

## Increased Activated Human T Cell Lymphotropic Virus Type I (HTLV-I) Tax11-19-Specific Memory and Effector CD8<sup>+</sup> Cells in Patients with HTLV-I-Associated Myelopathy/Tropical Spastic Paraparesis: Correlation with HTLV-I Provirus Load

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To discern the T cell subtype associated with T cell differentiation, the expression of CD45RA and CD27 was measured from total CD8<sup>high</sup> cells and from human T cell lymphotropic virus type I (HTLV-I) Tax11-19 peptide-specific CD8<sup>+</sup> cells in peripheral blood lymphocytes of patients with HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP). Phenotypically defined memory and/or effector cells (CD45RA<sup>-</sup>CD27<sup>+</sup>, CD45RA<sup>+</sup>CD27<sup>-</sup>, and CD45RA<sup>-</sup>CD27<sup>-</sup>) were increased in HAM/TSP CD8<sup>+</sup> cells, compared with those of HTLV-I-seronegative healthy control subjects. The percentage of human leukocyte antigen (HLA)-DR-positive cells was also increased in CD8<sup>+</sup> cells of HAM/TSP, compared with those in HLA-DR<sup>+</sup>CD8<sup>+</sup> cells of healthy control subjects. HTLV-I provirus load correlated with the frequency of Tax11-19-specific CD8<sup>+</sup> cells. The high frequency of memory and/or effector type HTLV-I Tax11-19-specific CD8<sup>+</sup> cells suggests that continuous restimulation driven by HTLV-I antigens *in vivo* may be associated with the pathogenesis of HAM/TSP.

Human T cell lymphotropic virus type I (HTLV-I) is the causative agent of HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [1, 2]. The vast majority of HTLV-I-infected individuals are clinically asymptomatic, in whom <1% develop HAM/TSP [3]. The relatively low prevalence of HAM/TSP among those infected with HTLV-I suggests that virus-host interactions play a role in the pathobiology of this inflammatory neurologic disease.

Increased HTLV-I provirus load [4–6] and augmented immune responses to HTLV-I have been reported in patients with HAM/TSP, compared with that in asymptomatic virus carriers. HTLV-I-specific CD8<sup>+</sup> cytotoxic T lymphocyte (CTL) activity in peripheral blood and cerebrospinal fluid (CSF) is elevated in HAM/TSP patients [7–9]. In HLA A2-expressing patients, a high proportion of these CTLs recognize the HTLV-I Tax 11-19 peptide [10, 11]. On the basis of limiting dilution assays, the precursor frequency of HTLV-I Tax 11-19-specific CTL was estimated to be 1:75 to 1:320 in CD8<sup>+</sup> lymphocytes [12]. In addition, the production of proinflammatory cytokines by HTLV-I Tax-specific CD8<sup>+</sup> lymphocytes was demonstrated by using intracellular staining techniques [13]. Pathologically, HAM/TSP is character-

ized by CD8<sup>+</sup> T cell perivascular cell infiltrates, predominantly in the thoracic spinal cord [14–16]. These CD8<sup>+</sup> cells expressed TIA-1, which is thought to be specific for cytotoxic cells [17]. Collectively, the evidence supports a central role of HTLV-I-specific CD8<sup>+</sup> T cells in the pathogenesis of HAM/TSP.

Recently, HTLV-I Tax peptide-loaded HLA-A2(\*0201)/mouse IgG chimeras (Tax-A2/Ig) were used to demonstrate HTLV-I Tax-specific A2-restricted CD8<sup>+</sup> cells in peripheral blood lymphocytes (PBL) from patients with HAM/TSP [18, 19]. HTLV-I Tax 11-19-specific CD8<sup>+</sup> cells from PBL of HLA-A\*0201 HAM/TSP patients were found to represent an extraordinarily high proportion of the total CD8<sup>+</sup> population (14%) [19]. The proportion of these HTLV-I Tax 11-19-specific CD8<sup>+</sup> cells with particular functions, however, has not been defined. For example, in human immunodeficiency virus (HIV) infection, it has been reported that the majority of peptide loaded HLA tetramer-binding T cells were probably memory precursors, because there was no correlation between peptide loaded HLA tetramer-positive cells and fresh CTL activity [20].

The cell surface markers CD27 and CD45RA have been demonstrated to be useful in identifying T cell subsets [21]. The simultaneous expression of CD45RA and CD27 separates functionally distinct subpopulations of human CD8<sup>high</sup> cells. On the basis of both phenotypic and functional properties, 4 distinct populations within the CD8<sup>high</sup> T cell subset have been described: naive T cells were phenotypically defined as CD45RA<sup>+</sup>CD27<sup>+</sup>; effector T cells were defined as CD45RA<sup>+</sup>CD27<sup>-</sup>; memory T cells were defined as CD45RA<sup>-</sup>CD27<sup>+</sup>; and CD45RA<sup>-</sup>CD27<sup>-</sup>

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**Table 1.** Profile of patients with human T cell lymphotropic virus type I (HTLV-I)-associated myelopathy (HAM)/tropical spastic paraparesis and of healthy control subjects.

Patient or subject	Age, years	Sex	Duration of illness, years
HAM-1	50	M	19
HAM-2	54	M	11
HAM-3	47	M	6
HAM-4	49	M	5
HAM-5	56	F	12
HAM-6	47	F	10
A2ND-1	38	M	—
A2ND-2	47	F	—
A2ND-3	45	M	—
A2ND-4	32	M	—
A2ND-5	53	M	—
A2ND-6	51	M	—

NOTE. A2ND, HTLV-I-seronegative healthy control subject possessing HLA-A2; F, female; M, male.

T cells were found to contain both memory and effector type cells [21].

In this study, we determined the expression of CD45RA and CD27 as T cell differentiation markers in total CD8<sup>high</sup> cells from peripheral blood mononuclear cells (PBMC) of patients with HAM/TSP and healthy control subjects. In addition, the high frequency of HTLV-I Tax11-19-specific CD8<sup>+</sup> cells demonstrated in PBL of HLA-A2 HAM/TSP patients afforded us the opportunity to define these differentiation cell surface markers on HTLV-I Tax-specific T cells. We also assessed the relationship between T cell subtype and HTLV-I provirus load, as measured by quantitative polymerase chain reaction (PCR). This study serves to define further the *in vivo* function of HTLV-I-specific T cell populations in PBL of HAM/TSP patients and the role that these cells may play in the pathogenesis of this disorder.

## Materials and Methods

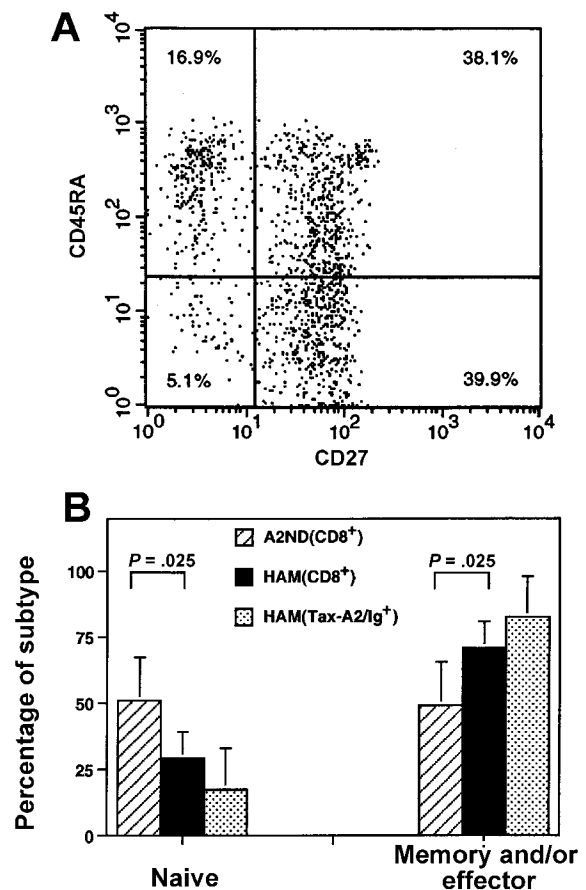
**Subjects.** Six patients with HAM/TSP (HAM-1 to HAM-6) and 6 HTLV-I-seronegative healthy control subjects (A2ND-1 to A2ND-6) were tested. All subjects were positive for HLA-A\*0201. The diagnosis of HAM/TSP was made according to neurological symptoms and serological testing for anti-HTLV-I antibody [22]. All patients had diagnostic cerebral and spinal magnetic resonance imaging. All patients had anti-HTLV-I antibodies in serum and had a slowly progressive spastic paraparesis. HAM-4 was infected with hepatitis virus type C. The clinical features of the patients included in this study are shown in table 1.

**Isolation of PBMC.** PBMC were isolated from peripheral blood samples on density gradient, using lymphocyte separation medium (ICN Biomedicals), and the cells were viably cryopreserved in liquid nitrogen until tested.

**Peptides and HLA-A2/Ig.** HTLV-I Tax11-19 peptide (LLFGY-PVYV) and HIV Gag77-85 (SLYNTVATL) were synthesized and were 95% purified by high-performance liquid chromatography (New England Peptide).

Tax A2/Ig was prepared as described elsewhere [19]. A2/Ig was loaded with 660-fold molar excess of peptide (HTLV-I Tax11-19 or HIV Gag77-85) and was incubated for 10 days at 4°C [19].

**Flow cytometric analysis.** To evaluate the expression of CD27 and CD45RA in Tax-specific CD8<sup>+</sup> cells, CD8<sup>+</sup> cells were initially separated by a positive selection procedure, using washed polystyrene magnetic beads coated with anti-human CD8 monoclonal antibody (MAb; Dynabeads; Dynal). The thawed PBMC were incubated with Dynabeads in PBS containing 2% fetal calf serum (FCS) for 30 min at 4°C with gentle mixing. Rosetted cells were



**Figure 1.** Different proportion of CD8<sup>+</sup> subtype between patients with human T cell lymphotropic virus type I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) and healthy control subjects. Naive CD8<sup>high</sup> cells consist of CD45RA<sup>+</sup>CD27<sup>+</sup>CD8<sup>+</sup> cells phenotypically. Memory and/or effector CD8<sup>+</sup> cells consist of CD45RA<sup>-</sup>CD27<sup>+</sup>, CD45RA<sup>+</sup>CD27<sup>-</sup>, and CD45RA<sup>-</sup>CD27<sup>-</sup> CD8<sup>+</sup> cells phenotypically. *A*, Representative pattern of CD45RA and CD27 expression on CD8<sup>high</sup> cells of a patient with HAM/TSP. Nos. indicate proportion of each subtype. *B*, Diagonal-striped bars indicate the mean percentage  $\pm$  SD of respective subtype in CD8<sup>high</sup> cells of 6 healthy control subjects (A2ND[CD8<sup>+</sup>]); solid bars indicate the mean percentage  $\pm$  SD of respective subtype in CD8<sup>high</sup> cells of 6 patients with HAM/TSP (HAM[CD8<sup>+</sup>]); dotted bars indicate the mean percentage  $\pm$  SD of respective subtype in Tax peptide-loaded HLA-A2/mouse IgG chimera (Tax-A2/Ig)-positive CD8<sup>+</sup> cells of 6 patients with HAM/TSP (HAM-[Tax-A2/Ig<sup>+</sup>]).

**Table 2.** Expression of CD45RA and CD27 in CD8<sup>high</sup> cells.

Patient or subject	CD45RA <sup>+</sup> CD27 <sup>+</sup> (naive)	CD45RA <sup>-</sup> CD27 <sup>+</sup> (memory)	CD45RA <sup>+</sup> CD27 <sup>-</sup> (effector)	CD45RA <sup>-</sup> CD27 <sup>-</sup> (effector/memory)
HAM-1	38.1	39.9	16.9	5.1
HAM-2	22.6	37.0	23.4	17.0
HAM-3	20.5	33.7	29.0	16.8
HAM-4	36.1	28.6	25.6	9.7
HAM-5	17.3	60.1	9.7	12.9
HAM-6	40.3	19.6	32.9	7.2
Mean ± SD	29.2 ± 10.1 <sup>a</sup>	36.5 ± 13.6 <sup>a</sup>	22.9 ± 8.4	11.5 ± 5.0 <sup>a</sup>
A2ND-1	74.2	14.0	9.3	2.5
A2ND-2	59.0	23.7	13.2	4.2
A2ND-3	60.0	26.4	11.0	2.6
A2ND-4	39.6	14.7	41.8	3.9
A2ND-5	28.5	24.1	38.9	8.5
A2ND-6	44.6	16.4	30.8	8.2
Mean ± SD	51.0 ± 16.5	19.9 ± 5.4	24.2 ± 14.7	5.0 ± 2.7

NOTE. Data are percentages of expression. A2ND, human T cell lymphotropic virus type I (HTLV-I)-seronegative healthy control subject possessing HLA-A2; HAM, HTLV-I-associated myelopathy.

<sup>a</sup> Mann-Whitney U test (*P* < .05).

harvested by using a magnetic particle concentrator (Dynal). The cells were resuspended in RPMI 1640 containing 1% FCS, and Detachabead (Dynal) was added. After incubation for 60 min at room temperature with gentle mixing, CD8<sup>+</sup> enriched cells were obtained. The purity of the fractionated cell populations was >96%. CD45RA and CD27 on Tax-A2/Ig-positive populations from unseparated PBL resulted in high nonspecific binding of HLA-A2/Ig in the CD8-negative population, presumably because of high nonspecific binding of monocytes and B cells.

Next, 1 × 10<sup>6</sup> of purified CD8<sup>+</sup> cells were incubated with 10 μL of 10 μg/μL human gamma globulin (Sigma) for 5 min at 4°C, to block nonspecific binding. The cells were incubated with 10 μL of HLA-A2/Ig for 40 min at 4°C. After washing (2 times) with PBS containing 0.5% FCS and 0.1% NaN<sub>3</sub>, 5 μL of phycoerythrin (PE)-labeled goat anti-mouse IgG1 (GAM; Caltag Laboratories) was incubated for 20 min at 4°C, to detect HLA-A2/Ig chimeras binding to cell surfaces. Anti-human CD27-fluorescein isothiocyanate (FITC; Pharmingen) or anti-human CD45RA-TRICOLOR (TC) MAbs (Caltag Laboratories) were used to detect respective cell surface molecules and were incubated for 20 min at 4°C. Triple-color-labeled cells were washed twice, were resuspended in washing buffer, and were analyzed by flow cytometry (FACS Calibur; Becton Dickinson).

We also evaluated expression of perforin. To do so, 1 × 10<sup>6</sup> of purified CD8<sup>+</sup> cells were incubated with anti-human CD27-PE (Pharmingen) and with anti-human CD45RA-TC. After washing (2 times), labeled cells were fixed and permeabilized with Cytotfix/

Cytoperm solution (Pharmingen) for 20 min at 4°C. After washing (2 times) with 1×Perm/Wash solution (Pharmingen), the cells were incubated with anti-human Perforin-FITC MAb (Pharmingen) for 30 min at 4°C. The labeled cells were washed twice and were suspended with PBS containing 0.5% FCS and 0.1%NaN<sub>3</sub>, and were analyzed by flow cytometry.

To evaluate the expression of CD27 and CD45RA on total CD8<sup>high</sup> lymphocytes, triple staining with anti-human CD27-FITC, anti-human CD8-PE (Caltag Laboratories), and anti-human CD45RA-TC on PBMC was carried out. Expression of HLA-DR (Becton Dickinson) on Tax11-19-specific CD8<sup>+</sup> cells was analyzed using triple staining with anti-human HLA-DR FITC, Tax-A2/Ig-GAM-R-PE complex, and CD8-TC on PBMC.

**Cytotoxicity assay.** CD8<sup>high</sup>-enriched cells were prepared by depleting CD4<sup>+</sup> T cells, monocytes, NK cells, and B cells by use of mouse anti-human CD4, CD14, CD16, and CD19 MAbs (Caltag Laboratories) and Dynabeads coated with anti-Pan Mouse IgG MAb (Dynal). Then, CD8<sup>high</sup>-enriched cells were separated to CD27<sup>+</sup>CD8<sup>+</sup> cells and CD27<sup>-</sup>CD8<sup>+</sup> cells, using mouse anti-human CD27 MAb (Caltag Laboratories) and Dynabeads coated with anti-Pan Mouse IgG MAb. Perforin expression of purified CD27<sup>+</sup>CD8<sup>+</sup> or CD27<sup>-</sup>CD8<sup>+</sup> cells was evaluated by flow cytometry, as described above.

The CTL assay was determined by using Europium (Aldrich Chemical), as described elsewhere [23, 24]. Effector cells (CD27<sup>+</sup>CD8<sup>+</sup> or CD27<sup>-</sup>CD8<sup>+</sup> cells) were incubated with targets at varying effector-to-target ratios (E:T). The specific lysis was calcu-

**Table 3.** Expression of CD45RA and CD27 on human T cell lymphotropic virus type I (HTLV-I) Tax peptide-loaded HLA-A2/mouse IgG chimera (Tax-A2/Ig)-positive CD8<sup>+</sup> cells.

Patient	Tax-A2/Ig <sup>+</sup> /CD8 <sup>+</sup> cells	CD45RA <sup>+</sup> CD27 <sup>+</sup> (naive)	CD45RA <sup>-</sup> CD27 <sup>+</sup> (memory)	CD45RA <sup>+</sup> CD27 <sup>-</sup> (effector)	CD45RA <sup>-</sup> CD27 <sup>-</sup> (effector/memory)
HAM-1	7.3	16.8	65.0	6.6	11.6
HAM-2	3.9	5.8	70.1	2.6	21.5
HAM-3	0.6	8.2	76.6	1.6	13.6
HAM-4	13.2	47.8	21.4	25.2	5.6
HAM-5	0.9	12.0	71.2	3.2	13.6
HAM-6	4.2	14.3	54.9	10.8	20.0
Mean ± SD	5.0 ± 4.7	17.5 ± 15.4	59.9 ± 20.2	8.3 ± 8.9	14.3 ± 5.8

NOTE. Data are percentages of expression. HAM, HTLV-I-associated myelopathy.

lated as (experimental release – spontaneous release)/(maximum release – spontaneous release) × 100. The assay was performed in triplicate.

**Quantitative PCR.** HTLV-I provirus load was measured by using ABI PRISM 7700 Sequence Detector (Perkin Elmer/Applied Biosystems), as described elsewhere [6]. The primer set for HTLV-I pX region was 5'-ACAAAGTTAACCATGCTTATTATCAGC-3' positioned at nt 7276–7302 and 5'-ACACGTAGACTGGGTATCCGAA-3' positioned at nt 7355–7334. (Nucleotide coordinates are numbered according to the HTLV-I reference sequence on the Genbank database.) The primer set for  $\beta$ -actin was 5'-CACACTGTGCCCATCTACGA-3' positioned at nt 2146–2165 and 5'-CTCAGTGAGGATCTTCATGAGGTAGT-3' positioned at nt 2250–2225. The TaqMan fluorescent probe was 5'-TTCCCAGGGTTTGGACAGAGTCTTCT-3' positioned at nt 7307–7332 for HTLV-I pX region and was 5'-ATGCCCTCCCCATGCCATCCTGCGT-3' positioned at nt 2171–2196 for  $\beta$ -actin. DNA standards were extracted from HTLV-I-negative PBMC for  $\beta$ -actin and TARL-2 [25] for pX to make a standard curve. All samples were performed in triplicate. The thermal cycler conditions were as follows: 50°C for 2 min (for the activation of uracil-N-glycosylase [UNG]), 95°C for 10 min (for the inactivation of UNG and the activation of Taq polymerase), and 45 cycles at 95°C for 15 s (denaturation) and 60°C for 1 min (annealing and extension).

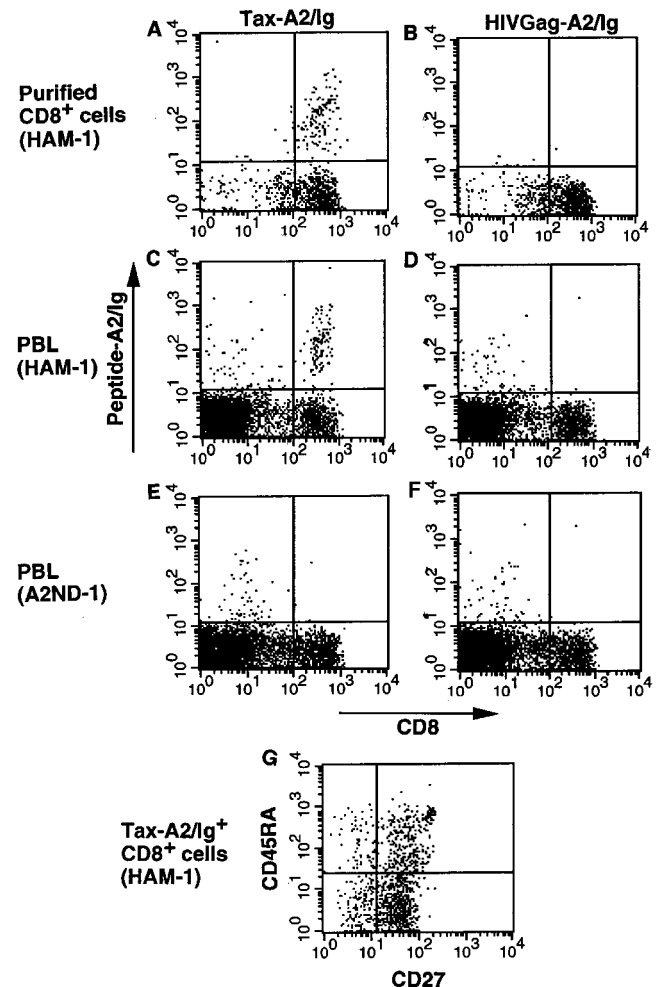
The amount of HTLV-I proviral DNA was calculated as copy number of HTLV-I (pX) per 100 PBMC = [(copy number of pX)/(copy number of  $\beta$ -actin/2)] × 100.

## Results

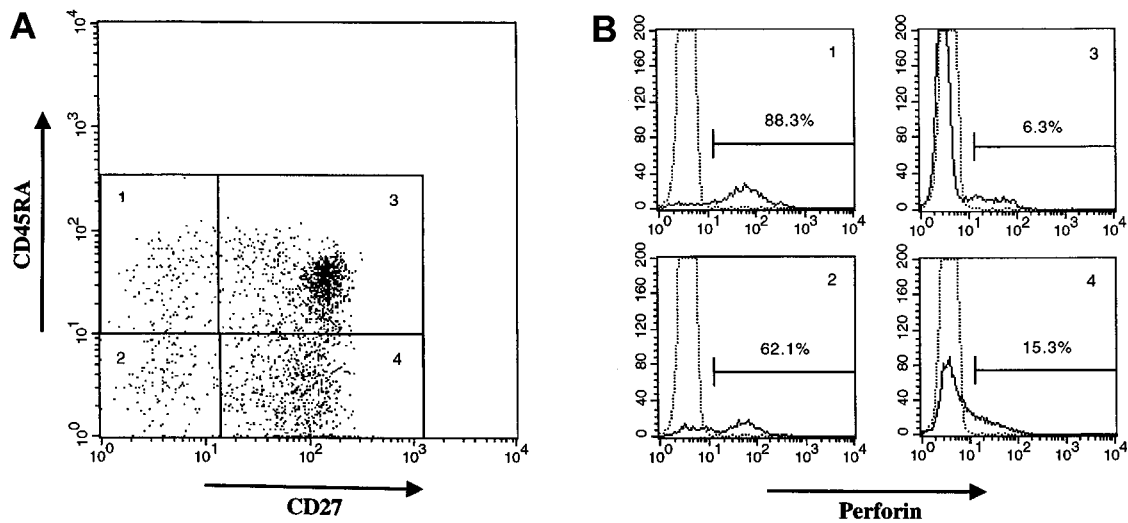
**Analysis of CD27 and CD45RA expression on total CD8<sup>high</sup> cells.** PBMC from patients with HAM/TSP and from healthy control subjects were stained with anti-CD27, anti-CD45RA, and anti-CD8 MAbs. Cells were gated on the CD8 population from which CD27 and CD45RA expression were analyzed (representative pattern of expression in figure 1A). As shown in table 2, the mean percentage of CD45RA<sup>+</sup>CD27<sup>+</sup> (phenotypically defined memory cells) and CD45RA<sup>+</sup>CD27<sup>-</sup> (memory and effector cells) in HAM/TSP was increased, compared with that in healthy control subjects. In contrast, the CD45RA<sup>-</sup>CD27<sup>+</sup> (naive cells) in HAM/TSP was decreased, compared with that in healthy control subjects. These results are summarized in figure 1B. The difference in the percentage of naive cells and memory and/or effector cells between HAM/TSP and healthy control subjects was statistically significant ( $P = .025$ , Mann-Whitney  $U$  test; figure 1B).

**Analysis of CD27 and CD45RA expression on Tax-A2/Ig CD8<sup>+</sup> cells.** To determine the distribution of differentiation markers on HTLV-I Tax peptide-specific CD8<sup>+</sup> cells, triple staining with anti-CD45RA, anti-CD27 MAbs, and HLA-A2/Ig was performed. Magnetic beads were used to purify CD8<sup>+</sup> cells for the analysis of expression of CD45RA and CD27 on Tax-A2/Ig-positive populations. All HAM/TSP samples contained Tax-A2/Ig-positive CD8<sup>+</sup> cells (table 3). The percentage of Tax-A2/Ig-positive CD8<sup>+</sup> cells was similar in both CD8<sup>+</sup>-enriched and -unenriched samples.

(Representative histograms are shown in figure 2A and 2C). The percentage of Tax-A2/Ig-positive cells in total CD8<sup>+</sup> cells ranged from 0.6% to 13.2% (mean ± SE, 5.0% ± 1.9%; median, 4.7%; table 3). There was no significant binding of Tax-A2/Ig to CD8<sup>+</sup> cells of HTLV-I-seronegative healthy control subjects (figure 2E). CD45RA and CD27 expression was analyzed after gating on Tax-A2/Ig-positive cells. The pattern of CD45RA and CD27 expression in Tax-A2/Ig-positive CD8<sup>+</sup> cells from a representative HAM/TSP patient is shown in figure 2G. More than half the Tax-A2/Ig



**Figure 2.** Specificity of peptide-loaded HLA-A2/mouse IgG chimeras (A2/Ig). High frequency of Tax-A2/Ig-binding CD8<sup>+</sup> cells were observed in samples from human T cell lymphotropic virus type I (HTLV-I)-associated myelopathy/tropical spastic paraparesis (HAM/TSP). Percentage of Tax-A2/Ig-positive CD8<sup>+</sup> cells was almost the same in CD8<sup>+</sup> separated samples (A), compared with that in samples before CD8<sup>+</sup> cell separation (C). There was no significant signal of Tax-A2/Ig-binding CD8<sup>+</sup> cells of HTLV-I-seronegative healthy control subjects possessing HLA-A2 (A2ND; E). Human immunodeficiency virus (HIV) Gag-A2/Ig was used as a negative control for Tax-A2/Ig, and no significant signal was observed (B, D, and F). Histogram (G) shows the expression of CD45RA and CD27 of Tax-A2/Ig-positive CD8<sup>+</sup> cells of HAM-1. PBL, peripheral blood lymphocytes.



**Figure 3.** Expression of perforin in CD8<sup>high</sup> T cell subsets. *A*, Purified CD8<sup>high</sup> cells, which were stained with CD45RA, CD27, and perforin. Four subsets were phenotypically defined as effector (*quadrant 1*), effector/memory (*quadrant 2*), naive (*quadrant 3*), and memory (*quadrant 4*). *B*, Histograms representing staining for intracellular perforin of T cell subsets from the 4 quadrants in *A*. Dotted line indicates isotype-matched control (negative control). There was significantly more perforin expression in T cell population with effector and effector/memory phenotype (*quadrants 1* and *2*) than in T cells with a naive or memory phenotype (*quadrants 3* and *4*).

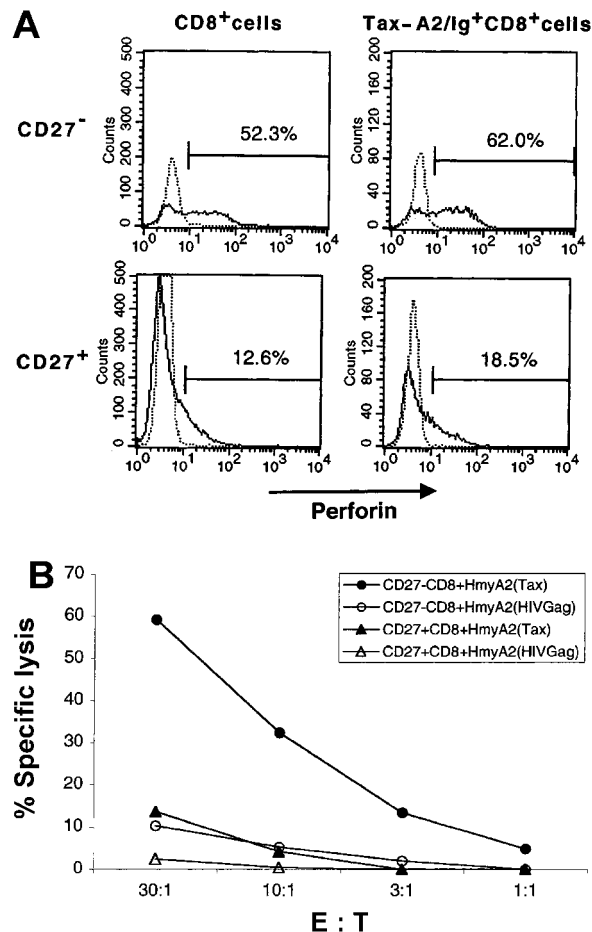
Ig-positive CD8<sup>+</sup> cells (mean, 59.9%) consisted of CD45RA<sup>-</sup>CD27<sup>+</sup> memory cells, whereas the CD45RA<sup>+</sup>CD27<sup>+</sup> naive cell population decreased (table 3), compared with that in the naive population, from total CD8<sup>+</sup> cells from HAM/TSP patients or healthy control subjects (table 2). The mean percentage of memory and/or effector cells (CD45RA<sup>+</sup>CD27<sup>-</sup>, CD45RA<sup>-</sup>CD27<sup>+</sup>, and CD45RA<sup>-</sup>CD27<sup>-</sup>) of Tax-A2/Ig-positive CD8<sup>+</sup> cells represented 82.5% of the total PBL population (table 3; summarized in figure 1*B*).

**Perforin expression and cytotoxicity of CD27<sup>+</sup>CD8<sup>+</sup> and CD27<sup>-</sup>CD8<sup>+</sup> cells.** Perforin expression in CD8<sup>+</sup> T cells was analyzed, to address the question of whether the CD45RA and CD27 phenotypic analyses that have been used to characterize T cell populations reflect biological function. It has been reported that there is a good correlation between the expression of perforin and the functional cytolytic activity in which perforin expression could be used as markers for activated cytolytic lymphocytes *in vivo* [26]. As shown in figure 3, T cell populations that have been phenotypically defined as CD45RA<sup>+</sup>CD27<sup>-</sup> effector cells and CD45RA<sup>-</sup>CD27<sup>+</sup> memory and effector cells contained significantly more perforin (88% and 62%, respectively) than did phenotypically defined CD45RA<sup>+</sup>CD27<sup>+</sup> naive cells and CD45RA<sup>-</sup>CD27<sup>+</sup> memory cells (6% and 15%, respectively). To confirm that these perforin-positive cells have cytolytic activity *ex vivo*, CD27<sup>-</sup>CD8<sup>high</sup> cells and CD27<sup>+</sup>CD8<sup>high</sup> cells from PBMC of HAM/TSP patients were purified and then were analyzed for perforin expression and cytotoxicity. The CD27<sup>-</sup> subset (phenotypically defined as effector or effector/memory) in both total CD8<sup>high</sup> cells and Tax-A2/Ig-positive CD8<sup>+</sup> cells contained ~4 times more perforin than did the CD27<sup>+</sup> subset (phenotypically defined as memory or naive cells;

figure 4*A*). CD27<sup>-</sup> subset showed strong cytotoxicity (59.3% at E:T, 30:1) to HTLV-I Tax peptide-pulsed targets, compared with that of CD27<sup>+</sup> subset (figure 4*B*). These cytolytic activities were not observed in the HIV Gag peptide-pulsed targets. These result indicated that CD27<sup>-</sup>CD8<sup>high</sup> effector type cells had abundant perforin expression and were able to lyse target cells *ex vivo*, which supports the use of phenotypic characterization of these T cell subsets as a measure of T cell function.

**HLA-DR expression on total CD8<sup>high</sup> cells and Tax-A2/Ig CD8<sup>+</sup> cells.** To determine whether Tax11-19-specific CD8<sup>+</sup> cells from HAM/TSP patients were activated, we analyzed HLA-DR expression, a known activation maker, in various T cell populations. The mean percentage of HLA-DR-positive cells in total CD8<sup>high</sup> cells of HAM/TSP (28.5%) patients was significantly increased ( $P = .0039$ ), compared with that in healthy control subjects (9.4%; table 4). Moreover, Tax-A2/Ig-positive CD8<sup>+</sup> cells in HAM/TSP patients demonstrated an even higher HLA-DR expression (53.9%), compared with HLA-DR-positive cells in the total CD8<sup>high</sup> population (28.5%) of HAM/TSP patients (table 4). The difference was statistically significant ( $P = .0039$ , Mann-Whitney *U* test). The expression of HLA-DR on Tax-A2/Ig-positive CD8<sup>+</sup> cells correlated with the proportion of memory and/or effector T cell population in Tax-A2/Ig-positive CD8<sup>+</sup> cells ( $r = .859$ ;  $P = .0286$ ; figure 5) and inversely correlated with the proportion of virus-specific CD8<sup>+</sup> cells with a naive phenotype (data not shown).

**Correlation between HTLV-I provirus load and T cell subtype.** It was of interest to determine whether the high frequency of Tax 11-19-specific CD8<sup>+</sup> cells that was demonstrated in HAM/TSP patients correlated with the HTLV-I provirus load



**Figure 4.** Perforin expression and cytotoxicity of CD27<sup>+</sup>CD8<sup>+</sup> and CD27<sup>-</sup>CD8<sup>+</sup> cells. CD27<sup>+</sup>CD8<sup>high</sup> and CD27<sup>-</sup>CD8<sup>high</sup> cells were purified from peripheral blood mononuclear cells (PBMC) of HAM-4. *A*, Histograms representing the amount of intracellular perforin in sorted CD27<sup>-</sup> and CD27<sup>+</sup> cells from total CD8<sup>high</sup> cells and Tax peptide-loaded HLA-A2/mouse IgG chimera (Tax-A2/Ig)-positive CD8<sup>+</sup> cells. Dotted line indicates isotype-matched control (negative control). *B*, Cytolytic activities, which were measured at various effector-to-target (E:T) ratios. Graph represents cytotoxicity of purified CD27<sup>-</sup>CD8<sup>high</sup> cells against Tax peptide-pulsed target cells (●) and human immunodeficiency virus (HIV) Gag peptide-pulsed target cells (○) and cytotoxicity of purified CD27<sup>+</sup>CD8<sup>high</sup> cells against Tax peptide-pulsed target cells (▲) and HIV Gag peptide-pulsed target cells (△). HmyA2, human plasma cell line transfected with HLA A2.

known to be elevated in patients with disease [6]. The HTLV-I provirus load in this cohort of HAM/TSP patients ranged from 0.74 copies to 68.01 copies per 100 PBMC (mean ± SE, 26.15 ± 9.39 copies/100 PBMC; median, 23.14 copies/100 PBMC). There was a significant correlation between the HTLV-I provirus load and the frequency of Tax-A2/Ig CD8<sup>+</sup> cells ( $r = .926$ ;  $P = .0079$ ; figure 6*A*). In addition, the HTLV-I provirus load correlated with Tax-A2/Ig CD8<sup>+</sup> cells expressing the effector phenotype ( $r = .846$ ;  $P = .0337$ ; figure 6*B*) and inversely correlated with the proportion of memory type Tax-A2/

Ig CD8<sup>+</sup> cells ( $r = -.868$ ;  $P = .0248$ ; figure 6*C*). These results suggest that both the high frequency and the state of differentiation of HTLV-I Tax11-19-specific CD8<sup>+</sup> cells that were demonstrated in PBL of HAM/TSP patients are associated with a high virus burden.

## Discussion

Continuously high CTL activity against HTLV-I has been observed in patients with HAM/TSP [10, 11]. It is believed that HTLV-I-specific CD8<sup>+</sup> T cells may play a pivotal role in the pathogenesis of HAM/TSP. The factors that cause accumulation of these antigen-specific cells, as well as the factors that drive differentiation of HTLV-I-specific CD8<sup>+</sup> T cells in vivo, however, are unclear. It was of interest to determine whether these cells represent naive T cells, effector T cells, or memory T cells. Recently, a novel method (divalent peptide-loaded HLA-A2/Ig) that allows for the visualization of T cells specific for HTLV-I Tax11-19 was developed [19]. This method allows not only for the quantification of the frequency of antigen-specific T cells but also for the direct characterization of these cells. It has been reported that combined staining with CD45RA and CD27 MAb was useful to discern differentiation stages in CD8<sup>+</sup> T cell development [21]. In this study, we demonstrated that the T cell subtype associated with T cell differentiation and activation markers in HTLV-I Tax11-19-specific CD8<sup>+</sup> T cells in HAM/TSP by phenotypic analysis and association with HTLV-I provirus load.

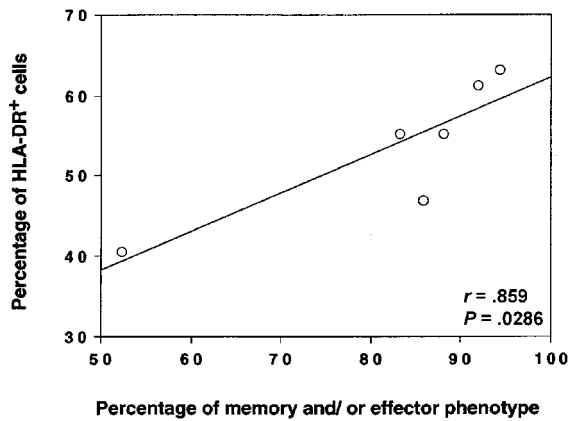
Hamann et al. [21] demonstrated that human CD8<sup>+</sup> subtypes, distinguished by expression of CD45RA and CD27, was related to CTL function, such as cytotoxicity, expression of perforin,

**Table 4.** Expression of HLA-DR on CD8<sup>high</sup> cells and human T cell lymphotropic virus type I (HTLV-I) Tax peptide-loaded HLA-A2/mouse IgG chimera-positive CD8<sup>+</sup> cells.

Patient or subject	HLA-DR	
	CD8 <sup>+</sup>	Tax-A2/Ig <sup>+</sup>
HAM-1	27.6	55.3
HAM-2	29.2	63.3
HAM-3	14.7	61.3
HAM-4	35.1	40.7
HAM-5	30.7	55.4
HAM-6	33.4	47.1
Mean ± SD	28.57 ± 7.3 <sup>a</sup>	53.9 ± 8.6 <sup>a</sup>
A2ND-1	14.6	
A2ND-2	9.0	
A2ND-3	2.7	
A2ND-4	5.2	
A2ND-5	11.0	
A2ND-6	14.1	
Mean ± SD	9.4 ± 4.8 <sup>a</sup>	

NOTE. Data are percentages of expression. A2ND, human T cell lymphotropic virus type I (HTLV-I)-seronegative healthy control subject possessing HLA-A2; HAM, HTLV-I-associated myelopathy.

<sup>a</sup> Mann-Whitney *U* test ( $P < .05$ ).

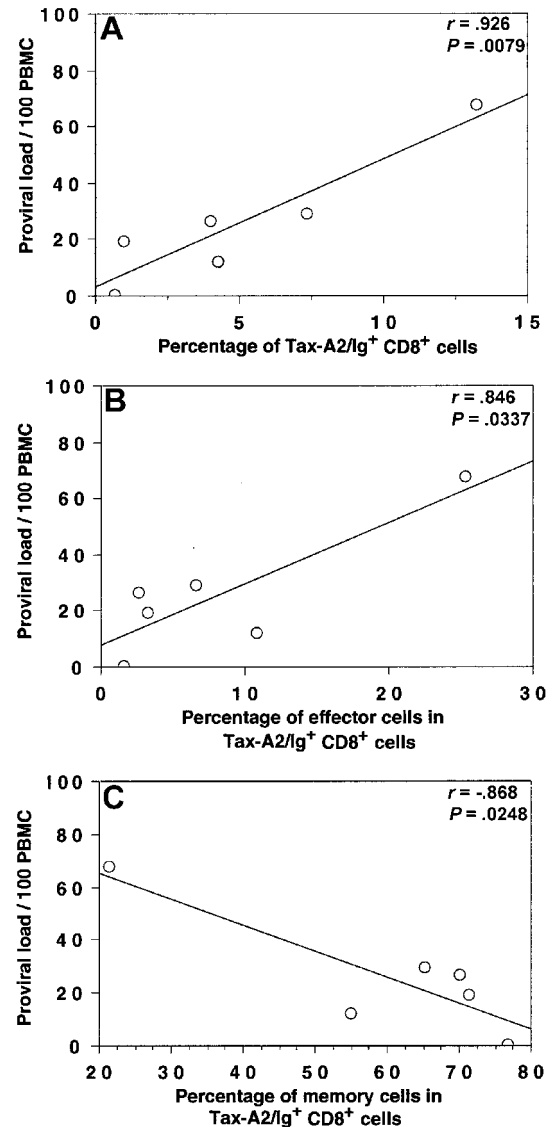


**Figure 5.** Correlation between Tax peptide-loaded HLA-A2/mouse IgG chimera (Tax-A2/Ig)-positive HLA-DR<sup>+</sup> CD8<sup>+</sup> cells and proportion of memory and/or effector subtype in Tax-A2/Ig-positive CD8<sup>+</sup> cells in human T cell lymphotropic virus type 1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). Percentage of HLA-DR<sup>+</sup> cells in Tax-A2/Ig-positive CD8<sup>+</sup> cells have a statistically significant positive correlation with the proportion of memory and/or effector subtype (CD45RA<sup>-</sup>CD27<sup>+</sup>, CD45RA<sup>+</sup>CD27<sup>-</sup>, and CD45RA<sup>-</sup>CD27<sup>-</sup>CD8<sup>+</sup> cells) in HAM/TSP PBMC.

granzyme A, and CD95 ligand. They suggested a model of human CD8<sup>+</sup> T cell differentiation [27] and phenotypically defined naive cells as CD45RA<sup>+</sup>CD27<sup>+</sup>, memory cells as CD45RA<sup>-</sup>CD27<sup>+</sup>, effector cells as CD45RA<sup>+</sup>CD27<sup>-</sup>, and CD45RA<sup>-</sup>CD27<sup>-</sup> as both effector/memory-type cells. We also confirmed that this phenotypic analysis actually related to biological function, using perforin-detection assay and cytotoxicity assay (figures 3 and 4). A comparison of the T cell subtypes in total CD8<sup>high</sup> cells of HAM/TSP patients and healthy control subjects demonstrated a significant reduction in the naive T cell population with a concomitant increase in the memory and/or effector cell population in HAM/TSP patients (table 2). This observation may result from the ability of an HTLV-I persistent infection to stimulate continuously the immune system in HAM/TSP patients. In vitro spontaneous proliferation of PBMC without exogenous antigens [28–30] and elevated levels of proinflammatory cytokines, such as interleukin (IL)-1 [31], interferon (IFN)- $\gamma$  [32], and tumor necrosis factor (TNF)- $\alpha$  [33], support this hypothesis.

We also examined the T cell subtype in HTLV-I Tax11-19-specific CD8<sup>+</sup> T cells. HTLV-I Tax 11-19-specific CD8<sup>+</sup> cells from PBL of HLA-A2 HAM/TSP patients represent a high proportion of the total CD8<sup>+</sup> [19]. In this study, we determined that >80% of HTLV-I Tax 11-19-specific CD8<sup>+</sup> cells of HAM/TSP patients were phenotypic memory and/or effector type cells (table 3), which can represent  $\leq$ 6% of the total CD8<sup>+</sup> population in some patients. The biological cytotoxic function of T cells within this population was correlated with T cell phenotype (figures 3 and 4). Interestingly, a small percentage of the HTLV-I Tax 11-19-specific CD8<sup>+</sup> cells demonstrated the CD45RA<sup>+</sup>CD27<sup>+</sup> T cell phenotype (a marker for naive cells). It is unlikely that these cells

are functionally naive, because they are derived from an antigen-specific T cell population, although it has been reported that CD45RA<sup>-</sup>CD27<sup>+</sup> (memory cells) may convert to CD45RA<sup>+</sup>CD27<sup>+</sup> (naive cells) [27, 34]. These cells would be phenotypically and functionally indistinguishable from “true” naive T cells. Al-



**Figure 6.** Correlation between human T cell lymphotropic virus type I (HTLV-I) provirus load and Tax peptide-loaded HLA-A2/mouse IgG chimera (Tax-A2/Ig) CD8<sup>+</sup> cells in HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP). HTLV-I provirus load was measured by quantitative polymerase chain reaction, and HTLV-I copy number per 100 peripheral blood mononuclear cells (PBMC) was represented. *A*, Statistically significant positive correlation between HTLV-I provirus load and frequency of Tax-A2/Ig CD8<sup>+</sup> cells in total CD8<sup>high</sup> cells. *B*, Positive correlation between HTLV-I provirus load and proportion of effector type (CD45RA<sup>+</sup>CD27<sup>-</sup>) cells in Tax-A2/Ig CD8<sup>+</sup> cells. *C*, Inverse correlation between HTLV-I provirus load and proportion of memory type (CD45RA<sup>-</sup>CD27<sup>+</sup>) cells in Tax-A2/Ig CD8<sup>+</sup> cells.

ternatively, T cell phenotype (particularly for naive cells) may not always correlate with T cell function.

Analysis of differentiated stages of antigen-specific CD8<sup>+</sup> cells is useful for evaluating the effectiveness of treatment. We examined quantitative and qualitative alteration of HTLV-I Tax 11-19-specific CD8<sup>+</sup> cells from HAM/TSP patients during treatment, using an immunomodulator. The number of HTLV-I Tax 11-19-specific CD8<sup>+</sup> cells decreased after treatment, along with a concomitant decrease in the proportion of effector type of HTLV-I Tax 11-19-specific CD8<sup>+</sup> cells and an increase in the proportion of virus-specific memory CD8<sup>+</sup> cells (authors' unpublished data).

We also demonstrated that >50% of HTLV-I Tax 11-19-specific CD8<sup>+</sup> cells were HLA-DR positive (table 4), which correlated with the proportion of memory and/or effector type cells (figure 5). We previously reported that HTLV-I Tax 11-19-specific CD8<sup>+</sup> expressed intracellular IFN- $\gamma$  and TNF- $\alpha$  and that high levels of HTLV-I Tax 11-19-specific CD8<sup>+</sup> cells persisted for 8 years in a patient with HAM/TSP [19]. Collectively, these results suggest that HTLV-I Tax 11-19-specific CD8<sup>+</sup> cells may be continuously differentiated and activated in vivo by HTLV-I Tax. These results are consistent with an Epstein-Barr virus (EBV) study that used HLA/EBV-peptide tetramers [35]. The majority of the EBV antigen-specific CD8<sup>+</sup> cells had activated/memory phenotype in patients with acute infectious mononucleosis (AIM). After recovery from AIM, although the frequency of the EBV antigen-specific CD8<sup>+</sup> cells decreased, this population of antigen-specific cells continued to be easily detectable for  $\geq 3$  years [35].

It is important to note that the results in this study have demonstrated a significant correlation between the frequency of HTLV-I Tax 11-19-specific CD8<sup>+</sup> cells and HTLV-I provirus load. High HTLV-I provirus load has been demonstrated in HAM/TSP patients [4–6] and may be related to the inflammatory process of this disease. HAM/TSP patients were shown to have a 16-fold increase in the amount of HTLV-I proviral DNA in PBL than were HTLV-I-infected asymptomatic carriers who were not members of the same family [6], and HTLV-I provirus load significantly correlated with concentration of neopterin in CSF [6] and soluble IL-2-receptor in serum samples [36]. We measured HTLV-I proviral DNA with an accurate quantitative PCR method and have shown a statistically significant correlation between the provirus load and the frequency of Tax-A2/Ig CD8<sup>+</sup> cells (figure 6), specifically with cells that have an effector phenotype (figure 6). This result is consistent with previous reports that demonstrated HTLV-I-specific IFN- $\gamma$ <sup>+</sup> CD8<sup>+</sup> cells (an effector function measured by intracellular cytokine detection assay) significantly correlating with HTLV-I provirus load in HAM/TSP patients [37]. In a recent study that demonstrated that, in HAM/TSP patients treated with the cytosine analogue lamivudine, the frequency of anti-Tax CTL declined with the decrease in HTLV-I viral DNA and then increased again as the viral DNA copy number increased [38].

Our results also demonstrate an inverse relationship between the proportion of Tax-A2/Ig CD8<sup>+</sup> cells with a memory phenotype and the HTLV-I provirus load (figure 6), which suggests that HTLV-I Tax-specific effector type CD8<sup>+</sup> cells may be either continuously differentiated from their "memory" pool [27] or have different costimulation requirements. The positive correlation of HTLV-I provirus load and Tax-A2/Ig CD8<sup>+</sup> support the hypothesis that the CD8<sup>+</sup> T cell immune system may respond to HTLV-I in vivo and cannot control HTLV-I infection effectively [39]. However, other mechanisms, such as increased longevity of T cells with an effector phenotype and infection of a subset of activated T cells may be other explanations that need further study. With regard to HTLV-I asymptomatic carriers, the frequency of Tax-specific CD8<sup>+</sup> [7, 19] cells and provirus load [6] have been shown to be lower than those of HAM/TSP patients, and HTLV-I-specific IFN- $\gamma$ <sup>+</sup> CD8<sup>+</sup> cells did not correlate with HTLV-I provirus load [37]. HTLV-I-specific IFN- $\gamma$ <sup>+</sup> CD8<sup>+</sup> cells in HTLV-I-asymptomatic carriers may control HTLV-I infection, and this suppression may result in low HTLV-I provirus load and the frequency of Tax-specific CD8<sup>+</sup> cells observed in many asymptomatic carriers.

In summary, we demonstrated that HTLV-I Tax11-19-specific CD8<sup>+</sup> cells are associated with distinct stages of T cell differentiation and activation. HTLV-I Tax 11-19-specific CD8<sup>+</sup> cells predominantly consist of activated memory and/or effector type cells. The persistence of a high frequency of memory and/or effector-type HTLV-I Tax11-19-specific CD8<sup>+</sup> cells suggests that the cellular immune system may be continuously driven by HTLV-I-infected cells in vivo that serve to drive this "memory" T cell population into effector T cells that may be involved in the pathogenesis of HAM/TSP.

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