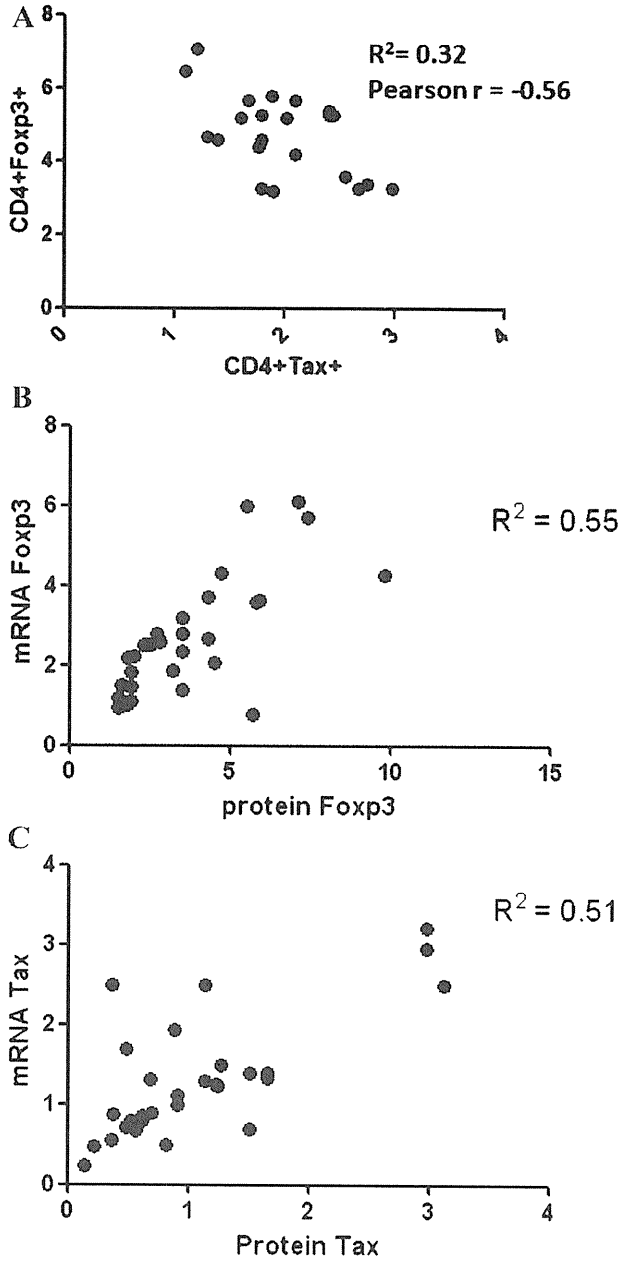

Fig. 4. Correlation between Foxp3 and Tax in HAM/TSP patients. A correlation between $CD4^+Tax^+$ and $CD4^+Foxp3^+$ post-treatment percentage is shown. Data obtained from pre-treatment and post-treatment samples were plotted together in a correlation graph of mRNA and protein levels as well. **A**: A negative and statistically significant correlation between $CD4^+Foxp3^+$ and $CD4^+Tax^+$ cell population was found in post-treatment samples ($r = -0.56$; $P < 0.01$). **B**: Results of Foxp3 mRNA and protein levels calculated as MFI in flow cytometry data showed a positive and statistical significant correlation ($r = 0.55$; $P < 0.001$). **C**: A positive and statistical significant correlation was also found in the case of Tax protein and mRNA data ($r = 0.51$; $P < 0.001$).

and protein levels of Foxp3 after betamethasone treatment. Glucocorticoids action on HTLV-I viral component has not been reported.

Tax protein is the major viral component associated with the development and progression of HAM/TSP, being its mRNA identified as one of the best biomarker of this disease [Yamano et al., 2002; Oh and Jacobson, 2008]. An association was found between betamethasone therapy and a significant decrease in both $CD4^+Tax^+$ T cell population and Tax mRNA in treated patients compared with the pre-treated condition. The results show that HAM/TSP patients treated with betamethasone have reduced levels of infected T $CD4^+$ cells and Tax mRNA with no significant differences with Carriers. Thus, carriers could be a suitable group for comparing the effectiveness of betamethasone treatment in HAM/TSP patients in terms of Tax levels and clinical improvement. In treated patients the decrease in the number of $CD4^+Tax^+$ cells is inversely proportional to the increase of $CD4^+Foxp3^+$ T cells. Only a single patient did not follow this trend not showing a clinical improvement after treatment. This patient had very low percentage of $CD4^+Tax^+$ T cells and Tax mRNA, similar with those detected in Carriers. This patient showed a slight decrease of $CD4^+Foxp3^+$ cells but Foxp3 mRNA levels did not change with betamethasone treatment. These results suggest that the increase in Foxp3 mRNA and decrease of Tax mRNA are simultaneously required for clinical improvement of HAM/TSP patients.

In vitro studies showed a Tax-dependent $CD4^+CD25^+$ Tregs reduction through the suppression of Foxp3 expression [Yamano et al., 2005]. Repression of Tax transcription and transactivation functions by Foxp3 through targeting both NF-κB and CREB pathways have been published [Grant et al., 2006]. Based on the results, betamethasone might employ the same mechanism to reduce Tax levels. Glucocorticoids inhibit this pathway to shut down the immune response. Therefore, betamethasone therapy could produce both an increase in Foxp3 levels and a repression of Tax transcriptional pathways, with the concomitant reduction of this viral protein. Tax and Foxp3 are suggested as potential biomarkers for assessing treatment in HAM/TSP.

No significant differences in mRNA levels of the immunological markers CTLA-4, GITR, IL-10, and TGF-β in HAM/TSP were found in treated patients. These results suggest that cytokines related with the inducible Treg population are not involved in glucocorticoid-dependent Foxp3 increase.

Since all HAM/TSP patients, with one exception, showed gait recovery after glucocorticoid treatment, these results suggest that glucocorticoids might be able to produce both inmunological and neurogical changes by increasing Treg population and decreasing the spasticity condition, respectively. Although corticosteroids have been the most widely used therapy for HAM/TSP, few clinical trials of them have been recently published [Izumo et al., 1996; Croda et al., 2008]. These were designed as uncontrolled case series, showing that this therapy appears to be clinically beneficial with a transient effect. The role of glucocorticoids as anti-spastic drugs was unknown. The beneficial effects of corticosteroids in HAM/TSP could be

due to IFN-γ overproduction [Casseb and Penalva, 2000]. Anti-inflammatory properties of corticosteroids might have an impact on myelin membrane inflammation process observed in HAM/TSP, mainly in those with few years of disease when inflammation is more prominent [Araujo et al., 1995].

The results of this study should be interpreted with caution, since we performed a short clinical trial, unblinded and not placebo controlled. Consequently, for clinical trial in HAM/TSP is important to know the impact of epidemiological variables of patients (e.g., age, duration of symptoms, age of onset, disability scores, time of progression, etc.), and the establishment of biomarkers to assess the effectiveness of treatment. Thus, only a double-blinded clinical trial, and placebo-controlled study could ultimately determine the role of corticosteroids in HAM/TSP.

Taken together, the findings suggest a dual action of glucocorticoids. An immunological action reflected by a concomitant increase in Foxp3 and decrease of Tax and a neurological action determined by a reduction of spasticity in HAM/TSP patients. Further research on pathways leading to Tax repression as consequence of glucocorticoids therapy and the effect of this drug on the central nervous system, could help to understand the mechanisms related to betamethasone action in the context of HAM/TSP.

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PLoS NEGLECTED TROPICAL DISEASES

HTLV-1 Tax Specific CD8+ T Cells Express Low Levels of Tim-3 in HTLV-1 Infection: Implications for Progression to Neurological Complications

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Abstract

The T cell immunoglobulin mucin 3 (Tim-3) receptor is highly expressed on HIV-1-specific T cells, rendering them partially "exhausted" and unable to contribute to the effective immune mediated control of viral replication. To elucidate novel mechanisms contributing to the HTLV-1 neurological complex and its classic neurological presentation called HAM/TSP (HTLV-1 associated myelopathy/tropical spastic paraparesis), we investigated the expression of the Tim-3 receptor on $CD8^+$ T cells from a cohort of HTLV-1 seropositive asymptomatic and symptomatic patients. Patients diagnosed with HAM/TSP down-regulated Tim-3 expression on both $CD8^+$ and $CD4^+$ T cells compared to asymptomatic patients and HTLV-1 seronegative controls. HTLV-1 Tax-specific, HLA-A*02 restricted $CD8^+$ T cells among HAM/TSP individuals expressed markedly lower levels of Tim-3. We observed Tax expressing cells in both $Tim\text{-}3^+$ and $Tim\text{-}3^-$ fractions. Taken together, these data indicate that there is a systematic downregulation of Tim-3 levels on T cells in HTLV-1 infection, sustaining a profoundly highly active population of potentially pathogenic T cells that may allow for the development of HTLV-1 complications.

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Competing Interests: L.C. Ndhlovu, R. Brad Jones, M.A. Ostrowski and D.F. Nixon are named as inventors on a patent application related to modulation of Tim-3 in viral infections.

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Introduction

The vast majority of HTLV-1-infected individuals with low and stable HTLV-1 proviral load levels are clinically asymptomatic for life [1]. However, 1–3% of subjects develop progressive neurological complications related to HTLV-1 infection, classically denominated as HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP) [2,3,4]. The infection can also lead to a debilitating malignancy, known as HTLV-1 associated adult T cell leukemia (ATL) in approximately 2–5% of infected individuals [4,5,6,7].

The immune response, and in particular the cellular immune response, plays an important role in the control of HTLV-1 infection [8,9,10,11,12]. *In vitro* studies further demonstrate that $CD8^+$ T cell responses are able to directly lyse HTLV-1-infected $CD4^+$ T cells [9,11,13]. In patients with HAM/TSP, $CD8^+$ T cells are capable of producing multi-cytokine responses and are able to release cytotoxic molecules [14,15]. Recent studies have selected out patients with HLA-A*02 and HLA-Cw08 genes as being associated with lower HTLV-1 proviral load and a reduced risk of progression to HAM/TSP [16,17].

While these data support an important protective role for the $CD8^+$ T cell immune response with the potential for viral control, other studies suggest that HTLV-1-specific $CD8^+$ T cells may paradoxically contribute to the neuromuscular immunopathology through autoimmune mechanisms, leading to the clinical manifestation of HAM/TSP [18]. Furthermore, patients with HAM/TSP also present with high numbers of HTLV-1 Tax-specific CD8+ T cells in the cerebrospinal fluid [15,19,20,21,22] that are thought to play a immunopathogenic role, either by release of neurotoxic cytokines, such as TNF-α and IFN-γ [23,24], or by direct

Author Summary

The retrovirus, Human T lymphotropic virus type 1 (HTLV-1) infects 10–20 million people worldwide. The majority of infected individuals are asymptomatic; however, approximately 3% develop the debilitating neurological disease, HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). There is also currently no cure, vaccine or effective therapy for HTLV-1 infection. The precise role of CD8+ killer T cells in the control or contribution of HTLV-1 disease progression remains unclear. The T-cell immunoglobulin mucin domain-containing (Tim) proteins are type 1 transmembrane proteins. Three human Tim proteins (Tim-1, -3, and -4) exist and display markedly diverse expression patterns and functions. Tim-3 is upregulated on $CD8^+$ T cells during chronic viral infections leading to a population of poorly functioning T cells. We investigated the expression of Tim-3 on T cells from patients with asymptomatic and symptomatic HTLV-1 infection and compared this with HTLV-1 uninfected donors. Patients diagnosed with HAM/TSP down-regulated Tim-3 expression on T cells when compared to asymptomatic patients and uninfected controls. Our study provides evidence of a novel mechanism for the persistent inflammation observed in HTLV-1 infected patients with neurological deficits and significantly advances our understanding of how the Tim-3 pathway functions.

cytotoxicity. It is evident from these studies that the precise role of $CD8^+$ T cells in the control or pathogenesis of HTLV-1 disease progression remain unclear. Further knowledge of the mechanisms leading to T cell induced immunopathology in HTLV-1 infection will be important in determining successful immune-based therapies and provide insights for effective vaccine designs.

During chronic viral infections, virus-specific $CD8^+$ T cells undergo an altered pattern of differentiation and can become exhausted [25,26]. $CD8^+$ T cell exhaustion is a transcriptionally altered state of T cell differentiation distinct from functional effector or memory $CD8^+$ T cells [27]. $CD8^+$ T cell exhaustion leads to profound T cell dysfunction and the inability of the T cells to control retroviral replication [28,29,30]. Conversely, downregulation of exhaustion markers could lead to a highly functional population of T cells. T cell immunoglobulin and mucin domain-containing protein 3 (Tim-3), is upregulated on $CD8^+$ T cells during chronic viral infections [29,30,31,32,33,34,35,36]. Programmed death receptor-1 (PD-1) is also known as another immune exhaustion biomarker expressed in chronic viral infections [28,37,38,39,40,41,42,43]. High levels of PD-1 and Tim-3 on virus-specific T cells have been shown to lead to poor proliferative capacity and, in some cases, ineffective Th1 cytokine production [29,39,44]. A sustained downregulation of these receptors would lead to an exacerbated constitutively active T cell population. The phenotypic profile of immune exhaustion markers on T cells is unknown in seropositive HTLV-1 individuals. In this study, we show for the first time that HTLV-1 associated complications may be related to the highly responsive inflammatory Tax-specific T cells in HTLV-1-infected individuals. These results support the idea that HTLV-1 infection induces mechanisms resulting in a limited T cell exhaustion profile, leading potentially to neuro-immunopathology and disease complications.

Materials and Methods

Ethics Statement

The research involving human participants reported in this study was approved by the institutional review board of the University of Sao Paulo (IRB #0855/08) Sao Paulo, Brazil. Informed consent was obtained for all subjects. All clinical investigation were conducted according to the principles expressed in the Declaration of Helsinki (http://www.wma.net/en/30publications/10policies/b3/index.html).

Humans Subjects

Patients were serially recruited in the HTLV-1 Outpatient Clinic at the University of Sao Paulo, Brazil in two stages with written informed consent approved by the University of Sao Paulo's Institutional Review Board (#0855/08). The diagnosis of HAM/TSP based on criteria outlined by the WHO [45] (Table 1). The majority of the patients were female (63%) with a median age of 48 (IQR: 22–66) years. We enrolled age and sex matched healthy uninfected volunteers without clinical and laboratory evidence of HTLV-1-associated disease, from the same demographics as the infected subjects. All HTLV -1 seropositive subjects tested negative for Hepatitis B, Hepatitis C, and HIV infections. No other inflammatory diseases or disorders were present in any of

Table 1. Patients description.

ID Number	Gender	Age (years)	Clinical Presentation	PBMC (cps/1000)	HLA-A*02 Status
237	M	39	asymptomatic	20	pos
410	F	43	asymptomatic	14	pos
411	F	47	asymptomatic	84	pos
405	F	22	asymptomatic	15	pos
403	F	53	asymptomatic	604	pos
240	F	N/A	asymptomatic	0	pos
425	M	29	asymptomatic	43	
416	M	48	asymptomatic	140	pos
221	M	N/A	asymptomatic	9	pos
424	M	46	asymptomatic	106	
418	M	66	asymptomatic	<1	
419	F	33	asymptomatic	72	
421	M	54	asymptomatic	23	
423	F	42	asymptomatic	72	
218	F	46	HAM/TSP	2	pos
402	F	50	HAM/TSP	152	pos
224	F	57	HAM/TSP	1923	pos
412	F	53	HAM/TSP	117	pos
312	F	N/A	HAM/TSP	161	pos
413	F	61	HAM/TSP	1510	pos
420	M	64	HAM/TSP	12	
422	F	64	HAM/TSP	ND	
HD1	N/A	N/A	Healthy		
HD2	F	46	Healthy		
HD3	F	39	Healthy		
HD4	F	29	Healthy		
HD5	F	60	Healthy		
HD6	M	37	Healthy		
HD7	F	45	Healthy		

ND = not detected, N/A = not available.

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the participants. Blood samples were processed with Ficoll-Paque PLUS (Amersham Pharmacia Biotech, Uppsala, Sweden) gradient centrifugation, and peripheral-blood mononuclear cells (PBMC) were isolated and cyropreserved in fetal bovine serum (FBS) containing 10% DMSO in liquid nitrogen.

Pentamers, Peptides and Cytokines

Conjugated Pentamers were obtained commercially from Proimmune (Oxford, UK). The HLA-A*02 restricted HTLV-1 Tax (LLFGYPVYV) and CMV (NLVPMVATV) peptides were obtained from New England Peptide (Gardner, MA). In some experiments rIL-2 [80 IU/ml] (Roche Diagnostics, Mannheim, Germany) and rIL-15 [50 ng/ml] (R&D Systems, Minneapolis, MN) were used during *in vitro* culture studies.

Flow Cytometry Assessment

Cryopreserved PBMC were rapidly thawed in warm RPMI 1640 with 10% FBS, washed in FACS buffer (PBS, with 0.5% bovine serum albumin, 2 mM EDTA (Sigma-Aldrich, St. Louis, MO)). For staining, 5×10^5 cells were incubated with conjugated antibodies against Tim-3 (R&D Systems, Minneapolis, MN), PD-1 (Biolegend, San Diego, CA), CD4, CD8, CD3 (all from BD Biosciences, San Jose, CA) for 30 min on ice. In some experiments, PMBC were then fixed and permeabilized prior to staining with conjugated anti-Tax (clone Lt-4) antibodies [46] or a control labeled IgG. Fluorescence minus one (FMO) samples were prepared for each fluorochrome to facilitate gating as well as conjugated isotype control antibodies. Anti-mouse IgG-coated beads were stained with each fluorochrome separately and used for software-based compensation. Analysis was performed using a FACSCanto instrument (BD Biosciences) and at least 100,000 events were collected and analyzed with FlowJo software (TreeStar, Ashland, OR).

To define pentamer positive cells: staining was initially performed immediately after thawing with biotin-labeled HLA-A2 Tax or CMV epitope specific pentamer fluorotags followed a secondary staining step with fluorophore conjugated antibodies against CD8 (BD), Tim-3 (R&D Systems), PD-1 (Biolegend) and CD3 (BD), and with labeled streptavidin. Cells were washed twice with PBS containing 1% FBS, then fixed in 2% paraformaldehyde and run on a customized BD FACSCanto within 12 hours.

Viral Load Assessment

HTLV-1 proviral DNA was extracted from PBMC using a commercial kit (Qiagen GmbH, Hilden Germany) and according to the manufacturer's instructions. The extracted DNA was used as a template to amplify a fragment of 158 bp from the viral tax region using previously published primers[47]. The SYBR green real-time PCR assay was carried out in 25 µl PCR mixture containing 10× Tris (pH 8.3; Invitrogen, Brazil), 1.5 mM $MgCl_2$, 0.2 µM of each primer, 0.2 mM of each dNTPs, SYBR Green (18.75 Units/r×n; Cambrex Bio Science, Rockland, ME) and 1 unit of platinum Taq polymerase (Invitrogen, Brazil). The amplification was performed in the Bio-Rad iCycler iQ system using an initial denaturation step at 95°C for 2 minutes, followed by 50 cycles of 95°C for 30 seconds, 57°C for 30 seconds and 72°C for 30 seconds. The human housekeeping β globin gene primers GH20 and PC04[48] were used as an internal control calibrator. For each run, standard curves for the value of HTLV-1 tax were generated from MT-2 cells of $\log_{10}$ dilutions (from 10^5 to 10^0 copies). The threshold cycle for each clinical sample was calculated by defining the point at which the fluorescence exceeded a threshold limit. Each sample was assayed in duplicate and the mean of the two values was considered as the copy number of the sample. The amount of HTLV-1 proviral load was calculated as follows: copy number of HTLV-1 (tax) per 1,000 cells = (copy number of HTLV-1 tax)/(copy number of β globin/2) ×1,000 cells. The method could detect 1 copy per 10^3 PBMC.

Elispot Assays

MAIPS4510 Elispot plates (Millipore, Danvers, MA) were coated with anti-IFN-γ (10 µg/ml) (Mabtech, Nacka Strand, Sweden) in PBS, 50 µl/well, either overnight at 4°C or for one hour at room temperature. After three washes with PBS, PBMC (1×10^5 cells/well) and the appropriate antigens were added (Tax peptide and CMV peptide), with a final volume of 200 µl/well. Plates were incubated at 37°C in 5% CO_2 for 16–20 hours. After washing with phosphate-buffered saline (PBS) plus 0.1% Tween 20 (PBST), biotinylated anti-IFN-γ 1 µg/ml) (Mabtech), antibodies were added to the appropriate wells in PBS 0.1% tween 1% BSA (PBSTB) for 30 minutes at room temperature. Plates were washed again three times with PBST, and alkaline phosphatase-conjugated streptavidin (Jackson Immunoresearch, West Grove, PA) was added (50 µl of 1:1,000 dilution in PBSTB) and incubated for 30 min at room temperature. Plates were washed in PBSTB, soaked for 1 hour in PBSTB and incubated with blue substrate (Vector Labs, Burlingame, CA) until spots were clearly visible, then rinsed with tap water. When plates were dry, spots were counted using an automated ELISPOT reader.

Statistical Analysis

Statistical analysis was performed by using GraphPad Prism statistical software (GraphPad Software, San Diego, CA). Non-parametric statistical tests were used. The Mann-Whitney U was used for comparison tests and the Spearman rank test were used for correlation analyses.

Results

Subjects

Peripheral venous blood was drawn from 22 HTLV-1 seropositive patients and 7 HTLV-1 seronegative matched donors, all screened for the presence of HLA-A*02 alleles, and peripheral blood mononuclear cells (PBMC) were extracted and cryopreserved.

Tim-3 and PD-1 Expression on CD8+ and CD4+ T Cells in Patients with HTLV-1 Infection

Tim-3 and PD-1 are two cellular molecules expressed on T cells implicated in immune exhaustion. We evaluated the expression and co-expression of Tim-3 and PD-1 on T cells derived from HTLV-1 seropositive (both asymptomatic carriers and patients with the diagnosis of HAM/TSP) and seronegative controls to determine whether they were modulated in HTLV-1 infection. We observed a significant decrease in the frequency of Tim-3^+ PD-1^- expressing $CD8^+$ and $CD4^+$ T cells among HTLV-1 seropositive subjects ($CD8^+$: median 8.01%, IQR 5.42–10.50; $CD4^+$: median 4.3%, IQR 3.50–5.99) compared to HTLV-1 seronegative controls ($CD8^+$ median 15.10%, IQR 10.50–17.60; $CD4^+$: median 6.84%, IQR 5.74–7.85) (Figure 1A and B). Patients with HAM/TSP (red circles) had significantly lower levels of Tim-3^+ PD-1^- expressing $CD8^+$ ($p = 0.002$) and $CD4^+$ ($p = 0.004$) T cells compared to healthy uninfected controls (open circles). In contrast, the frequency of Tim-3^- $PD1^+$ T cells trended to an increase in subjects with HTLV-1 infection ($CD8^+$: median 18.80%, IQR 10.42–24.90; $CD4^+$: median 20.70%, IQR 13.6–25.35) compared to healthy uninfected controls ($CD8^+$: median 9.22%, IQR 8.97–15.50; $CD4^+$: median 13.60%, IQR 12.7–18.6)

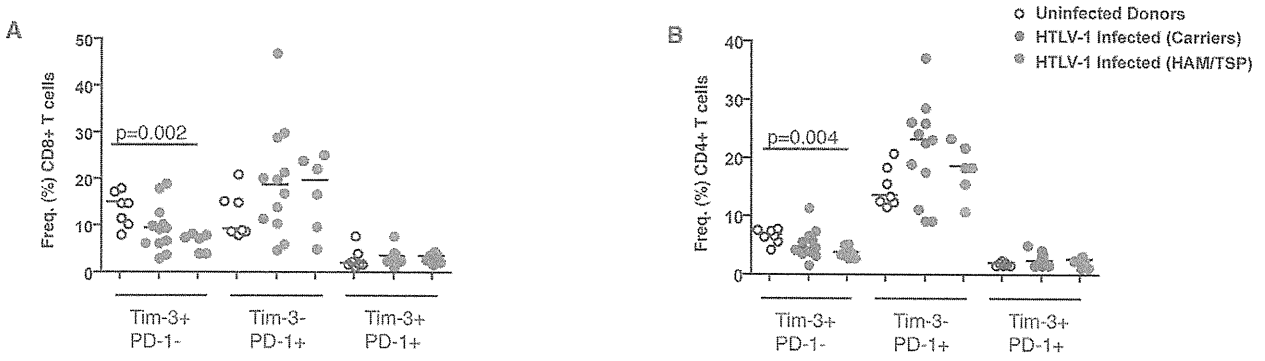

Figure 1. Tim-3 expression on T cells in HTLV-1 infection. Graphs show the frequencies of co-expression of Tim-3 and PD-1 on (A) CD8+ (left), and (B) CD4+ (right), T cells as assessed by multiparametric flow cytometry from PBMCs derived 18 HTLV-1 seropositive (12 asymptomatic and 6 with diagnosis of HAM/TSP) infected subjects and 7 HTLV-1 seronegative healthy uninfected donors from our initial recruitment. Statistically significant differences are reported as $p<0.05$.
doi:10.1371/journal.pntd.0001030.g001

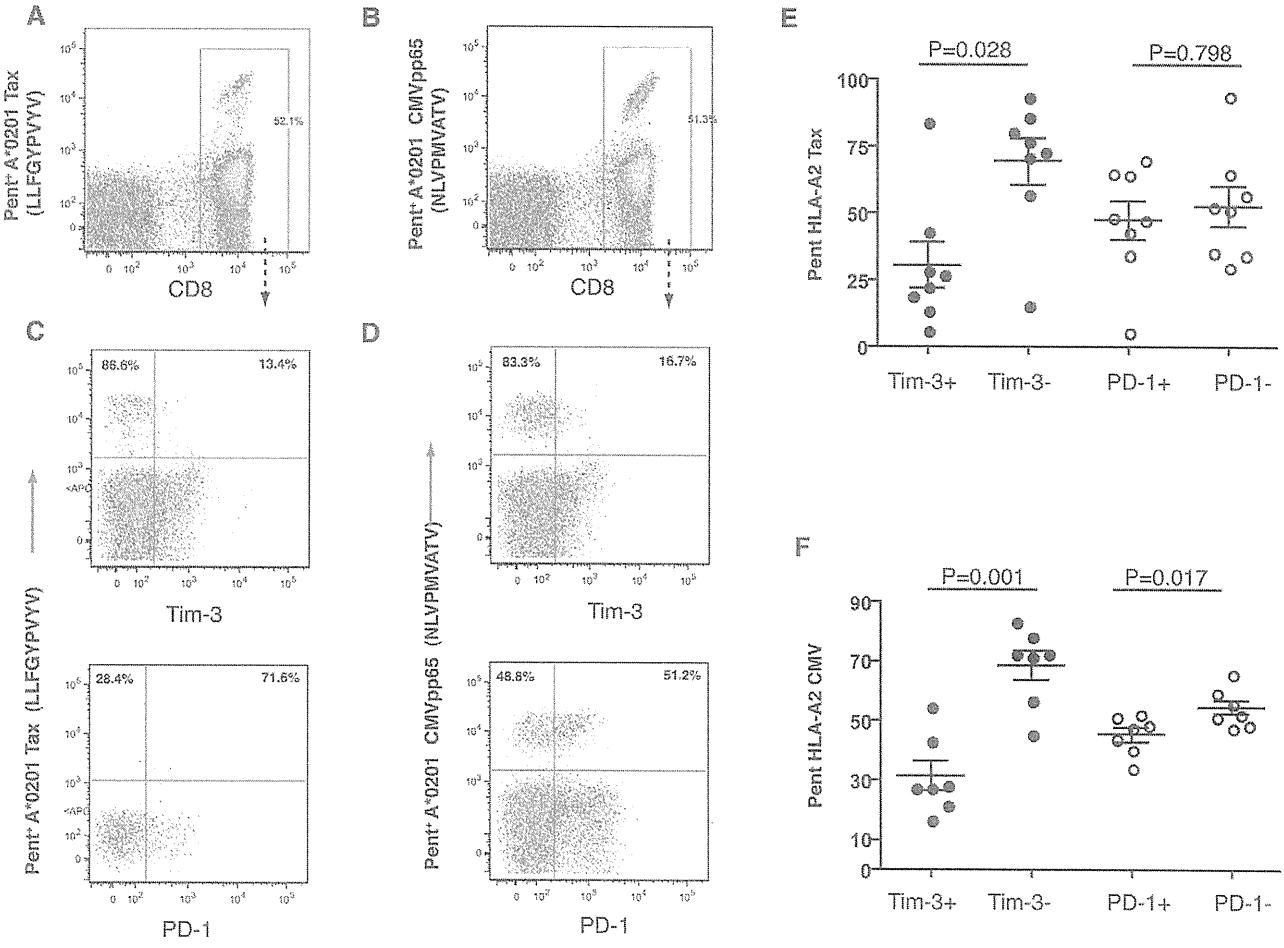

Figure 2. Tim-3 expression on HTLV-1-specific CD8+ T cells in HTLV-1 infection. PBMC from HLA-A*02+ chronically HTLV-1 infected individuals were stained with matched HLA pentamers presenting CMV and HTLV-1 epitopes, and with an anti-Tim-3 antibody. Shown are representative flow cytometry data from one HTLV-1-infected person using HLA-A*02 pentamers presenting the (A) HTLV-I-Tax 11–19 epitope and, (B) CMV-pp65 epitope 'NLVPMVATV'. (C, D) Plots show co-expression of Tim-3 (upper panel) and PD-1 (lower panel) with the respective HLA-A*02 pentamers (Tax (left) and CMVpp65 (right)) from the gated CD8+ T population depicted in Fig 2 A, B. The percentages of cells in the upper left and right quadrants of the flow plots demonstrated in Figure 2C, D reflect only the percentage of pentamer expressing cells. The compiled expression data of the frequency of Tax (E) and CMVpp65 (F) pentamer cells on either Tim-3+ or Tim-3- and PD-1+ or PD-1- CD8+ T cells from 8 subjects are shown in Figure 2 E and F. Statistical analyses comparing pooled responses were performed using the Mann-Whitney test.
doi:10.1371/journal.pntd.0001030.g002

(Figure 1A and B). Only a few T cells co-expressed both Tim-3 and PD-1, and no differences were observed between uninfected subjects and those with HTLV-1 asymptomatic infection or HAM/TSP patients. Using linear regression analysis we observed no association between the frequency of Tim-3 or PD-1 expression on $CD8^+$ T cells in HTLV-1 infected subjects and proviral load. ($p = 0.68$; $r = 0.1043$; or $p = 0.89$; $r = -0.03202$, respectively).

Distribution of Tim-3 Expression on HTLV-1-Specific T Cells

HLA- A*02 positive HTLV-1-infected patients have high amounts of circulating $CD8^+$ T cells specific for an immunodominant HLA- A*02 -restricted epitope, HTLV-1 Tax 11–19 [20,49,50]. In HAM/TSP patients, these HTLV-1's Tax-specific $CD8^+$ T cells correlate with HTLV-1 proviral load [23]. Among this cohort, we identified 15 HLA-A2 positive subjects (asymptomatic carriers, $n = 9$ and HAM/TSP, $n = 6$; Table 1), and evaluated the Tim-3 and PD-1 receptor expression on Tax-specific $CD8^+$ T cells. Eight patients had Tax-specific $CD8^+$ T cells (median 2.45%, IQR 1.11–5.31) as determined by specific pentamers. Among these patients we also observed HLA-A*02 -restricted CMVpp65 CD8+ T cells (median 2.49%, IQR 1.87–11.37). Interestingly, Tim-3 levels were dramatically reduced on $CD8^+$ Tax 11–19-specific T cells (median 24.77%, IQR 15.2–39.54) compared to the expression of PD-1 (median 48.06%, IQR 36.81–65) (Figure 2A,C and E). We also evaluated Tim-3 expression on HLA- A*02 CMV specific T cells and found a similar pattern of expression with Tim-3 levels reduced on $CD8^+$ CMV-specific T cells (median 27.62%, IQR 21.48–43.19) compared to PD-1 (median 47.70%, IQR 40.45–51.16) (Figure 2B,D and F).

Relationship between the Functionality of Tax 11-19-Specific $CD8^+$ T Cells and Tim-3 Levels

To determine whether there was an association with Tim-3 or PD-1 levels on Tax 11–19-specific $CD8^+$ T cells and their functionality, we evaluated the production of IFN-γ in response to the HLA-A*02-restricted Tax 11–19 immuno-dominant epitope and in comparison, the CMVpp65 epitope by an ELISPOT assay derived from PBMCs derived from 8 HLA- A*02 restricted infected individuals with Tax 11–19- and CMVpp65 specific $CD8^+$ T cells (Figure 3). We saw no correlation between IFN-γ secretion and global PD-1 or Tim-3 expression on either the $CD4^+$ or $CD8^+$ T cells, irrespective of disease status (data not shown). The frequency of PD-1 expression on Tax-specific or CMV-specific $CD8^+$ T cells

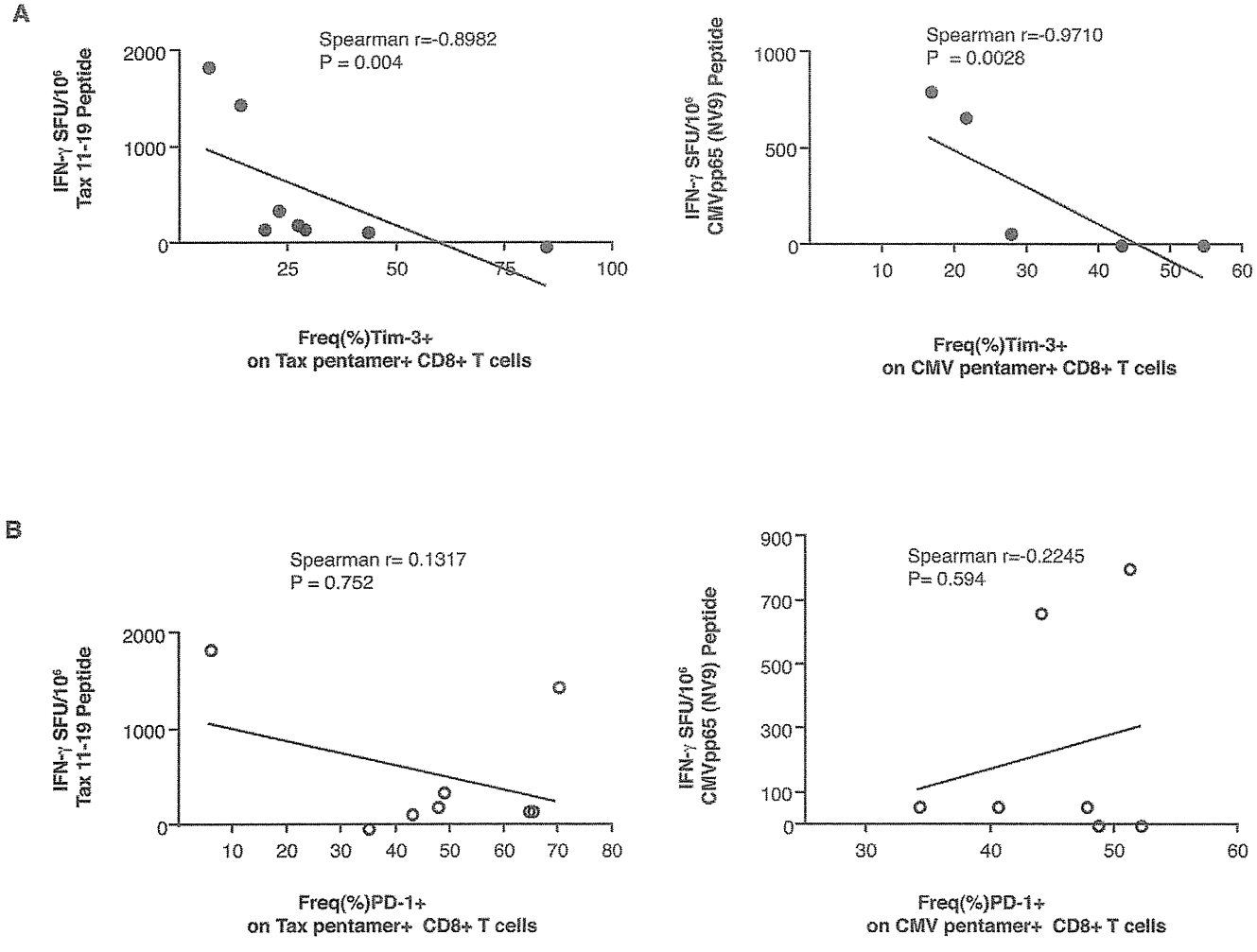

Figure 3. Association of Tax specific CD8+ T cells with effector responses. The graphs show the association between the frequency of Tim-3 (A) and PD-1 (B) expression on HLA-A*02 restricted Tax11-19 or CMV pp65 specific CD8+ T cells with the number of IFN-γ secreting cells ($SFU/10^6$) in response to Tax 11–19 peptide or the CMV pp65 epitope. The Spearman rank test was used for correlation analyses.
doi:10.1371/journal.pntd.0001030.g003

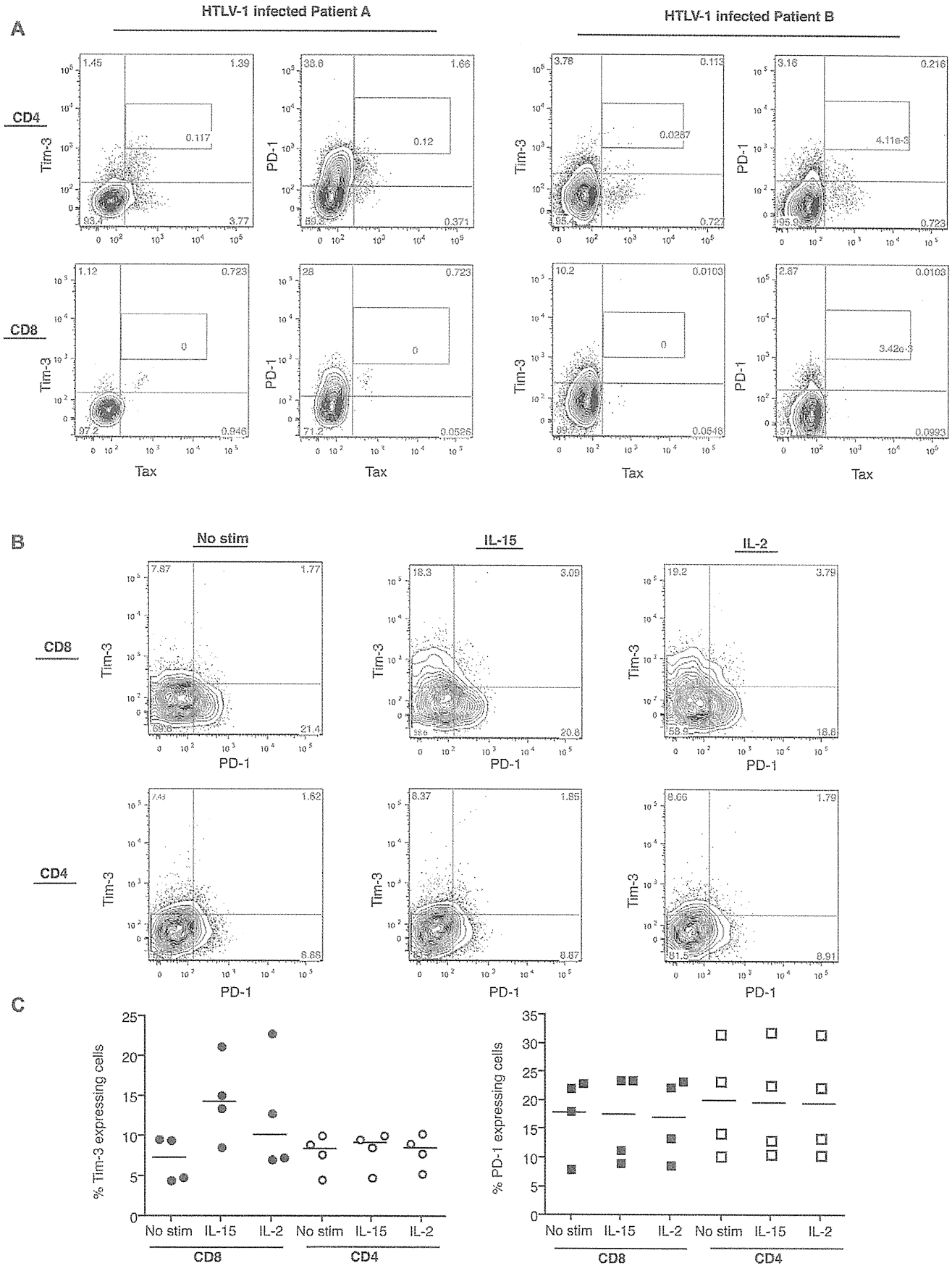
A
HTLV-1 infected Patient A
HTLV-1 infected Patient B
CD4
CD8
Tim-3
PD-1
Tax
B
No stim
IL-15
IL-2
CD8
CD4
Tim-3
PD-1
C
% Tim-3 expressing cells
% PD-1 expressing cells
No stim
IL-15
IL-2
CD8
CD4

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.
doi:10.1371/journal.pntd.0001030.g004

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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Author Contributions

Conceived and designed the experiments: LCN FEL DFN EGK. Performed the experiments: LCN FEL AMH ARJ GMC IGE-J FRB VAY WKN SSS RGSV. Analyzed the data: LCN FEL AMH ARJ KIC IGE-J VAY. Contributed reagents/materials/analysis tools: LCN DFN ACS EGK SSS WKN YT. Wrote the paper: LCN FEL. Technical and scientific input: RBJ MAO. Edited the manuscript: DFN EGK.

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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 $P < .05$.

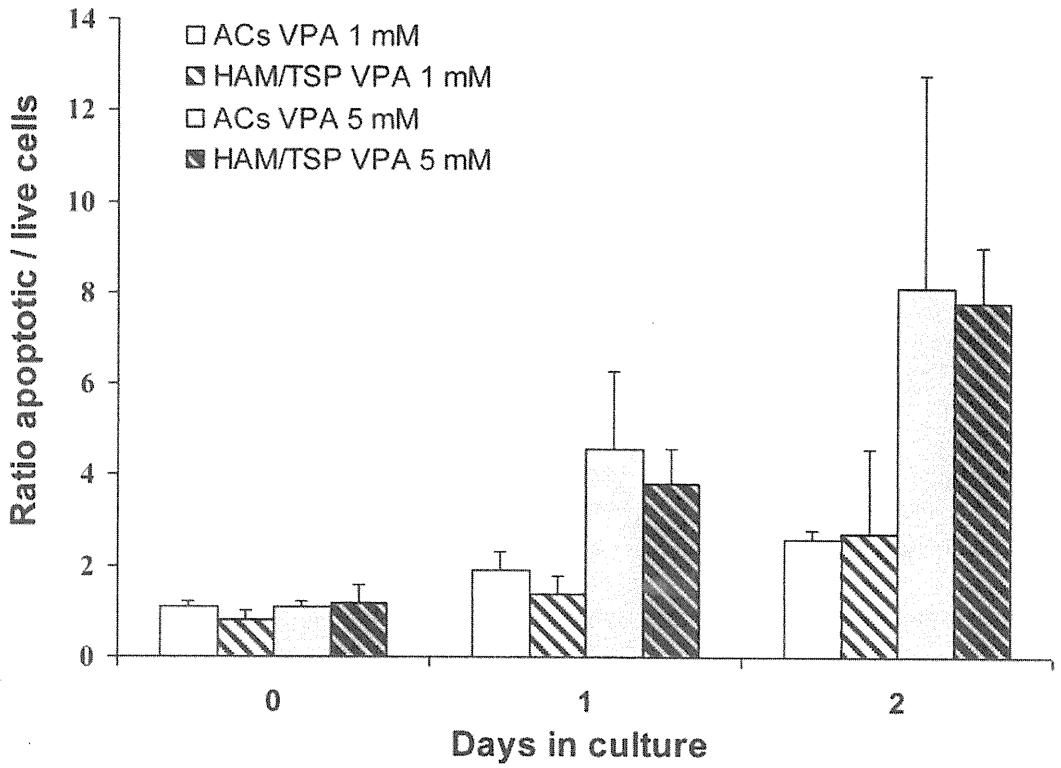

Figure 1. VPA is proapoptotic for ex vivo-cultured CD4+ T cells from HTLV-1 AC and HAM/TSP patients. $CD4^+$ cells isolated from 10 AC patients (non–cross-hatched bars) and 10 HAM/TSP patients (cross-hatched bars) were cultured for 6 (D0), 24 (D1), or 48 hours (D2) with 1mM VPA (white back bars) or 5mM (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

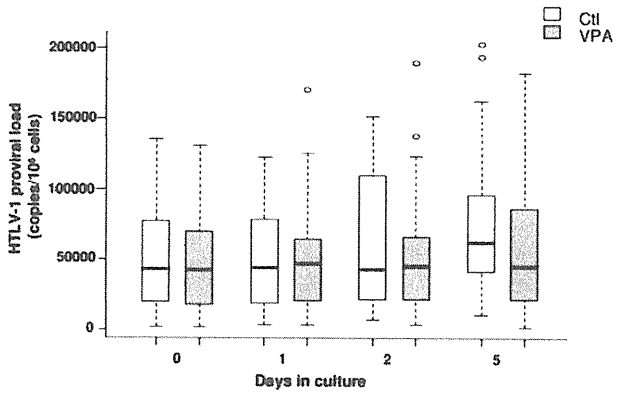

Figure 2. HTLV-1–proviral load remained stable throughout ex vivo culture of HTLV-1–infected $CD4^+$ T cells with or without valproate. $CD4^+$ T cells isolated from HTLV-1–infected individuals were cultured without (control [Ctl]) or with 5mM VPA (white and gray boxes, respectively). HTLV-1–proviral 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.

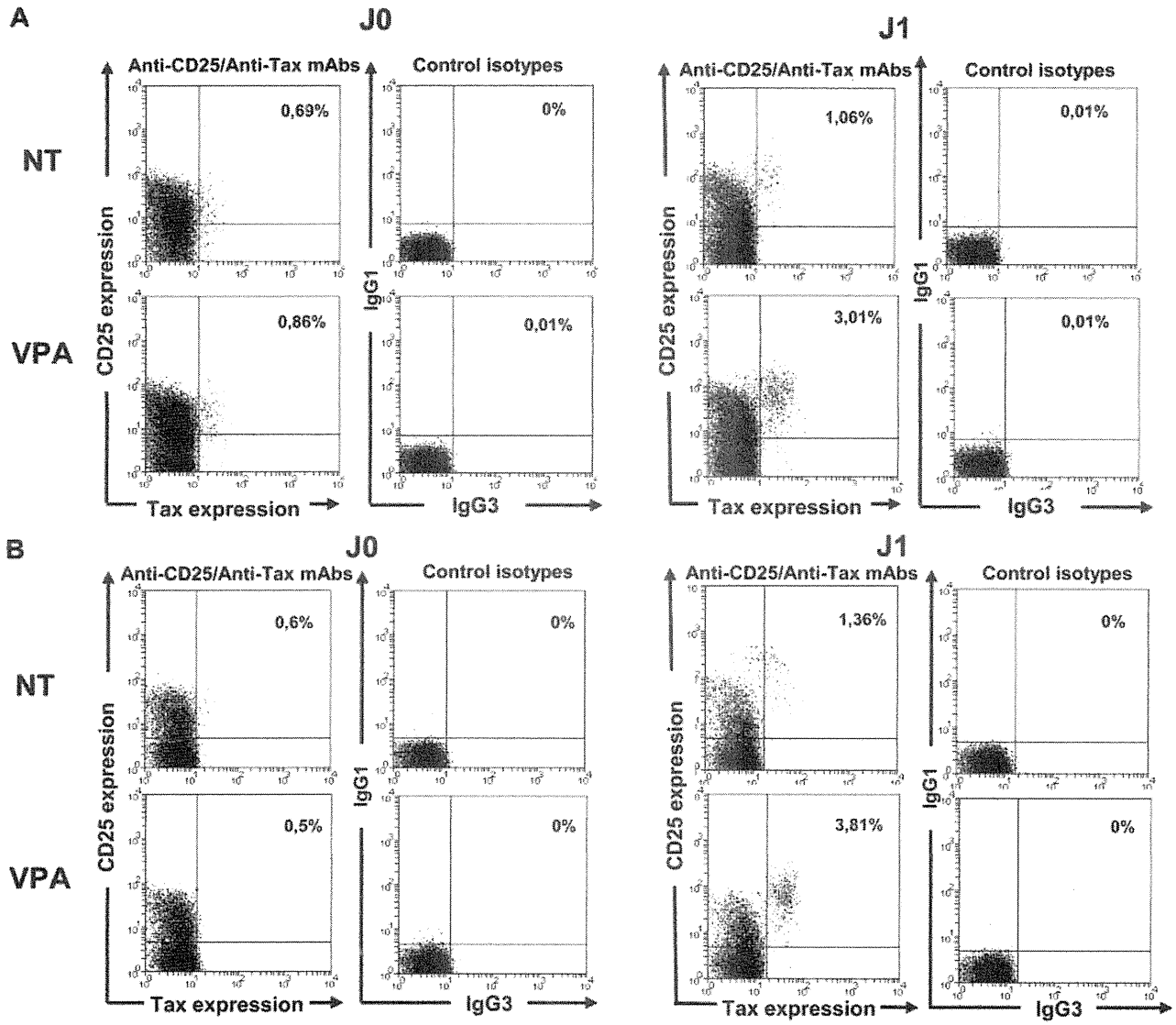

Figure 3. Tax induction in HTLV-1–infected CD4⁺ T cells treated with 1mM VPA. Intracellular Tax-protein expression in CD4⁺ T cells was analyzed by flow cytometry using quadruple CD3, CD4, CD25, and Tax (Lt-4 mAb) labeling and appropriate isotype controls. Experiments were performed on D0, D1, and D2 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

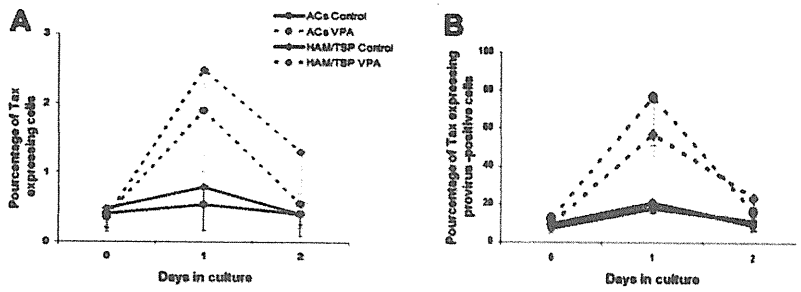

Figure 4. Valproate increases Tax-protein expression in HTLV-1–infected CD4+ T cells from asymptomatic carriers and HAM/TSP patients. Rates of Tax-protein–positive cells among HTLV-1–infected CD4+ T cells were calculated by normalizing the percentages of Tax-positive CD4+ cells, determined by CD3, CD4, 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).

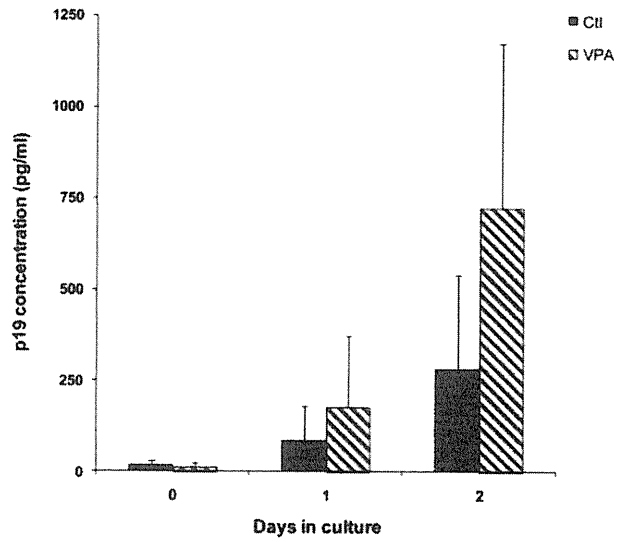

Figure 5. VPA activates the expression of the virus core-protein p19 in the culture supernatants of CD4+ T cells from HTLV-1–infected individuals. Culture supernatants were collected and expression of virus core-protein p19 was quantified by ELISA. Absolute p19 concentrations (in picograms per milliliter) 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

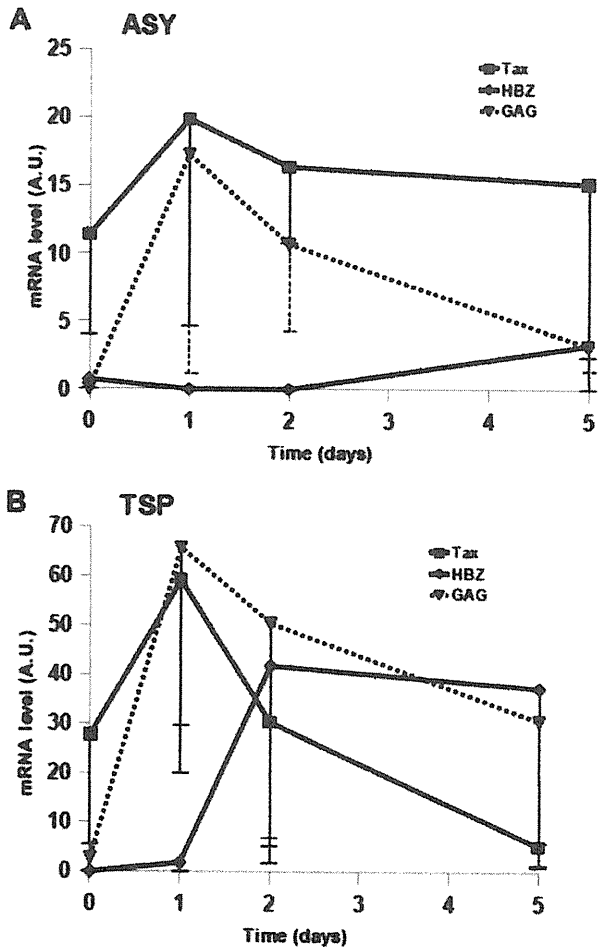

Figure 6. Kinetics of Tax-, Gag-, and HBZ-mRNA expressions in short-term cultures of CD4+ T cells from HTLV-1 asymptomatic carriers and HAM/TSP patients. Tax- (■, solid line), Gag- (▲, dotted line), and HBZ-mRNA (♦, solid line) in ex vivo cultured lymphocytes from AC (A) or HAM/TSP (B) patients were 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.

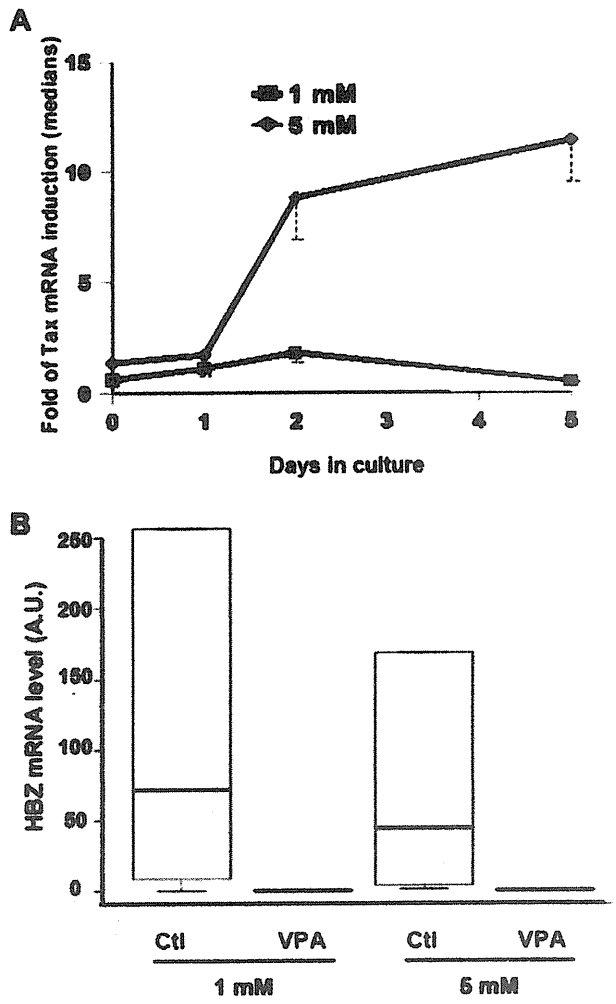

Figure 7. VPA effects on Tax and HBZ expressions in CD4⁺ T cells from HAM/TSP patients. (A) Patients' CD4⁺ T cells were cultured for the indicated times with 1mM (■) or 5mM VPA (◆). The curves illustrate VPA induction of Tax expression compared with the corresponding nontreated sample (medians and first 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.

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