Figure 4. Tim-3, PD-1 and Tax co-expression on T cells. (A) Plots demonstrate representative co-staining for Tax, PD-1 and Tim-3 on CD8+ and CD4+ T cells by flow cytometry following 24 hours incubation for the induction of Tax in two representative HTLV-1 infected patients. An isotype control was used to delineate the measurements for Tax expression. (B, C) Plots and graph depict the co-expression of Tim-3 and PD-1 by the indicated cytokines after 12 hr in vitro culture of 1×10^6 PBMC from 4 HTLV-1 infected patients. A representative donor is shown in B.
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also did not associate with the amount of IFN-γ secreted ($r=0.1317$; $P=0.7520$ and $r=0.2245$; $P=0.594$, respectively) (Figure 3B). However, we observed a statistically significant inverse correlation between the frequency of Tim-3 on both Tax-specific as well as CMV-specific $CD8^+$ T cells and the amount of IFN-γ secreted ($r=-0.8982$; $P=0.0046$; $r=0.9710$; $P=0.0028$; Figure 3A).

Co-Expression of Tim-3 and Tax on T Cells in HTLV-1 Infected Cells

Tax expression marks HTLV-1 viral replication in both $CD4^+$ and $CD8^+$ infected T cells. We aimed to determine whether the downregulation of Tim-3 we had observed was occurring only among infected cells, or in bystander cells as well. We therefore co-stained for Tax and Tim-3 expression on T cells from HTLV-1 infected subjects. We also stained for PD-1 expression as a control. The culture of PBMC overnight did not alter Tim-3 or PD-1 expression levels on the HTLV-1-infected T cells (data not shown). We observed that Tax was expressed on PBMC from some subjects following 24 hours of culture and was detected on both $Tim\text{-}3^+$ as well as $Tim\text{-}3^-$ $CD4^+$ T cells (Figure 4A). Similarly, Tax was present on both $PD\text{-}1^+$ and $PD\text{-}1^-$ T cells. We further identified a unique subset of Tax expressing CD4+ T cells that were $Tim\text{-}3^{hi}$ and lacked PD-1 in most of the subjects expressing Tax (Fig. 4A). No difference in the pattern of co-expression between HTLV-1 seropositive asymptomatic patients and those diagnosed with HAM/TSP was observed.

Elevated Tim-3 Expression by IL-2 and IL-15 Stimulated T Cells from HTLV-1 Infected Subjects

An increase in Tim-3 levels on T cells would potentially lead to a downregulation of T cell functionality. We therefore tested several gamma-chain associated cytokine mediators that could potentially modulate Tim-3 expression. We observed that IL-2, and especially IL-15, led to a prominent increase in the frequency of Tim-3 levels, specifically on the CD8+ T cell population after only 12 hours in culture (Figure 4B,C). No change in the levels of PD-1 expression were observed on both $CD8^+$ and $CD4^+$ T cells (Figure 4B,C).

Discussion

$CD8^+$ T cell dysfunction and/or exhaustion are common features of many chronic viral infections, including HIV-1 and HCV infections [29,30,31,32,33,34,35,36]. The mechanisms of T cell dysfunction are complex, but are in part mediated by a distinct set of inhibitory receptors [27,51]. A high, and sustained, expression of Tim-3 and PD-1, have emerged as hallmarks of T cell exhaustion in human viral infections, and blockade of these pathways can reinvigorate immune responses during persisting viral infections [29,30,33,34,36]. In this study, we report that $CD8^+$ and $CD4^+$ T cells in HTLV-1 infection express lower levels of Tim-3, and this was more pronounced in patients with HAM/TSP. Phenotypically, we observed that Tax HTLV-1-specific, HLA-A*02 -restricted $CD8^+$ T cells consistently retain a lower frequency of Tim-3. We propose that this low expression of Tim-3 on HTLV-1 Tax-specific T cells may lead to a persistent and deleterious effector T cell pool leading to more inflammation.

The pattern of expression of PD-1 in HTLV-1 infection has recently been shown to be elevated on T cells in HTLV-1 carriers and also on CMV and EBV specific T cells in asymptomatic carriers compared to healthy controls [52]. This opposing relationship of PD-1 and Tim-3 expression on T cells in patients with HTLV-1 infection suggests that the downregulation of Tim-3 expression potentially leads to more vigorous T cell activity in the HTLV-1-infected individual, whereas PD-1 may not fully reflect T cell dysfunction, but rather an activated status of the T cell response to infection. Indeed the association between the frequency of Tim-3 and PD-1 levels with IFN-γ secretion in response to either Tax or CMVpp65 epitopes show remarkably different correlations. In a study by Petrovas and colleagues, it was apparent that PD-1 expressing T cells are able to secrete cytokines in response to viral peptides [39]. Our data suggests that PD-1 and Tim-3 on antigen specific $CD8^+$ T cells are functionally different, and this may reflect a distinct stage of differentiation. PD-1 appears to mark early T-cell activation and exhaustion, while Tim-3 represents a more terminal stage of impairment.

The positive association between the frequency of HTLV-1's Tax-specific $CD8^+$ T cells and HTLV-1's Tax mRNA load and proviral load is well documented [8,53,54]. Studies evaluating the phenotype of $CD8^+$ T cells in HTLV-1 infection have been largely limited to characterizing the expression of T cell maturation and differentiation markers (CD28, CD45RO) [14]. Our data suggest that downregulation of Tim-3, rather than PD-1, marks global and Tax-specific $CD8^+$ T cells, which are hyperfunctional. This contrasts with HIV-1 and HCV infections, where the expression of Tim-3 is increased, leading to a population of $CD8^+$ T cells that are rendered dysfunctional both in terms of proliferative capacity and cytokine release as well as release of cytolytic granules [29,36].

Surface receptors known to regulate T cell function like CD244 and PD-1 have been shown to be upregulated either directly due to Tax or indirectly due to the cytokine milieu [52,55]. We postulate that either direct HTLV-1 viral components led to a downregulation of Tim-3, or as yet to be defined cytokine(s), suppress Tim-3 expression. In several human and murine studies, the manifestation of autoimmune diseases such as multiple sclerosis, have been attributed as a result of downregulated Tim-3 expression on T cells [56].

It still remains unclear how HTLV-1 infection sustains low levels of Tim-3 on T cells in infected patients and whether this is a cause or a consequence of disease progression. Multilayered mechanisms for this regulation may be occurring in the context of HTLV-1 infection. One strategy to reduce the T cells response would be through enhancement of the Tim-3 receptor for engagement with its cognate ligand. This could serve as a novel strategy to dampen the inflammatory inducing T cells. From our results, PD-1 engagement may not be as effective since both $PD\text{-}1^-$ and $PD\text{-}1^+$ cells retain the potential for $CD8^+$ T cell lytic function.

A novel strategy to reverse or prevent the onset of neurological complications would be through dampening effector T cell functions. From our results, it appears the γ-chain cytokines elicited higher levels of Tim-3 on specifically on $CD8^+$ T cells, and such a strategy could be harnessed to dampen T cell function in the HTLV-1 infected individual. Further work to understand the mechanisms for HTLV-1 disease progression and devise strategies to effectively prevent neurological complications will be needed.

Targeted modulation of the Tim-3 pathway provides a viable model for this intervention.

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Effects of valproate on Tax and HBZ expression in HTLV-1 and HAM/TSP T lymphocytes

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A determinant of human T-lymphotropic virus-1 (HTLV-1)–associated myelopathy/tropical spastic paraparesis (HAM/TSP) development is the HTLV-1–infected cell burden. Viral proteins Tax and HBZ, encoded by the sense and antisense strands of the pX region, respectively, play key roles in HTLV-1 persistence. Tax drives CD4$^+$-T cell clonal expansion and is the immunodominant viral antigen recognized by the immune response. Valproate (2-*n*-propylpentanoic acid, VPA), a histone deacetylase inhibitor, was thought to trigger Tax expression, thereby exposing the latent HTLV-1 reservoir to immune destruction. We evaluated the impact of VPA on Tax, Gag, and HBZ expressions in cultured lymphocytes from HTLV-1 asymptomatic carriers and HAM/TSP patients. Approximately one-fifth of provirus-positive CD4$^+$ T cells spontaneously became Tax-positive, but this fraction rose to two-thirds of Tax-positive–infected cells when cultured with VPA. Valproate enhanced Gag-p19 release. Tax- and Gag-mRNA levels peaked spontaneously, before declining concomitantly to HBZ-mRNA increase. VPA enhanced and prolonged Tax-mRNA expression, whereas it blocked HBZ expression. Our findings suggest that, in addition to modulating Tax expression, another mechanism involving HBZ repression might determine the outcome of VPA treatment on HTLV-1–infected-cell proliferation and survival. (*Blood.* 2011; 118(9):2483-2491)

Introduction

Human T-lymphotropic virus-1 (HTLV-1) is the etiologic agent of adult T cell leukemia (ATL) and HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP).[1,2] Although the majority of HTLV-1–infected individuals remain asymptomatic carriers (ACs), the lifetime cumulative risk of developing ATL or HAM/TSP is $< 5\%$. HTLV-1–provirus integrates into the genome of infected cells, predominantly CD4$^+$CD25$^+$ T lymphocytes, which represent the main reservoir in peripheral blood.[3] HAM/TSP, a central nervous system neuroinflammatory disease, is associated with perivascular and parenchymal infiltration of HTLV-1–infected T cells and activated cytotoxic T lymphocytes (CTLs).[4]

A major determinant of HAM/TSP development is the HTLV-1–infected cell burden. The peripheral blood HTLV-1–proviral load is higher in HAM/TSP patients than AC.[5,6] Follow-up studies of HAM/TSP cohorts showed that high provirus loads were associated with rapid disease progression.[7-9] Those studies also demonstrated a relative stability of the HTLV-1–proviral load throughout the disease. Set-point provirus load and subsequent HAM/TSP risk are influenced by the cellular immune-response efficiency,[10] although excessive activation of HTLV-1–specific CTLs might become deleterious and contribute to central nervous system tissue damage.[4] Host-pathogen interplay is characterized by very dynamic kinetics, resulting in an equilibrium between the virus-driven clonal expansion of infected T cells[11] and tight control exerted by the immune response.[10]

Tax, a transactivator protein encoded by the pX region of the HTLV-1 genome, plays a central role in disease pathogenesis. Tax activates viral transcription and also modulates many cellular signaling pathways involved in T cell activation, cycling, apoptosis or a combination.[12] Tax expression is promitotic and drives CD4$^+$ T-cell proliferation.[13] At the same time, Tax is the immunodominant target recognized by the CTL response.[14] Rapid immune elimination of Tax-expressing cells may explain the poor detection of *Tax*-gene products (ie, mRNA or protein) in freshly isolated peripheral blood mononuclear cells (PBMCs) from infected patients.[15-18] Short-term culture enables Tax detection and ex vivo conditions might allow Tax-expressing cells to escape immune selective pressure.[19]

The current model of HTLV-1 accumulation and persistence supposes 2 steps: first, Tax expression propels CD4$^+$ T cells into cell cycling, which is well documented; and second, silencing of virus expression allows escape from immune surveillance, which remains to be elucidated. Epigenetic mechanisms might participate in silencing of HTLV-1 gene transcription.[20] Use of histone deacetylase (HDAC) inhibitors, such as valproate (2-*n*-propylpentanoic acid, VPA), was postulated to transiently activate virus expression and thereby expose the latent virus reservoir to immune destruction.[21,22] Another avenue of research focuses on negative posttranscriptional regulators of virus expression, eg, pX-encoded Rex and p30^{II} proteins.[23] The HTLV-1 basic leucine

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zipper factor (HBZ), encoded by the provirus negative strand is suspected of down-regulating virus transcription and contributing to immune escape.[24] HBZ mRNA promotes $CD4^+$ T-lymphocyte proliferation and, unlike Tax mRNA, is consistently detected in ATL cells.[25] HBZ expression probably contributes to leukemogenesis.[26,27]

Herein, we evaluated the kinetics of *Tax*-, *Gag*-, and *HBZ*-gene expressions in $CD4^+$ T lymphocytes from ACs and HAM/TSP patients during short-term cultures with or without valproate. The HBZ-mRNA increase was delayed concomitant with decreasing *Tax*- and *Gag*-gene products. Notably, VPA had opposite effects on both transcriptions: enhancing sense transcriptions (ie, Tax and Gag expression), while impairing antisense transcription (ie, HBZ mRNA). Our findings suggest that HDACs and their inhibitors have complex interactions with HTLV-1 replication and clonal expansion of infected cells.

Methods

Patients

This study, conducted at University Hospital of Fort-de-France, Martinique (French West Indies), included 11 HAM/TSP patients and 12 ACs. HAM/TSP diagnosis was based on the 4 World Health Organization criteria: slowly progressive spastic paraparesis with symmetrical pyramidal signs, disturbed bladder function, no radiologic evidence of significant spinal cord compression, and intrathecal synthesis of anti–HTLV-1 antibodies. Since 1998, magnetic resonance imaging has replaced myelography to exclude spinal cord compression.[9] ACs had no neurologic symptoms. Peripheral blood samples were used in accordance with French bioethics laws concerning biologic collections. All experiments using patient samples were approved by the Ministère de la Recherche and the Comité de Protection des Personnes.

Cell culture and VPA treatment

We isolated PBMCs from EDTA-anticoagulated blood samples on Ficoll-density gradients and washed them in PBS. $CD8^+$ cells were removed using anti-CD8 paramagnetic microbeads (Miltenyi Biotec), following the manufacturer's instructions. $CD8^+$-cell–depleted PBMCs were then placed in culture wells (round-bottomed 24-well plate) at 10^6/mL in 1 mL of RPMI 1640 medium, supplemented with 10% FCS, glutamine (2mM), penicillin (100 IU/mL), and streptomycin (100 μg/mL; Eurobio). When appropriate, VPA was added to the medium at 1 or 5mM concentrations; the former concentration is pharmacologically relevant,[21] whereas the latter concentration was used to search for dose-response effects. Cells and culture supernatants were harvested after different times of incubation at 37°C in 5% CO_2, from day 0 (D0) up to D5, depending on the analysis performed.

Flow cytometry analysis of apoptosis

Cells were washed in PBS, resuspended in annexin V–binding buffer, and incubated for 15 minutes at room temperature with FITC-labeled annexin and propidium iodide (PI) reagents (BD Biosciences). We analyzed 10 000 events in dual-labeled samples by using a flow cytometer (FACSCalibur; BD Biosciences). Percentages of viable and apoptotic cells were determined using CellQuest software (Becton Dickinson Immunocytometry Systems) after appropriate compensations.

Flow-cytometry detection of Tax protein

Cells were washed in PBS and then incubated with peridinin-chlorophyll protein–labeled anti-CD3, allophycocyanin-labeled anti-CD4, and phycoerythrin-labeled anti-CD25 or isotype control monoclonal antibodies (mAbs; BD Biosciences) for 30 minutes at 4°C. Cells were fixed and permeabilized by using the Cytofix/Cytoperm Fixation/Permeabilization Solution kit (BD Biosciences), as recommended by the manufacturer. Cells were then incubated with 1/100-diluted anti-Tax protein Lt-4-FITC–conjugated mAb[28] or immunoglobulin G3 isotype-control mAb (Southern Biotechnology Associates) for 30 minutes at 4°C. The cells were washed twice in PBS before analysis of at least 10 000 events for each 4-fold–labeled sample with FACSCalibur and CellQuest software.

Detection of p19 virus-core protein by ELISA

Cell culture supernatants were collected, and virus-core p19 protein was analyzed by ELISA (Retrotek; Zeptometrix), according to the manufacturer's protocol. Absolute concentrations of p19 were determined with a standard purified-antigen dilution curve.

Analyses of Tax-, Gag-, and HBZ-gene expressions by quantitative RT-PCR

Cells were collected and cryopreserved as dry pellets until used. Nucleic acid was extracted using the AllPrep DNA/RNA Mini kit (QIAGEN). To obtain first-strand cDNA, total RNA isolated from each sample was subjected to reverse transcription by SuperScript II reverse transcriptase (Invitrogen) in the presence of oligo(dT)12-18 primer (Invitrogen). Real-time PCR was run in triplicate by using LightCycler 480 SYBR Green I Master Mix on LightCycler 480 thermocycler (Roche Applied Science). Respective forward and reverse primers used were *Tax* (forward primer, 5′-CCAACACCATGGCCCACTT-3′; reverse primer, 5′-GATGGGGTCCCAGGTGATCT-3′), Gag (forward primer, 5′-AGCCCCCAGTTCATGCAGACC-3′; reverse primer, 5′-GAGGGAGGAGCAAAGGTA-3′), and HBZ (forward primer, 5′-ATGGCGGCCTCAGGGCTGT-3′, reverse primer, 5′-TGGAGGGCCCCGTCGCAG-3′). Relative mRNA quantification was performed using Cp (crossing point) determined by the second derivative peak of each amplification curve and normalized to reference genes β-actin (forward primer, 5′-CCAACCGCGAGAAGATGA-3′; reverse primer, 5′-CCA GAG GCG TAC AGG GAT AG-3′) and hypoxanthine-guanine phosphoribosyltransferase-1 (forward primer, 5′ TGACACTGGCAAAACAATGCA-3′; reverse primer, 5′-GGTCCTTTTCACCAGCAAGCT-3′).[29]

Measurement of HTLV-1–provirus load

HTLV-1–proviral load was quantified using the real-time TaqMan polymerase chain reaction method, as described previously.[6] In brief, SK110/SK111 primers were used to amplify a 186-bp fragment of the *pol* gene, and the dual-labeled TaqMan probe (5′-5-carboxyfluorescein and 3′-5-carboxytetramethylrhodamine) was located at bp 4829-4858 of the HTLV-1 reference sequence (HTLVATK). Albumin DNA was quantified in parallel to determine the input cell number and was used as an endogenous reference. Standard curves were generated using 10-fold serial dilutions of a double standard plasmid (pcHTLV-ALB) containing 1 copy of the target regions of the HTLV-1 *pol* and cellular albumin genes. All samples were run in duplicate. The HTLV-1–provirus load was reported as ([HTLV-1 average copy number]/[albumin average copy number]) × 2 × 10^6 and is expressed as the number of HTLV-1 copies/10^6 cells.

Estimation of gene expression per HTLV-1–infected cell

Assuming that almost all infected cells in non-ATL cases harbor only 1 provirus, it can be considered that provirus load reflects the percentage of infected cells. Proviral load was used to estimate the percentage of HTLV-1–provirus positive cells expressing Tax protein and, when appropriate, to normalize gene expression between different samples and incubation times.

Statistical analyses

Data on paired and unpaired observations were compared, respectively, with Wilcoxon's signed-rank test and the Mann-Whitney *U* test. Correlations between continuous variables were assessed with Spearman's rank-order statistic. Statistical significance was set at $P < .05$.

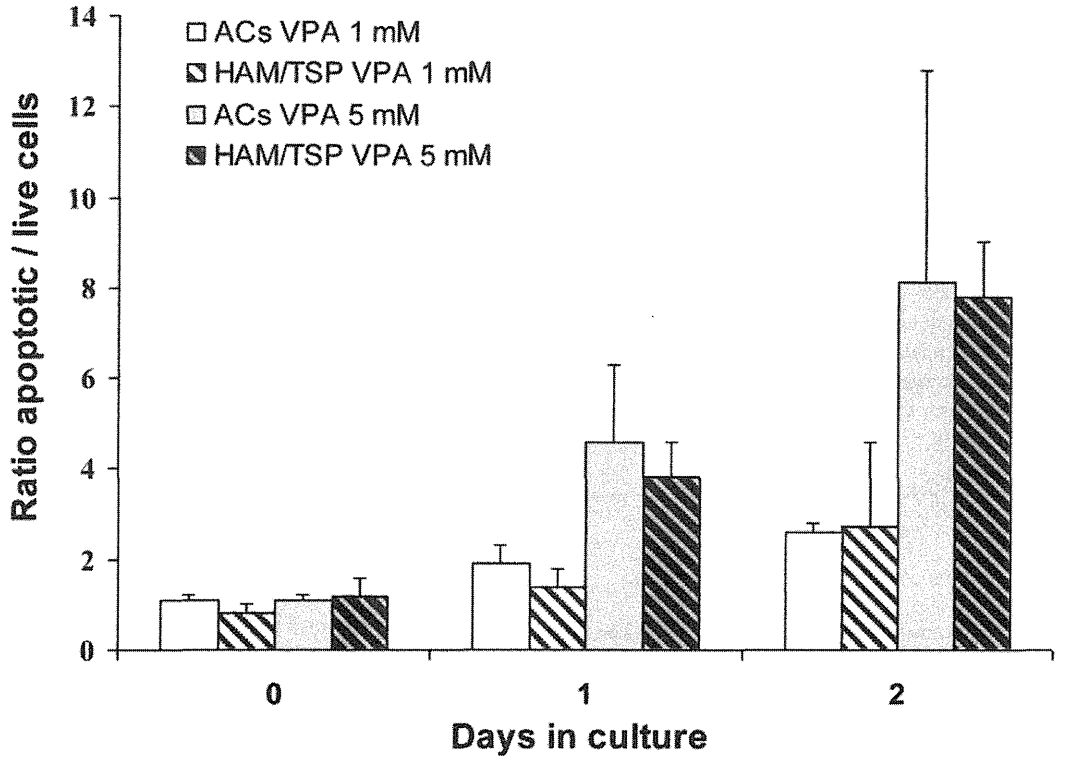

Figure 1. VPA is proapoptotic for ex vivo-cultured CD4+ T cells from HTLV-1 AC and HAM/TSP patients. CD4+ cells isolated from 10 AC patients (non–cross-hatched bars) and 10 HAM/TSP patients (cross-hatched bars) were cultured for 6 (D0), 24 (D1), or 48 hours (D2) with 1mM VPA (white back bars) or 5mM (gray back bars) VPA. Apoptotic cells were identified by flow cytometry based on annexin-PI labeling, and the ratios of the apoptotic rates of VPA-treated to nontreated cells were calculated. Mean ratio ± 1 SD are plotted.

Results

VPA was proapoptotic for lymphocytes isolated from AC and HAM/TSP patients

We first verified VPA impact on cell viability of short-term cultured CD8+-cell–depleted lymphocytes from HTLV-1–infected individuals. The VPA proapoptotic effect increased gradually from D0 to D2 and was dose-dependent, being higher at 5mM than at 1mM VPA (Figure 1). On D2 of culture of AC lymphocytes, the mean ± SD percentages of annexin-positive cells were 16 ± 2% in 1mM VPA-treated versus 6 ± 1% in nontreated wells and 29 ± 15% in 5mM VPA-treated versus 4 ± 1% in nontreated wells (Wilcoxon signed-rank test: $P = .008$ and .008, respectively). D2 rates of HAM/TSP patients' apoptotic cells were 12 ± 6% in 1mM VPA-treated versus 5 ± 2% in nontreated wells and 31 ± 2% in 5mM VPA-treated versus 4 ± 1% in nontreated wells (Wilcoxon signed-rank test: $P = .03$ and .008, respectively). As shown in Figure 1, VPA-induced apoptosis levels in cells from AC and HAM/TSP patients at each time, and VPA concentration were comparable (Mann-Whitney U test, $P > .5$).

VPA did not affect HTLV-1–provirus load during ex vivo culture of HTLV-1–infected CD8+-cell–depleted PBMCs

Mean ± SD and median HTLV-1–provirus loads in freshly isolated (D0) and CD8+-cell–depleted PBMCs from all samples evaluated were 50 589 ± 36 636 and 43 500 copies/10^6 cells. No correlation was found between initial percentages of HTLV-1–infected cells, assessed as D0 provirus load, and VPA-induced apoptosis observed on culture D2 (Spearman's rank-correlation test, $P = .9$). The percentages of HTLV-1–infected cells, assessed as HTLV-1–provirus load, remained stable over 5 days of culture with or without VPA (Figure 2). The difference observed on D5 was not significant (Wilcoxon signed-rank, test $P = .1$).

VPA enhanced Tax- and Gag-protein levels in short-term cultures of HTLV-1–infected CD8+-cell–depleted PBMCs

We next analyzed the expression of intracellular Tax by flow cytometry for 20 HTLV-1–infected samples. Tax-labeling efficiency using the anti–Tax Lt-4-FITC–conjugated mAb was first

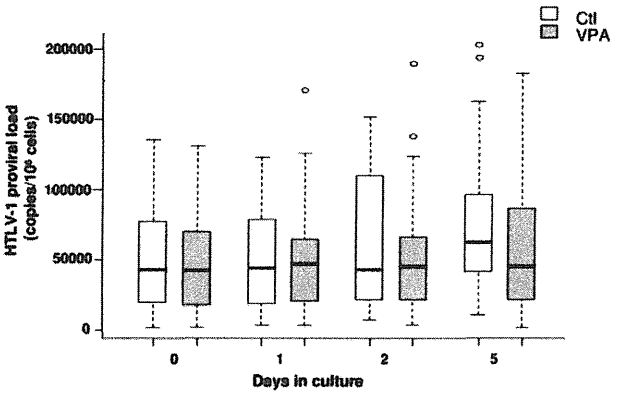

Figure 2. HTLV-1–proviral load remained stable throughout ex vivo culture of HTLV-1–infected CD4+ T cells with or without valproate. CD4+ T cells isolated from HTLV-1–infected individuals were cultured without (control [Ctl]) or with 5mM VPA (white and gray boxes, respectively). HTLV-1–proviral load was measured on D0, D1, D2, and D5 for the 20 subjects. Horizontal bars show the bold median and, from bottom to top, the 10th, 25th, 75th, and 90th percentiles.

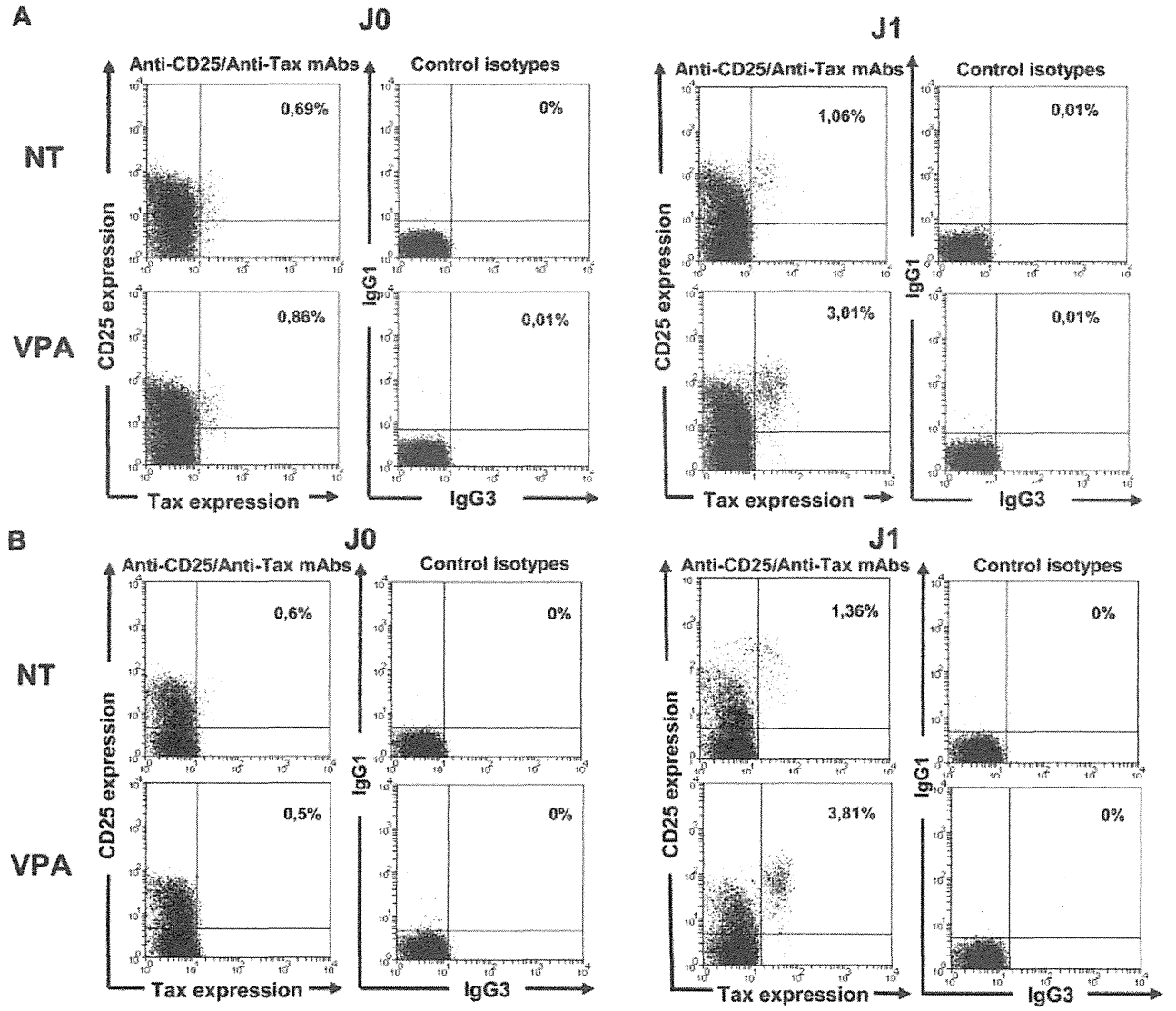

Figure 3. Tax induction in HTLV-1–infected CD4⁺ T cells treated with 1mM VPA. Intracellular Tax-protein expression in CD4⁺ T cells was analyzed by flow cytometry using quadruple CD3, CD4, CD25, and Tax (Lt-4 mAb) labeling and appropriate isotype controls. Experiments were performed on D0, D1, and D2 of culture of lymphocytes from 10 HTLV-1 ACs (A) and 10 HAM/TSP patients (B). D0 and D1 results from 1 representative experiment are shown.

verified using MT2 cells (data not shown). Quadruple labeling showed that Tax was almost exclusively detected in CD25-expressing $CD3^+CD4^+$ lymphocytes (Figure 3A-B).

Tax was detected in < 0.5% of $CD4^+$ T cells freshly isolated (D0) or cultured for 6 hours with or without VPA. Mean percentages of Tax protein-expressing $CD4^+$ T cells in VPA-treated and nontreated wells rose to 3.4% and 1.1%, respectively, on D1 (Wilcoxon signed-rank test, $P = .01$) and 1.4% and 0.4%, respectively, on D2 (Wilcoxon signed-rank test, $P = .02$). Mean peak percentage (D1 or D2) of Tax expression was 3.9% of VPA-treated and 1.1% of nontreated $CD4^+$ T cells (Wilcoxon signed-rank test, $P = .001$). Median values are shown in Figure 4A. Results were similar for both VPA concentrations (data not shown). When considering HTLV-1–provirus loads measured at each time, the estimated median rate of provirus-positive $CD4^+$ T cells spontaneously expressing Tax increased from < 8% on D0 to 19% on D1 and returned to 10% on D2. When VPA was added to the culture medium, the median Tax-detection rate rose to 63% on D1 but was 18% on D2. The difference between VPA-treated and nontreated samples was significant on D1 but not D2 (Wilcoxon signed-rank test, $P = .00001$ and .059, respectively). Tax-expression kinetics with and without VPA was similar in samples from 10 AC and 10 HAM/TSP patients (Figure 4B).

Expression of HTLV-1–core p19 protein was monitored in cell culture supernatants, and levels were corrected considering the proportion of nonapoptotic cells (Figure 5). VPA stimulated p19-protein production that differed significantly from nontreated cells on D2 (Wilcoxon signed-rank test, $P = .04$).

Opposite VPA effects on the kinetics of HTLV-1 sense and antisense gene expressions in $CD8^+$-cell–depleted PBMCs from HTLV-1–infected subjects

To explore further the VPA effect on Tax and HBZ expressions at the transcriptional level, we investigated Tax-, Gag-, and HBZ-mRNA expression kinetics by quantitative RT-PCR.

We first quantified viral mRNA expression in $CD8^+$-cell–depleted PBMCs from AC or HAM/TSP patients cultured without

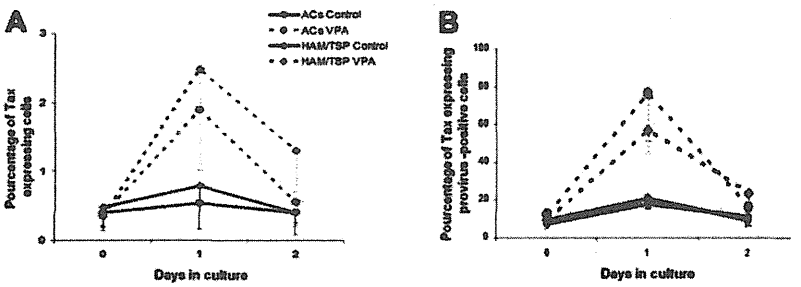

Figure 4. Valproate increases Tax-protein expression in HTLV-1–infected CD4+ T cells from asymptomatic carriers and HAM/TSP patients. Rates of Tax-protein–positive cells among HTLV-1–infected CD4+ T cells were calculated by normalizing the percentages of Tax-positive CD4+ cells, determined by CD3, CD4, and Tax triple labeling (A), with the percentage of infected cells in the CD4+ population, as assessed by HTLV-1–proviral load (B). Estimated median (and first quartile) percentages of Tax-positive cells among HTLV-1–infected CD4+ cells on D0 to D2 of culture is represented for 10 ACs (●) and 10 HAM/TSP patients (♦) treated with 5mM VPA (dashed lines) or nontreated (Ctrl, solid lines), respectively.

VPA (Figure 6). For both groups, Gag and HBZ expressions were low at culture onset. We observed that in AC, Tax-mRNA level correlated with provirus load (Spearman's coefficient, $R = 0.812$; $P = .008$) but not for HAM/TSP patients ($R = 0.195$, $P = .59$). As expected, we also observed increased Tax expression during the first 24 hours of culture. Maximum of Tax-expression peaks were reached after 1 day of culture for both groups, independently of clinical status (Figure 6A-B). Concomitantly with the Tax increase, the Gag-mRNA level rose during D1 of culture. Tax- and Gag-mRNA kinetics were parallel, and their expressions were correlated (R = 0.546, $P = .0007$ for AC patients; $R = 0.565$, $P = .0001$ for TSP/HAM patients).

Median HBZ-mRNA expressions increased after 5 days of culture in CD8+-cell–depleted PBMCs from AC patients' cells (Figure 6A) and after only 2 days of culture in HAM/TSP patients' cells (Figure 6B). It is worth noting that the HBZ-mRNA level on D5 correlated with proviral load in AC patients ($R = 0.800$, $P = .01$) but not in HAM/TSP patients, and increased HBZ expression, issued from 3′-long terminal repeat (LTR)-dependent transcription, seemed to correspond to decreased gene expression derived from 5′-LTR–dependent transcription (Tax and Gag).

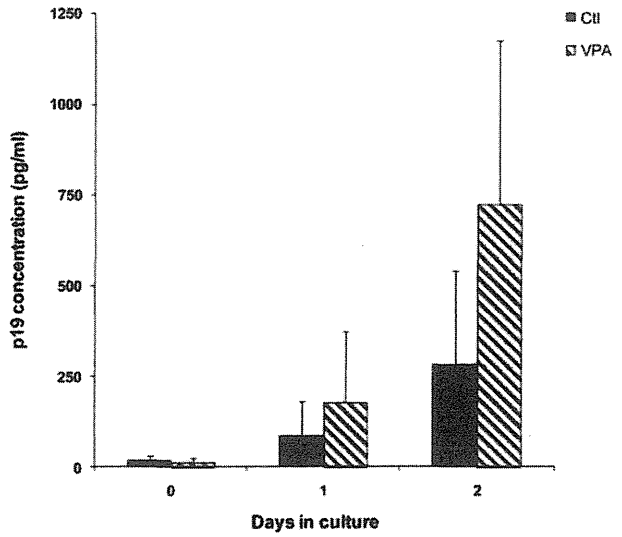

Figure 5. VPA activates the expression of the virus core-protein p19 in the culture supernatants of CD4+ T cells from HTLV-1–infected individuals. Culture supernatants were collected and expression of virus core-protein p19 was quantified by ELISA. Absolute p19 concentrations (in picograms per milliliter) were determined by normalization of absorbance values to a standard curve. p19-protein levels were corrected by considering the proportion of nonapoptotic cells, determined from annexin-PI flow cytometry analysis of each sample. Mean ± 1 SD concentration at different incubation times is represented for the 20 HTLV-1–infected samples cultured without (black bars) or with 5mM VPA (cross-hatched bars).

We next assessed the VPA effect on *Tax*-, *Gag*-, and *HBZ*-gene expressions. CD8+-cell–depleted PBMCs from HTLV-1–infected subjects were incubated with 1 or 5mM VPA. We observed an increased Tax mRNA in treated lymphocytes, as observed by flow cytometry (Figure 3). When 1mM VPA was used, no difference was observed in the Tax-induction ratio (Tax level with VPA/Tax level without VPA) between cells from AC and HAM/TSP patients (data not shown). However, with 5mM VPA, the Tax-induction ratio was significantly higher in HAM/TSP patients' cells than in AC patients' cells after 2 days of culture (Wilcoxon signed-rank test: $P = .004$ and .005, respectively, vs nontreated cells). When

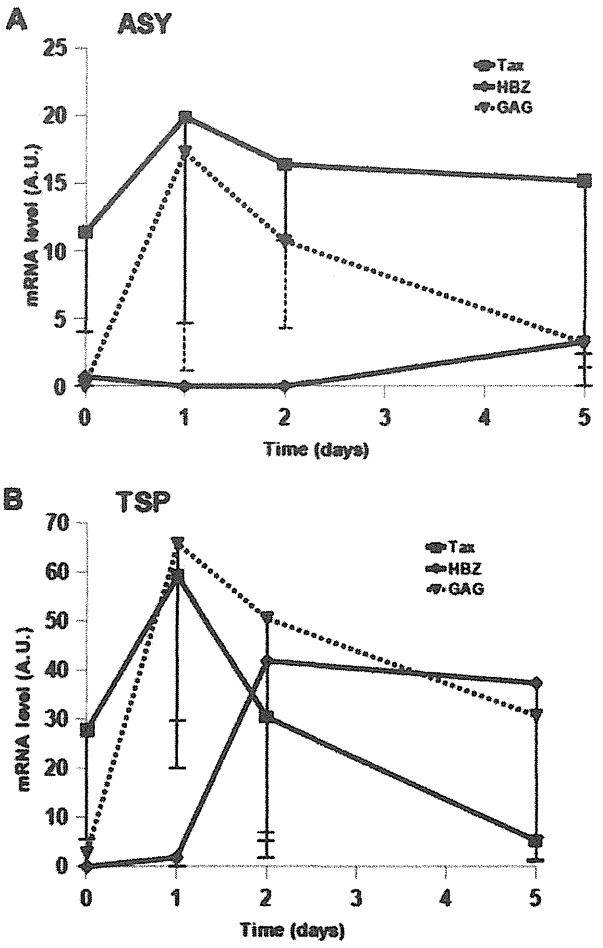

Figure 6. Kinetics of Tax-, Gag-, and HBZ-mRNA expressions in short-term cultures of CD4+ T cells from HTLV-1 asymptomatic carriers and HAM/TSP patients. Tax- (■, solid line), Gag- (▲, dotted line), and HBZ-mRNA (♦, solid line) in ex vivo cultured lymphocytes from AC (A) or HAM/TSP (B) patients were quantified (as described under "Methods"). The medians (and first quartiles) expressed in arbitrary units (AU) for 8 AC and 10 HAM/TSP patients are shown.

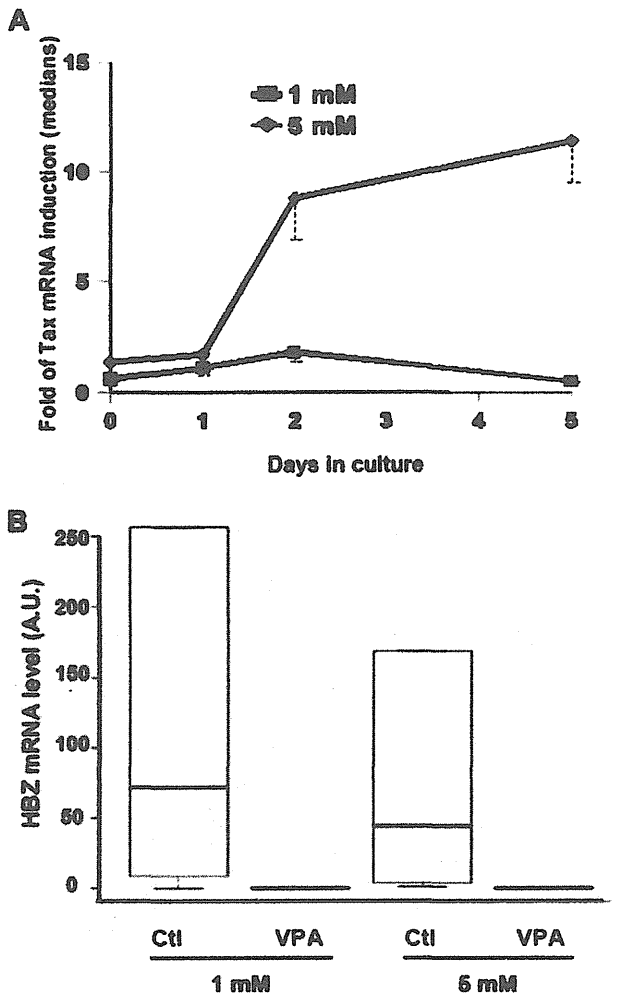

Figure 7. VPA effects on Tax and HBZ expressions in CD4+ T cells from HAM/TSP patients. (A) Patients' CD4+ T cells were cultured for the indicated times with 1mM (■) or 5mM VPA (◆). The curves illustrate VPA induction of Tax expression compared with the corresponding nontreated sample (medians and first quartiles of 5 patients for each concentration). (B) Box plot of the HBZ-mRNA levels, expressed in arbitrary units (AU), in CD4+ T lymphocytes from HAM/TSP patients after 5 days of culture with or without the indicated VPA concentration (5 patients per concentration). Horizontal lines are the bold medians and, from bottom to top, 10th, 25th, 75th, and 90th percentiles.

we compared VPA Tax-induction ratios in ex vivo cultures of T lymphocytes from HAM/TSP patients, both VPA concentrations had similar effects during the first 24 hours of culture (Figure 7A). For cells treated with 1mM VPA, the median Tax-induction peaked after 48 hours of culture and decreased between D2 and D5. In contrast, in 5mM VPA-treated T lymphocytes from HAM/TSP patients, Tax expression was strongly induced from D2 to D5, compared with that of nontreated cells (Wilcoxon signed-rank test: $P = .003$ and .0465, respectively; Figure 7A). Moreover, this induction in 5mM VPA-treated cells was significantly higher than in 1mM VPA-treated cells (Wilcoxon signed-rank test, $P = .01$ on D2 and .008 on D5). Furthermore, in the presence of 5mM VPA, Tax expression remained high during ex vivo culture of HAM/TSP patients' cells. No such phenomenon was observed in 5mM VPA-treated AC patients' cells.

Although VPA increased the Gag-mRNA level in both HTLV-1–infected groups (data not shown), induction was only statistically significant in HAM/TSP patients' lymphocytes after 24 hours of ex vivo culture with 1mM VPA and from D2 to D5 for the 5mM dose (Wilcoxon signed-rank test: $P = .03$, .009, .003, and .01 for D2, D3, D4, and D5, respectively).

We next examined VPA impact on HBZ expression (Figure 7B). Intriguingly, VPA seemed to have an opposite effect from that observed on *Tax* and *Gag* genes controlled by 5′-LTR transcription. Indeed, VPA treatment inhibited HBZ expression during late culture times. This inhibition was especially evident on D5, even with the lowest VPA concentration, for HAM/TSP patient's cells (Wilcoxon signed-rank test: $P = .05$ and .05; Figure 7B). No statistically significant VPA effect was seen on HBZ expression in AC patients' cells, probably because the initial HBZ level in nontreated cells was too low to observe any down-modulating effect of VPA.

Discussion

Reduction of the HTLV-1–provirus load might prevent long-term development of HAM/TSP or slow its progression. Therapeutic protocols designed to affect HTLV-1–infected cell proliferation or virus replication are still ineffective.[30] A novel approach, called gene-activation therapy, has been proposed, based on preclinical trials in the bovine leukemia virus model[31] and preliminary data on HAM/TSP.[21] The principle is to activate viral gene expression by HDAC inhibitors and thereby expose virus-positive cells to the host immune response.[22]

Attention has focused on VPA, the sodium salt of 2-propylpentanoic acid, which is well tolerated and displays adequate pharmacokinetics. This compound induces histone hyperacetylation and activates HTLV-1 5′-promoter–driven transcription.[21] VPA enhances Tax-protein expression during short-term culture of HTLV-1–infected cells.[32] Mosley et al estimated that VPA exposure increased the percentages of Tax-expressing provirus-positive CD4+ cells from 13% to 22%. We confirmed this observation, but with Tax-expressing provirus-positive cells rising from one-fifth spontaneously to two-thirds after adding VPA to the culture. Almost all Tax-expressing CD4+ T cells were CD25-positive. The $CD3^+CD4^+CD25^+$ subset is the major reservoir of HTLV-1–provirus and Tax peptide–HLA class I complexes and might stimulate and expand HTLV-1 Tax-specific CD8+ T cells.[3] Together, these quantitative and qualitative data are consistent with the theoretical hypothesis of unmasking of the latently infected cell pool and CTL clearance. However, concerns have been raised about therapeutic applications. A variety of proteins are regulated by HDAC-mediated acetylation and it was suggested that, as a side-effect of VPA treatment, the CD8+ cell antiviral function might be altered.[32] Moreover, memory CD8+ T cells contribute to virus reservoir in vivo and might be destroyed by autologous HTLV-1–specific CTL in a fratricidal response.[33] Finally, the major risk would be to trigger virus replication, as evidence by viral p19 matrix-antigen release, stimulate Tax-driven clonal expansion, and favor, if uncontrolled, subsequent central nervous system-tissue invasion.

To address this question, more information on the mechanisms involved in controlling HTLV-1–gene expression is required. HBZ is a potent suppressor of Tax-mediated virus-gene transcription by interacting with activating transcription factor/cAMP responsive element-binding protein (CREB) and CREB-binding protien/p300 on the 5′-LTR promoter.[24,34,35] The HBZ role in the tightly regulated pattern of *HTLV-1*–gene expression also is suggested by HBZ repression of Gag-p19 synthesis in stable virus-producing cell clones.[36] Kinetic study of gene expressions in cells transiently

transfected with the HTLV-1–provirus plasmid and in newly infected PBMCs showed that Gag/Pol, Tax/Rex, and Env mRNA are detected first and at their highest levels, whereas *HBZ* transcription was significantly lower and peaked later.[37] However, the experimental systems used by Li et al[37] remained far removed from the physiopathologic conditions of established HTLV-1 infections.

Herein, we described similar kinetics in freshly isolated and short-term cultured cells from HTLV-1–infected individuals. Tax- and Gag-mRNA expressions peaked at high levels on culture D1, before declining progressively concomitant with the rise of HBZ expression. Although the chronology in HAM/TSP cells is consistent with the hypothesis of a feedback loop coordinating Tax and HBZ expressions, we cannot exclude that in ACs the observed concomitant Tax-expression decrease and HBZ-expression increase are not related to cellular mechanisms or perhaps involve other HTLV-1 auxiliary proteins, for example, p30 or Rex.[23] Indeed, as shown in Figure 6, expression of Tax and HBZ were regulated differently in infected cells from AC and TSP/HAM patients.

We analyzed VPA impact on the balance of Tax/HBZ-mRNA expression in freshly cultured cells. VPA significantly enhanced but also prolonged Tax-mRNA levels. It should be noted that, in the presence of 5mM VPA, *Tax*-gene–expression kinetics was profoundly modified, with Tax rising constantly during lymphocyte culture, suggesting dysregulation of the processes responsible for its expression in cultured lymphocytes from HAM/TSP patients but not from ACs. Gag-mRNA–level kinetics under VPA was consistent with Tax findings. Surprisingly, VPA blocked the expression of HBZ. VPA's opposite effects on Tax and HBZ expressions might be explained by its isoenzyme-selective down-modulator properties. Indeed, HDAC complexes differ between the 5′- and 3′-HTLV-1 promoters, with HDAC1 and HDAC2 binding preferentially at the 5′-LTR and HDAC3 binding at the 3′-LTR.[38] VPA, in addition to the weakly inhibiting catalytic activity of class I HDAC[39] induces proteasomal degradation of HDAC2, unlike other inhibitors, eg, trichostatin A.[40] VPA preferentially releases HDAC2-dependent transcriptional repression and therefore might favor Tax binding at the 5′-LTR and sense-strand transcription. VPA modulation of HDAC levels is selective and does not affect HDAC3.[40] Alternatively, we cannot exclude that activation of sense transcription by Tax and VPA would impair antisense transcription, either by competing for transcription factors (ie, activating transcription factor/CREB factors) or interfering with its initiation. Indeed, we showed previously that deletion of the 5′-LTR (ie, sense transcription) promoted transcription from the 3′-LTR (ie, antisense transcription).[41]

HBZ mRNA has a growth-promoting effect on T lymphocytes, as demonstrated by mutation analyses of *HBZ* gene and short-hairpin RNA knockdown experiments.[25,42] The HBZ-mRNA–expression level has been shown to correlate with proviral load and was linked to survival of virus-infected cells in a rabbit model.[37] In vivo HBZ expression was correlated to proviral load[43,44] and HAM/TSP severity.[44] We consistently observed significantly higher spontaneous HBZ-mRNA levels in samples from HAM/TSP patients than in samples from ACs. $CD8^+$ cell depletion enabled evaluation of the intrinsic VPA impact on infected lymphocytes. Despite increased Tax production, percentages of HTLV-1–infected cells did not increase overtime in VPA-treated samples. That observation suggests that VPA-induced repression of HBZ expression counterbalances Tax stimulation of virus replication and T-cell proliferation.

Initial proof-of-concept studies provided evidence of complex relationships between VPA administration and HTLV-1–proviral loads. VPA treatment of HAM/TSP patients increased peripheral blood proviral load during the first weeks of the trial,[21] an observation recently confirmed in the simian T-cell leukemia virus type-1 (STLV-1) model.[45] Addition of azidothymidine (AZT) to block infectious propagation prevented the transient rise of virus production, and combined VPA and AZT treatment rather than VPA alone, strongly decreased the STLV-1–proviral load. Afonso et al[45] suggested that virus-expressing cells were killed by STLV-1–specific CTLs, which are protected by AZT from fratricidal destruction. In a recent 2-year clinical trial, VPA alone did not alleviate HAM/TSP symptoms (S.O. and L.W., manuscript in preparation). Results reported herein suggest that, in addition to Tax expression and the Tax-mediated CTL response, another mechanism involving HBZ repression might affect the net outcome of VPA therapy. Moreover, a more recent paper confirmed that HBZ plays a central role in HTLV-1 persistence and the authors suggested that, despite Tax being the immunodominant antigen, the CD8+-T cells specific to HBZ are the most effective at controlling HTLV-1.[46] The VPA-induced HBZ decrease also could enable the infected cells to escape this efficient immune response, thereby limiting the therapeutic impact on the virus reservoir within treated patients.

VPA induced moderate and dose-dependent apoptosis of cultured $CD4^+$ lymphocytes. Apoptosis rates were similar in lymphocytes from HAM/TSP patients and ACs, and there was no evidence of specific elimination of HTLV-1–infected cells. However, this drug requires further studies designed to test its effect on HTLV-1–transformed cells. Indeed, VPA has been shown to induce the death of chronic lymphocytic leukemia cells,[47] and several trials are currently exploring its activity against various types of cancer, including hematologic malignancies.[48] Depsipeptide, another HDAC inhibitor, has demonstrated efficacy against primary ATL cells.[49] Moreover, in a murine model of human ATL,[50] HDAC inhibitors are able to trigger growth arrest and death of HTLV-1–infected cell lines and ATL cells via activation of the death-receptor pathway and potentialization of tumor necrosis factor-related apoptosis-inducing ligand response.[51] More generally, epigenetic drugs are known to regulate expression of tumor-suppressor genes and activities of transcriptional factors involved in cancer initiation and progression. In the HTLV-1 model of leukemogenesis, HBZ is critical for immune escape and proliferation of ATL cells.[27] The possibility of targeting HBZ expression with VPA at therapeutically useful concentrations opens a new avenue of research for the prevention or treatment of HTLV-1–associated diseases.

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Authorship

Contribution: G.B. performed experiments and contributed to the experimental design and data interpretation; A.G. performed experiments and contributed to data interpretation and paper writing; A.L. and M.D. contributed to the experimental design; I.K.-S. performed experiments; S.O. and D.S. performed clinical assessment and recruitment of patients; Y.T. provided essential reagents; L.W., J.-M.M., and J.-M.P. contributed to paper writing; and R.C. designed the study, interpreted data, and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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MAJOR ARTICLE

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

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

INTRODUCTION

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

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

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

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

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

MATERIALS AND METHODS

Patients

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

Cell Surface Staining

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

Intracellular IFN-γ Detection

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

Table 1. Clinical Characteristics of the Study Groups

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

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

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

CD107a Degranulation Assay

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

Quantitative Polymerase Chain Reaction of the HTLV-I Proviral Load

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

Intracellular HTLV-I Tax Staining

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

Statistical Analysis

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

RESULTS

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

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

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

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

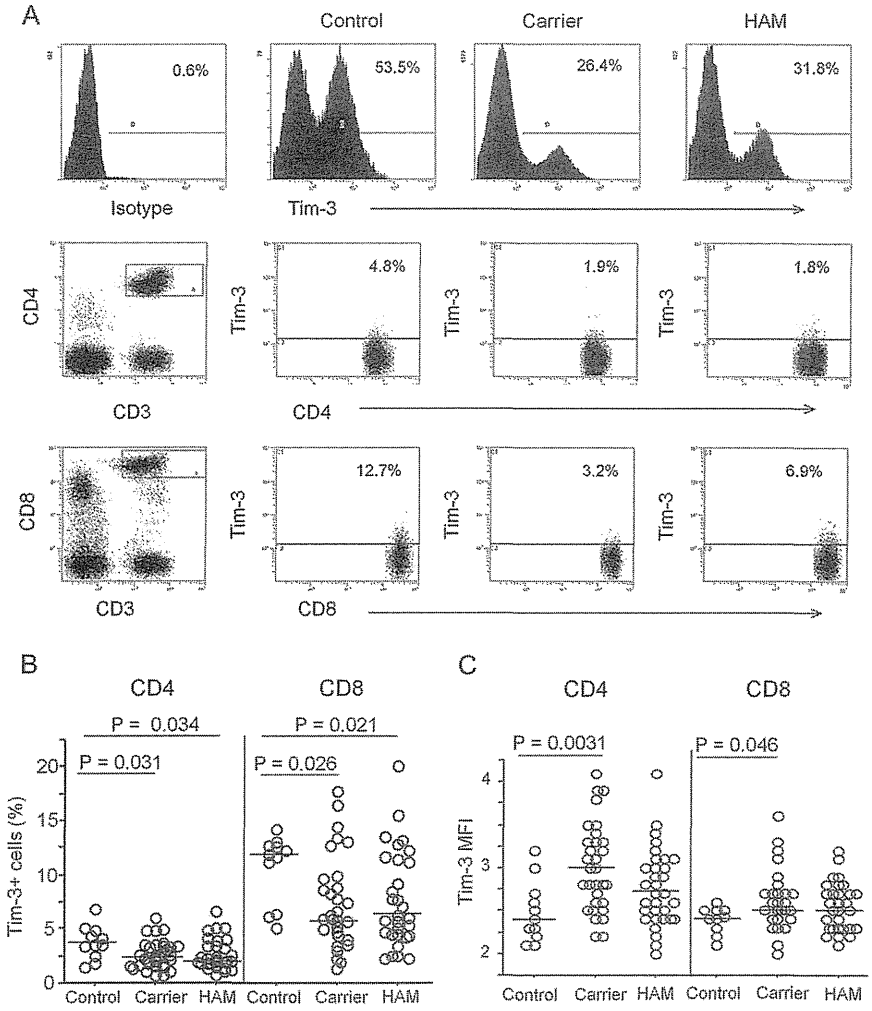

Figure 1. Low frequency of Tim-3$^+$ cells within the CD4$^+$ and CD8$^+$ T cell populations in HTLV-I infection. PBMCs from 63 HTLV-I–infected (32 HAM/TSP patients and 31 carriers) and 11 uninfected subjects were stained with antibodies against CD3, CD4, or CD8 and Tim-3. The numbers indicate the percentage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

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

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

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

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

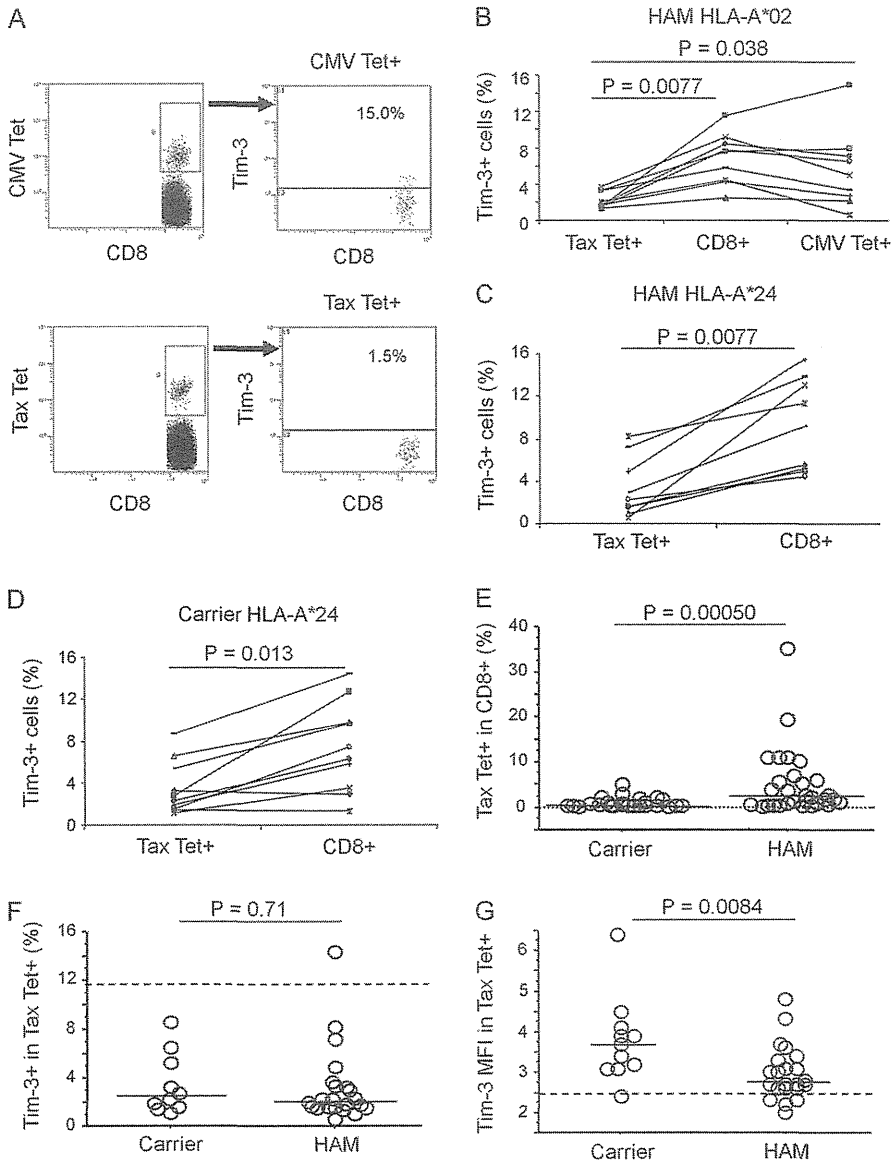

Figure 2. Low expression of Tim-3 on HTLV-I Tax-specific CTLs as compared with that on CMV-specific CTLs in HTLV-I infection. Tim-3 expression was determined in $CD8^+$, $CD8^+$Tax $tetramer^+$, and $CD8^+$CMV $tetramer^+$ cells of HAM/TSP patients and carriers. (*A*) A representative flow cytometry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

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

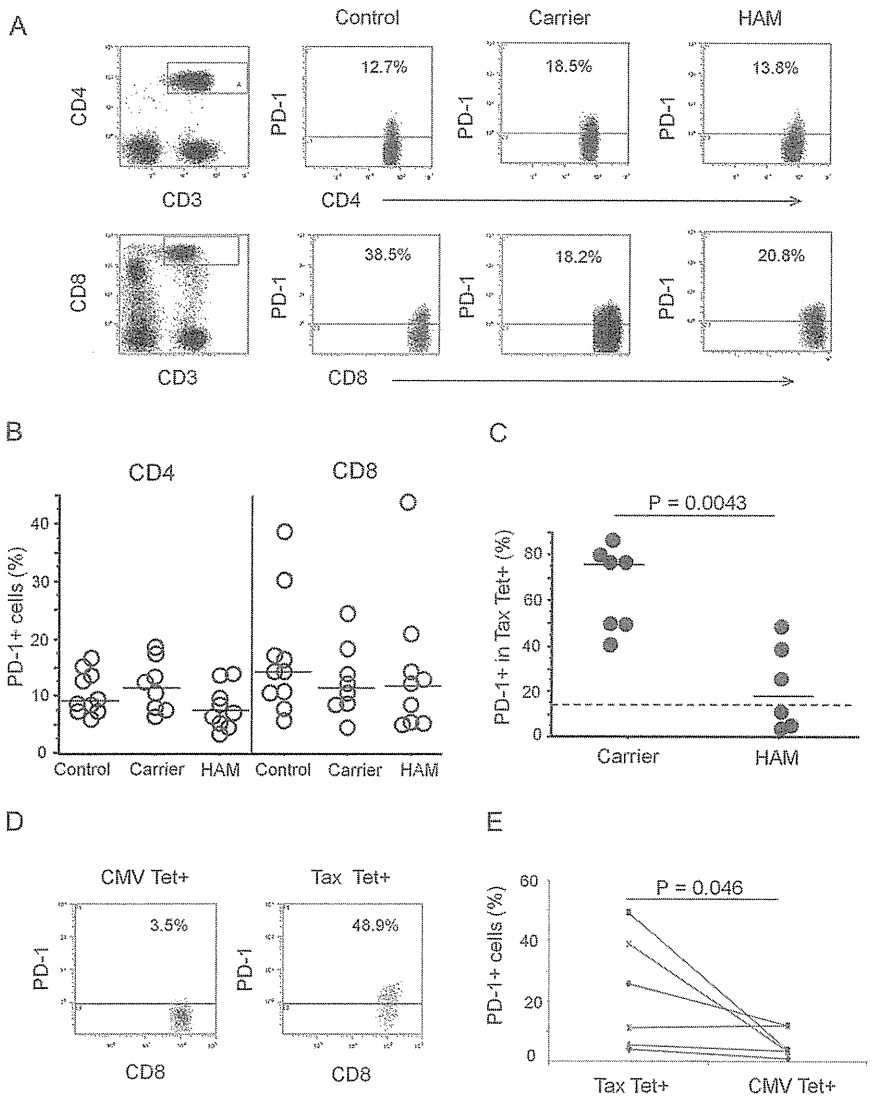

Figure 3. Increased PD-1 expression on HTLV-I Tax-specific CTLs as compared with that on CMV-specific CTLs. PD-1 expression was analyzed in PBMCs from 9 HAM/TSP patients, 8 carriers, and 10 controls after gating $CD3^+CD4^+$, $CD3^+CD8^+$, $CD8^+$Tax tetramer$^+$, or $CD8^+$CMV tetramer$^+$ cells. (*A*)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

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

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

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

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

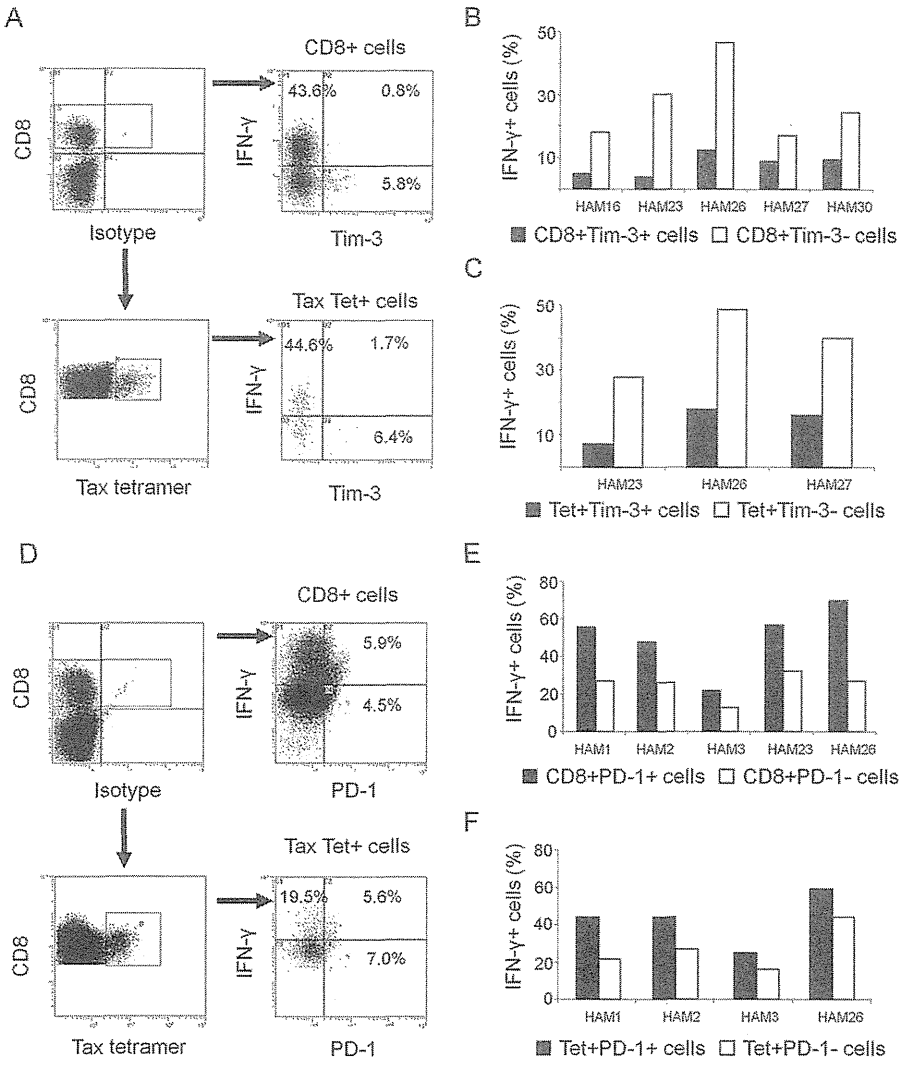

Figure 4. Reduced IFN-γ production by Tim-3$^+$ HTLV-I Tax-specific CTLs. PBMCs from 5 HAM/TSP patients were stimulated with PMA and ionomycin, and cultured for 6 hours in the presence of brefeldin A. IFN-γ production was determined by flow cytometry in CD8$^+$ and CD8$^+$Tax tetramer$^+$ cells with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

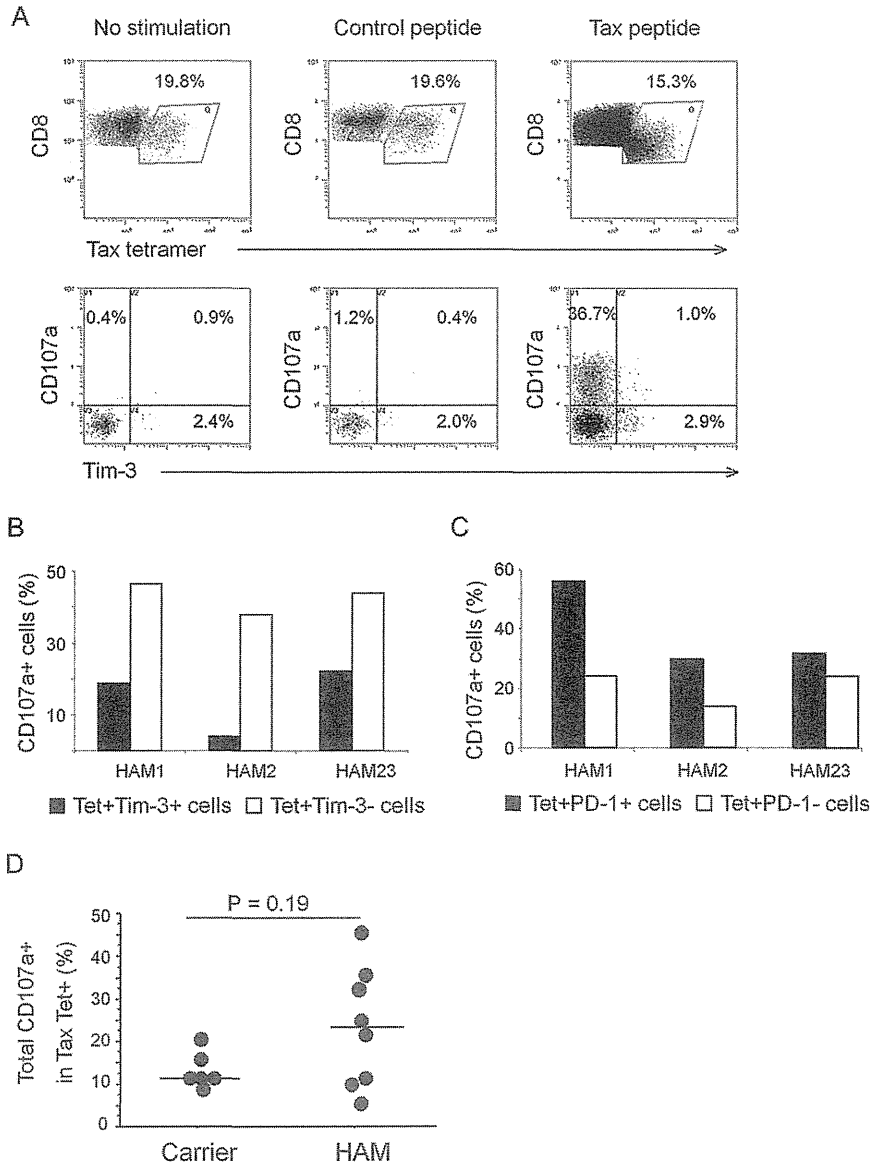

Figure 5. Reduced CD107a expression on Tim-3$^+$ HTLV-I Tax-specific CTLs. PBMCs from 8 HAM/TSP patients and 6 carriers were stimulated with HTLV-I Tax peptide or a control peptide, and cultured in the presence of an anti-CD107a antibody and brefeldin A for 4 hours. The expression of CD107a on CD8$^+$Tax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