22–1.46)	1	0.32 (0.05–1.16)
Symptomatic ICH, no. (%)	13 (3.1)	3 (1.7)	15 (3.2)	1 (0.8)	15 (2.7)	1 (2.5)	15 (2.6)	1 (4.0)
Multivariate OR (95% CI)	1	0.53 (0.11–1.79)	1	0.27 (0.01–1.47)	1	1.17 (0.06–6.88)	1	1.32 (0.07–7.70)
mRS 0–1, no. (%) (N=532)§	161 (40.5)	35 (26.1)	173 (39.4)	23 (24.7)	191 (38.2)	5 (15.6)	189 (36.8)	7 (38.9)
Multivariate OR (95% CI)	1	0.65 (0.40–1.03)	1	0.58 (0.31–1.04)	1	0.40 (0.13–1.05)	1	0.97 (0.32–2.91)
mRS 5–6, no. (%)	68 (16.1)	69 (38.8)	86 (18.3)	51 (39.5)	117 (20.9)	20 (50.0)	128 (22.3)	9 (36.0)
Multivariate OR (95% CI)	1	2.48 (1.55–3.94)‡	1	2.36 (1.36–4.09)†	1	3.23 (1.51–6.97)†	1	2.35 (0.81–6.44)
Mortality, no. (%)	20 (4.7)	23 (12.9)	25 (5.3)	18 (14.0)	34 (6.1)	9 (22.5)	41 (7.1)	2 (8.0)
Multivariate OR (95% CI)	1	2.04 (1.02–4.04)*	1	2.00 (0.93–4.24)	1	3.75 (1.45–9.09)†	1	1.54 (0.23–6.24)

NIHSS indicates National Institutes of Health Stroke Scale; ICH, intracerebral hemorrhage; mRS, modified Rankin Scale; CI, confidence interval; OR, odds ratio.
*$P<0.05$.
†$P<0.01$.
‡$P<0.001$.
§Assessed for patients who had premorbid mRS 0–1.

(modified Rankin Scale score 0–1), unfavorable outcome (modified Rankin Scale score 5–6) at 3 months, and death within 3 months. Symptomatic ICH was defined as that associated with neurological deterioration corresponding to an increase of ≥4 points from the baseline NIHSS score.

To evaluate the independent effect of the OUT group and each exclusion criterion on the clinical outcomes, a multivariate logistic regression model was estimated adjusting for sex, hypertension, dyslipidemia, atrial fibrillation, onset-to-treatment time, Alberta Stroke Programme Early CT Score, and internal carotid artery occlusion. The model was adjusted for: patients >80 years using NIHSS score, prior stroke, and diabetes; patients with NIHSS score ≥25, using age, prior stroke, and diabetes; and patients with prior stroke plus diabetes using age and NIHSS score.

Results

Of the 600 patients, 178 (85 men; age, 81.7±8.6 years) were categorized into the OUT group and the remaining 422 (292 men; 67.7±10.5 years) into the IN group. A higher percentage of patients in the OUT group were female, older, hypertensive, diabetic, and had higher initial NIHSS scores and internal carotid artery occlusion compared with the IN group (Supplemental Table I; http://stroke.ahajournals.org). Of the OUT group, 129 patients were >80 years old, 40 had severe stroke with an NIHSS score ≥25, and 25 had prior stroke plus diabetes.

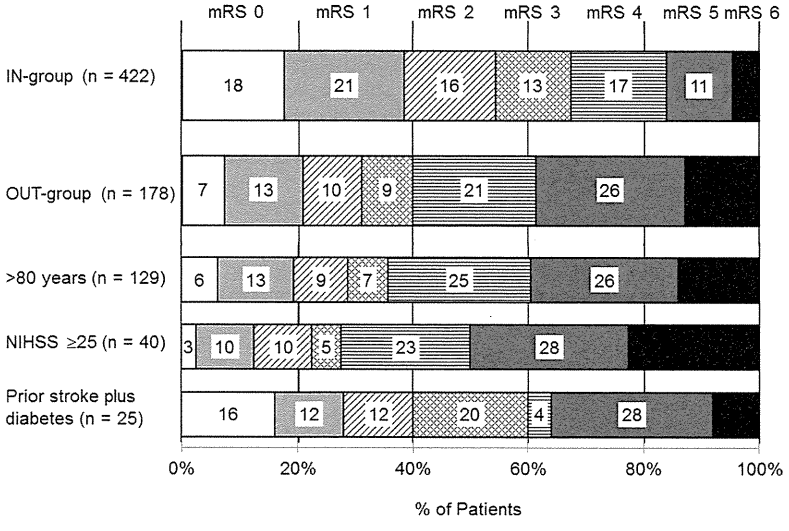

Figure. Modified Rankin Scale (mRS) distribution at 3 months.

After multivariate adjustment, any ICH was less common in patients in the OUT group than those in the IN group, but the frequency of symptomatic ICH did not differ significantly between the groups. Unfavorable outcome and death were more common in the OUT group than in the IN group and in patients with a NIHSS score ≥25 compared with those <25. Unfavorable outcome was also more common in patients >80 years than those ≤80 years (Table). The Figure shows the distribution of patients and their modified Rankin Scale scores at 3 months.

Discussion

More than 25% of ischemic strokes occur in patients ≥80 years old in Japan.[4] Advanced age was reported to be a strong predictor of poor outcomes and mortality independent of other clinical characteristics.[5] Randomized trials on rtPA did not include a sufficient number of patients with advanced age.[6–9] In the National Institute of Neurological Disorders and Stroke trial,[6] rtPA treatment was associated with increased likelihood of favorable outcome 3 months after stroke even in 49 patients aged >75 years with a NIHSS score >20 as compared with the placebo group. Risk of symptomatic ICH after thrombolysis did not increase, although clinical outcomes were worse in patients >80 years old as compared with younger patients in several studies.[10–13] An adjusted, controlled comparison based on 3472 patients >80 years old showed a better distribution of the modified Rankin Scale in thrombolysis patients than nonthrombolysis patients.[14] In this study, 0.6 mg/kg of alteplase may have caused the relatively small number of symptomatic ICH both in patients older than and ≤80 years old.

Diabetes mellitus was independently associated with symptomatic ICH after standard-dose rtPA therapy.[15] Infrequent development of ICH in our cohort and exclusion of patients with premorbid modified Rankin Scale ≥2 from the outcome analysis might weaken the impact of prior stroke plus diabetes on outcomes in this study. The small number of patients with prior stroke plus diabetes might also affect the statistical power.

This was not a randomized controlled study, subgroups were small, and physicians used judgment in selecting patients, all of which limit this study and introduce potential for error. In addition, data for patients with stroke who did not undergo thrombolysis were not collected and a comparison of patients with and without thrombolysis was not done.

In conclusion, 3-month outcomes after low-dose rtPA in patients outside the European indications were less favorable compared with those included in the indications. Low-dose intravenous rtPA therapy may be safely administered to patients outside the European indications without an increase of ICH by careful selection of patients. Patients with prior stroke and concomitant diabetes seem to be appropriate candidates for rtPA therapy.

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Disclosures

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SUPPLEMENTAL MATERIAL

Online Supplement Table. Baseline characteristics of patients within and outside the European indications

	OUT-group (N = 178)	IN-group (N = 422)	>80 years (N = 129)	NIHSS ≥25 (N = 40)	Prior stroke + diabetes (N = 25)
Male, n (%)	85 (47.8)	292 (69.2)‡	50 (38.8)‡	20 (50.0)	18 (82.0)
Age (years, mean ± SD)	81.7 ± 8.6	67.7 ± 10.5‡	85.8 ± 4.1‡	75.9 ± 10.0*	71.9 ± 7.7
Risk factors					
Hypertension, n (%)	121 (68.0)	245 (58.6)*	86 (66.7)	24 (60.0)	21 (84.0)*
Diabetes mellitus, n (%)	42 (23.6)	68 (16.2)*	17 (13.2)	2 (5.0)*	25 (100)‡
Dyslipidemia, n (%)	30 (17.0)	95 (22.8)	16 (12.5)†	4 (10.0)	12 (48.0)‡
Atrial fibrillation, n (%)	93 (52.8)	165 (39.9)	71 (55.9)‡	23 (57.5)	10 (40.0)
Stroke subtype					
Cardioembolism, n (%)	126 (70.8)	254 (60.2)	95 (73.6)	33 (82.5)	13 (52.0)
Large-artery atherosclerosis, n (%)	19 (10.7)	72 (17.1)	11 (8.5)	2 (5.0)	6 (24.0)
Small-vessel occlusion, n (%)	7 (3.9)	22 (5.2)	4 (3.1)	0 (0)	3 (12.0)
Others, n (%)	26 (14.6)	74 (17.5)	19 (14.7)	5 (12.5)	3 (12.0)
Onset-to-treatment time [min, median (IQR)]	150 (125-170)	145 (120-165)	145 (123-165)	151 (129-171)	155 (140-169)
Initial NIH Stroke Scale [median (IQR)]	16 (10-23.25)	11 (7-17)‡	15 (10-21)‡	27 (26-32.5)‡	11 (7-16.5)
ASPECTS [median (IQR)]	9 (8-10)	9 (8-10)	10 (8-10)	10 (7-10)	10 (8.25-10)
Internal carotid artery occlusion, n (%)	41 (23.2)	50 (12.0)‡	29 (22.7)†	10 (25.0)	5 (20.0)

NIHSS = National Institute of Health Stroke Score; SD = Standard deviation; IQR = interquartile range; ASPECTS = Alberta Stroke Programme Early CT Score

* $p < 0.05$, † $p < 0.01$, ‡ $p < 0.001$ vs. each opposite group

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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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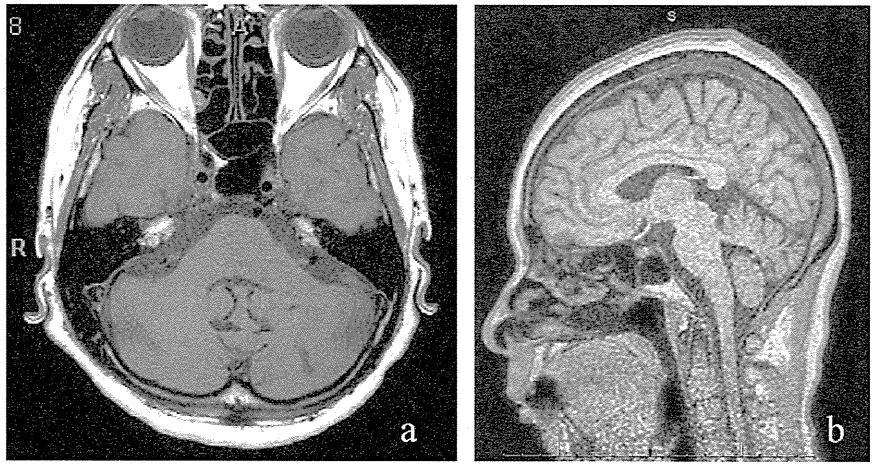

Fig. 1. Axial and sagittal T1-weighted MRI findings (a and b). Both images show moderate diffuse atrophy of the pons and cerebellum.

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].

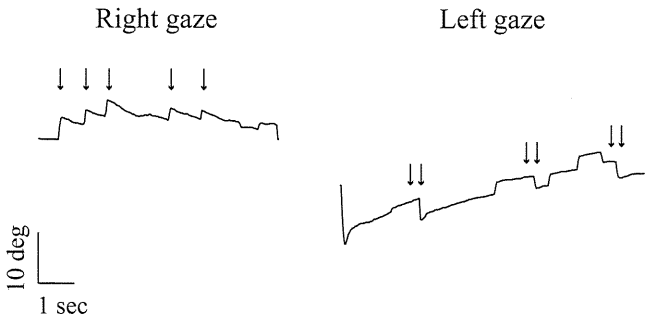

Fig. 2. The VOG recordings of horizontal gaze-evoked nystagmus. Arrows and double arrows indicate right and left gaze-evoked nystagmus, respectively.

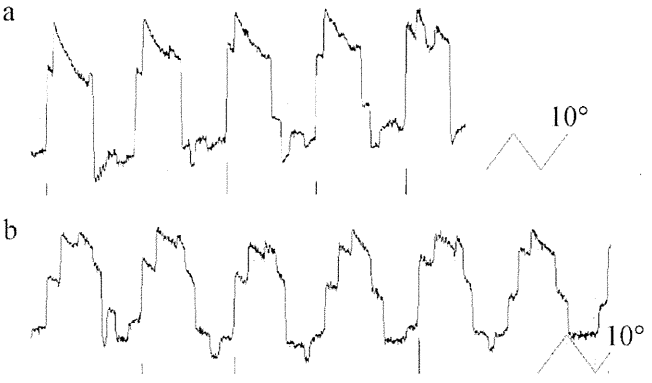

Fig. 3. Eye tracking test recordings for the patient. (a) ENG recording of horizontal saccade shows overshoot dysmetria. (b) ENG recording of horizontal sinusoidal smooth pursuit, which is superimposed on the nystagmus in the rightward and is saccadic in the leftward direction.

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 $Tim\text{-}3^+$ Cells Within $CD4^+$ and $CD8^+$ T cell Populations in HTLV-I Infection

$Tim\text{-}3^+$ 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 $Tim\text{-}3^+$ 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 $Tim\text{-}3^+$ 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 $Tim\text{-}3^+$ Tax-specific CTLs and HTLV-I proviral load, duration of illness, disease

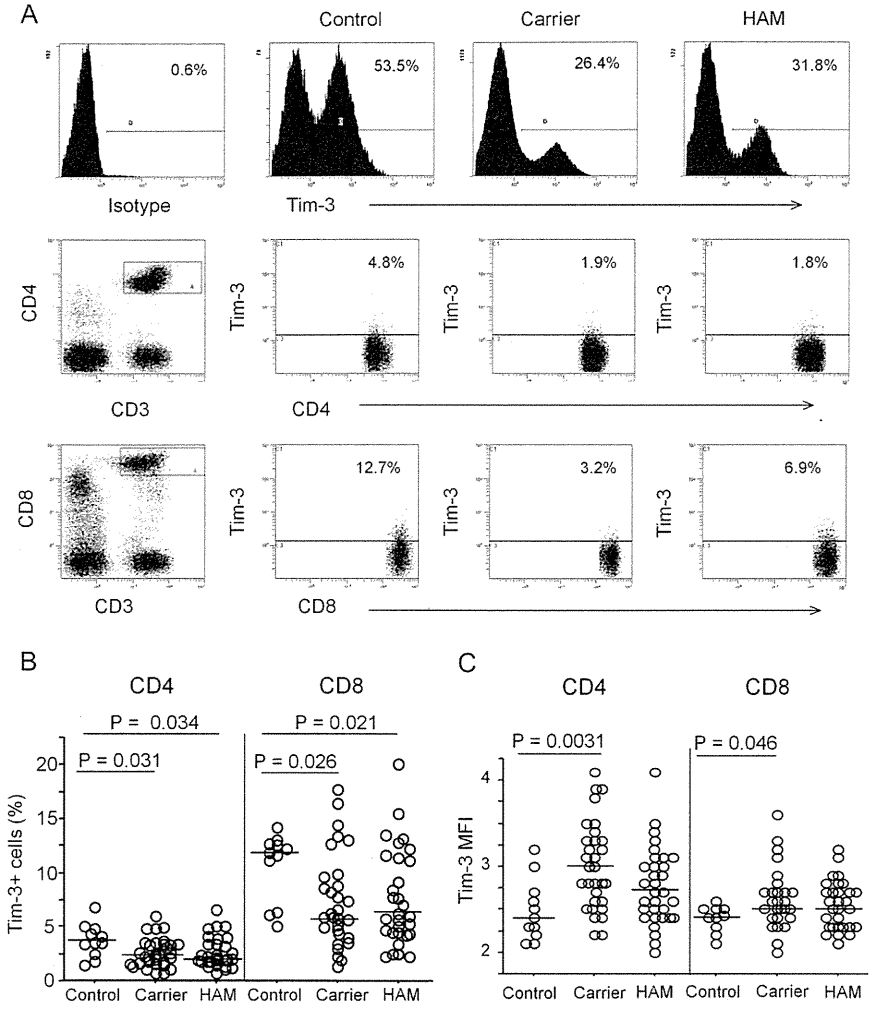

Figure 1. Low frequency of Tim-3$^+$ cells within the CD4$^+$ and CD8$^+$ T cell populations in HTLV-I infection. PBMCs from 63 HTLV-I–infected (32 HAM/TSP patients and 31 carriers) and 11 uninfected subjects were stained with antibodies against CD3, CD4, or CD8 and Tim-3. The numbers indicate the percentage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

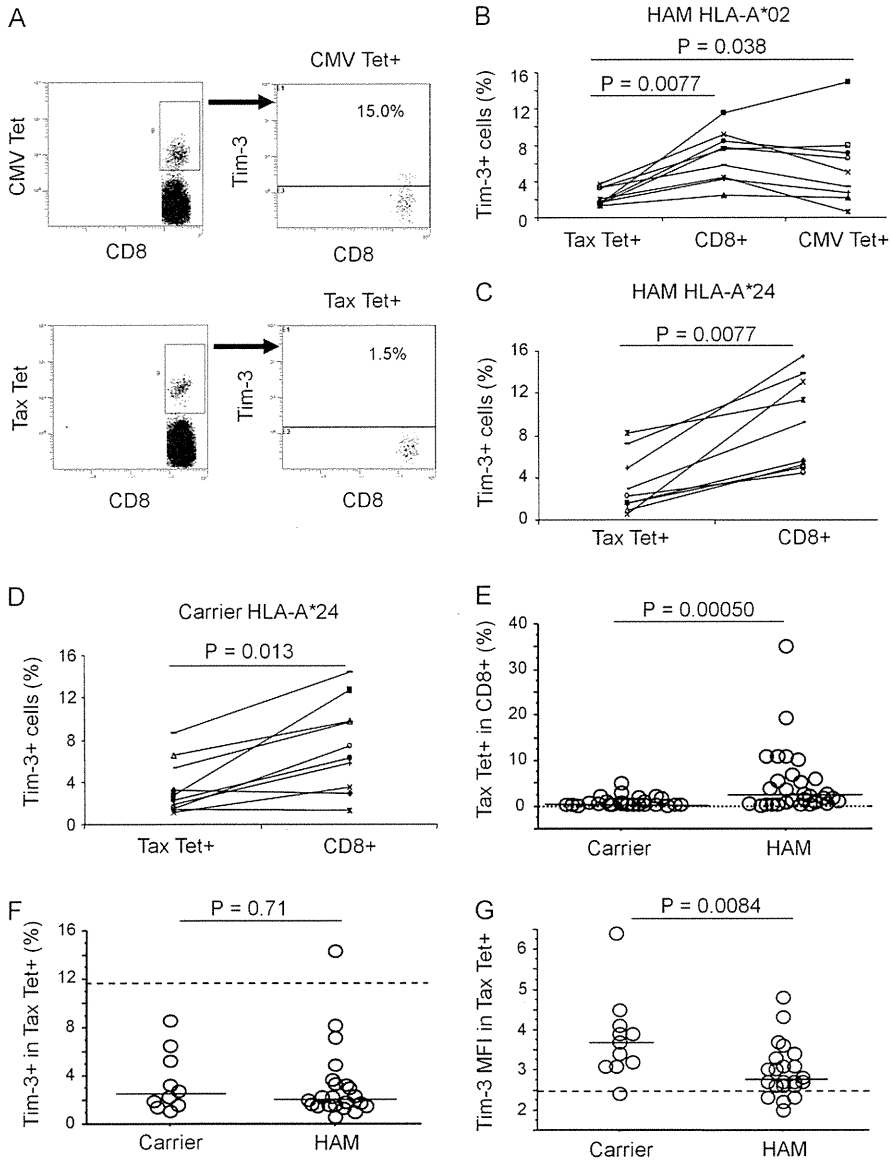

Figure 2. Low expression of Tim-3 on HTLV-I Tax-specific CTLs as compared with that on CMV-specific CTLs in HTLV-I infection. Tim-3 expression was determined in $CD8^+$, $CD8^+$Tax $tetramer^+$, and $CD8^+$CMV $tetramer^+$ cells of HAM/TSP patients and carriers. (*A*) A representative flow cytometry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

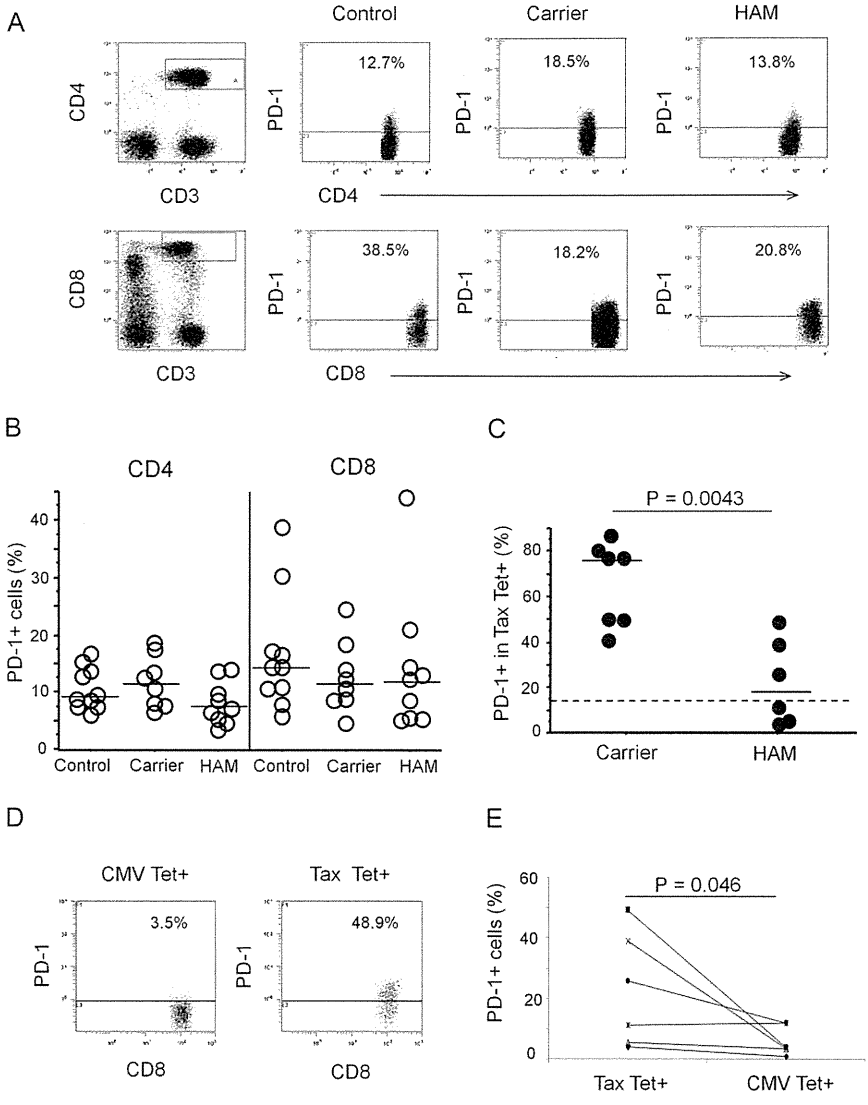

Figure 3. Increased PD-1 expression on HTLV-I Tax-specific CTLs as compared with that on CMV-specific CTLs. PD-1 expression was analyzed in PBMCs from 9 HAM/TSP patients, 8 carriers, and 10 controls after gating $CD3^+CD4^+$, $CD3^+CD8^+$, $CD8^+$Tax tetramer$^+$, or $CD8^+$CMV tetramer$^+$ cells. (*A*)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

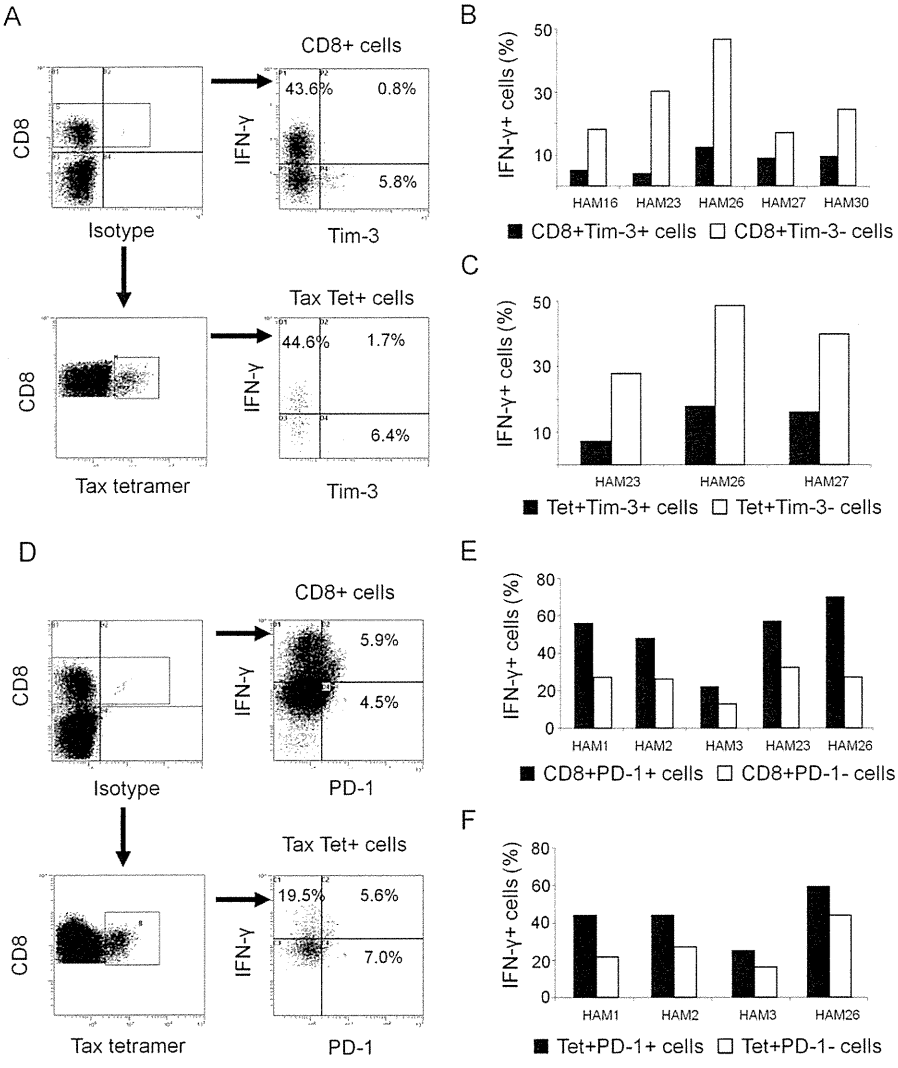

Figure 4. Reduced IFN-γ production by Tim-3$^+$ HTLV-I Tax-specific CTLs. PBMCs from 5 HAM/TSP patients were stimulated with PMA and ionomycin, and cultured for 6 hours in the presence of brefeldin A. IFN-γ production was determined by flow cytometry in CD8$^+$ and CD8$^+$Tax tetramer$^+$ cells with 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

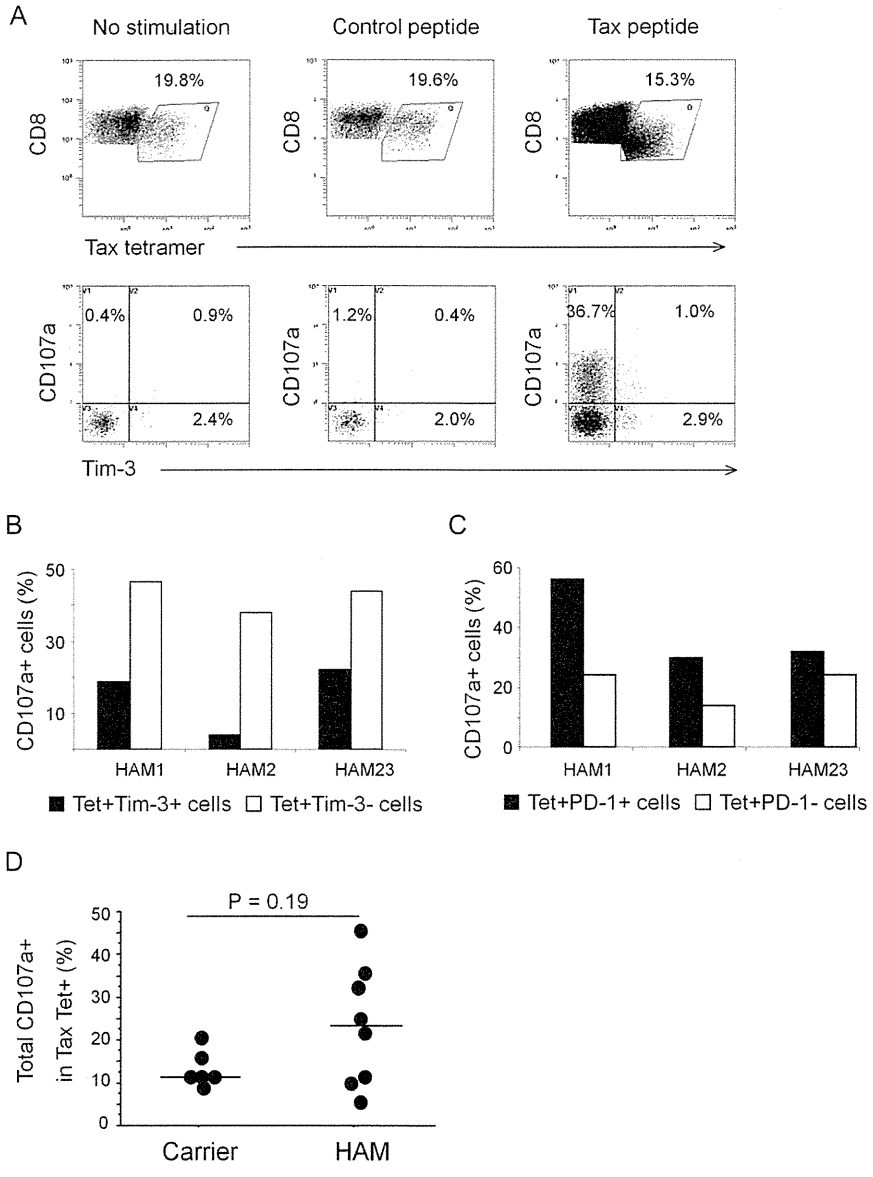

Figure 5. Reduced CD107a expression on Tim-3$^+$ HTLV-I Tax-specific CTLs. PBMCs from 8 HAM/TSP patients and 6 carriers were stimulated with HTLV-I Tax peptide or a control peptide, and cultured in the presence of an anti-CD107a antibody and brefeldin A for 4 hours. The expression of CD107a on CD8$^+$Tax 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*).

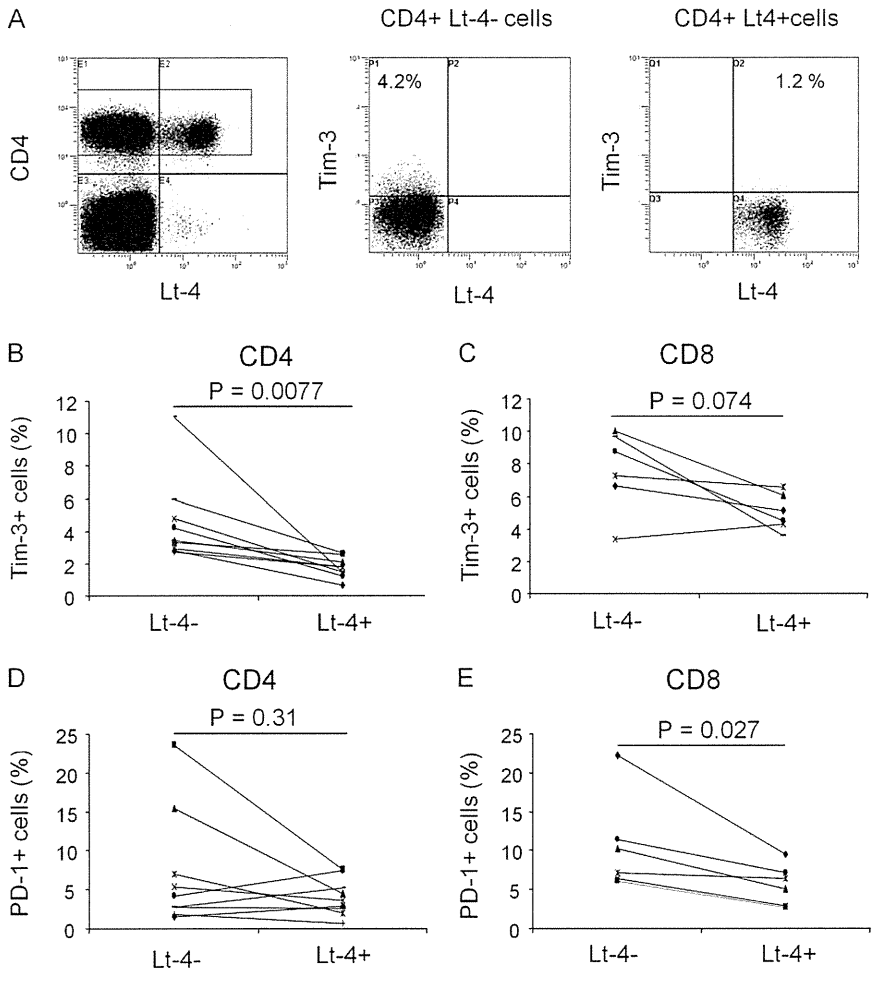

Figure 6. Low expression of Tim-3 on HTLV-I–infected cells. PBMCs from 9 HAM/TSP patients were cultured for 12 hours. Cells were double stained for the intracellular HTLV-I Tax protein, using the Lt-4 antibody, and the cell surface Tim-3 or PD-1. (*A*) After gating on $CD4^+$ cells, expression of Tim-3 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