OPEN ACCESS Freely available online

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

Lishomwa C. Ndhlovu[1*☯], Fabio E. Leal[2,3☯], Aaron M. Hasenkrug[1], Aashish R. Jha[1], Karina I. Carvalho[2], Ijeoma G. Eccles-James[1], Fernanda R. Bruno[2], Raphaella G. S. Vieira[1], Vanessa A. York[1], Glen M. Chew[1], R. Brad Jones[4], Yuetsu Tanaka[5], Walter K. Neto[6], Sabri S. Sanabani[6], Mario A. Ostrowski[4], Aluisio C. Segurado[3], Douglas F. Nixon[1¶], Esper G. Kallas[2,3¶]

1 Division of Experimental Medicine, Department of Medicine, University of California San Francisco, San Francisco, California, United States of America, **2** Division of Clinical Immunology and Allergy, Department of Infectious Diseases, School of Medicine, University of São Paulo, São Paulo, Brazil, **3** Department of Infectious Diseases, School of Medicine, University of São Paulo, São Paulo, Brazil, **4** Department of Immunology, University of Toronto, Toronto, Ontario, Canada, **5** Department of Immunology, University of the Ryukyus, Okinawa, Japan, **6** Molecular Biology Laboratory, Fundação Pró-Sangue, Hemocentro de São Paulo, São Paulo, Brazil

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.

Citation: Ndhlovu LC, Leal FE, Hasenkrug AM, Jha AR, Carvalho KI, et al. (2011) HTLV-1 Tax Specific CD8+ T Cells Express Low Levels of Tim-3 in HTLV-1 Infection: Implications for Progression to Neurological Complications. PLoS Negl Trop Dis 5(4): e1030. doi:10.1371/journal.pntd.0001030

Editor: Sunit Kumar Singh, Centre for Cellular and Molecular Biology (CCMB), India

Received October 19, 2010; **Accepted** February 27, 2011; **Published** April 26, 2011

Funding: Support for this work was provided by funds from the National Institutes of Health (NIH), University of California, San Francisco-Gladstone Institute of Virology & Immunology Center for AIDS Research (P30 AI027763). Additional support was provided by the Brazilian Program for STD and AIDS, Ministry of Health (914/BRA/3014 - UNESCO/Kallas), the São Paulo City Health Department (2004-0.168.922-7/Kallas), Fundação de Amparo a Pesquisa do Estado de São Paulo (04/15856-9/Kallas and 2010/05845-0/Kallas and Nixon), the John E. Fogarty International Center (D43 TW00003). The project described was also supported by Award Number R56AI083112 from the National Institute of Allergy and Infectious Diseases (LCN). Additional support was provided by the Montana State Complex Biological Systems Research Scholars Program, funded by the Howard Hughes Medical Institute to AH. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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.

* E-mail: lndhlovu@medsfgh.ucsf.edu; lndhlovu@hawaii.edu

☯ These authors contributed equally to this work.

¶ These authors also contributed equally to this work.

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.

doi:10.1371/journal.pntd.0001030.t001

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)

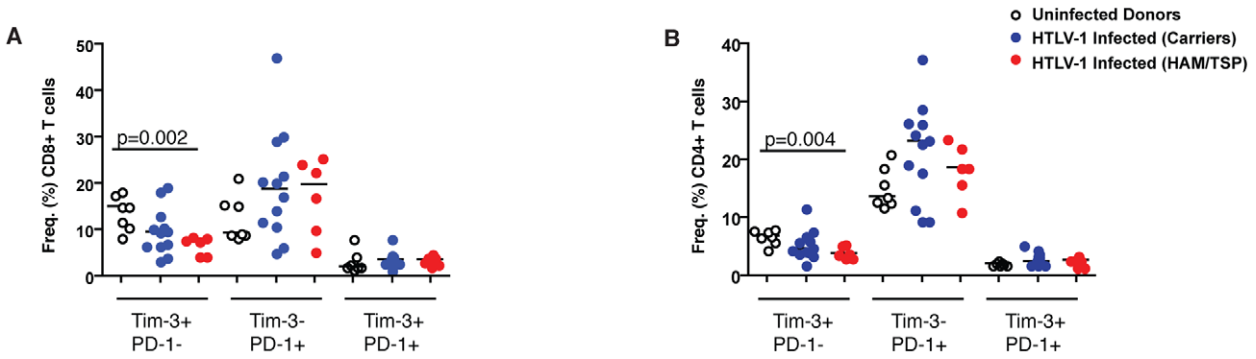

Figure 1. Tim-3 expression on T cells in HTLV-1 infection. Graphs show the frequencies of co-expression of Tim-3 and PD-1 on (A) CD8+ (left), and (B) CD4+ (right), T cells as assessed by multiparametric flow cytometry from PBMCs derived 18 HTLV-1 seropositive (12 asymptomatic and 6 with diagnosis of 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

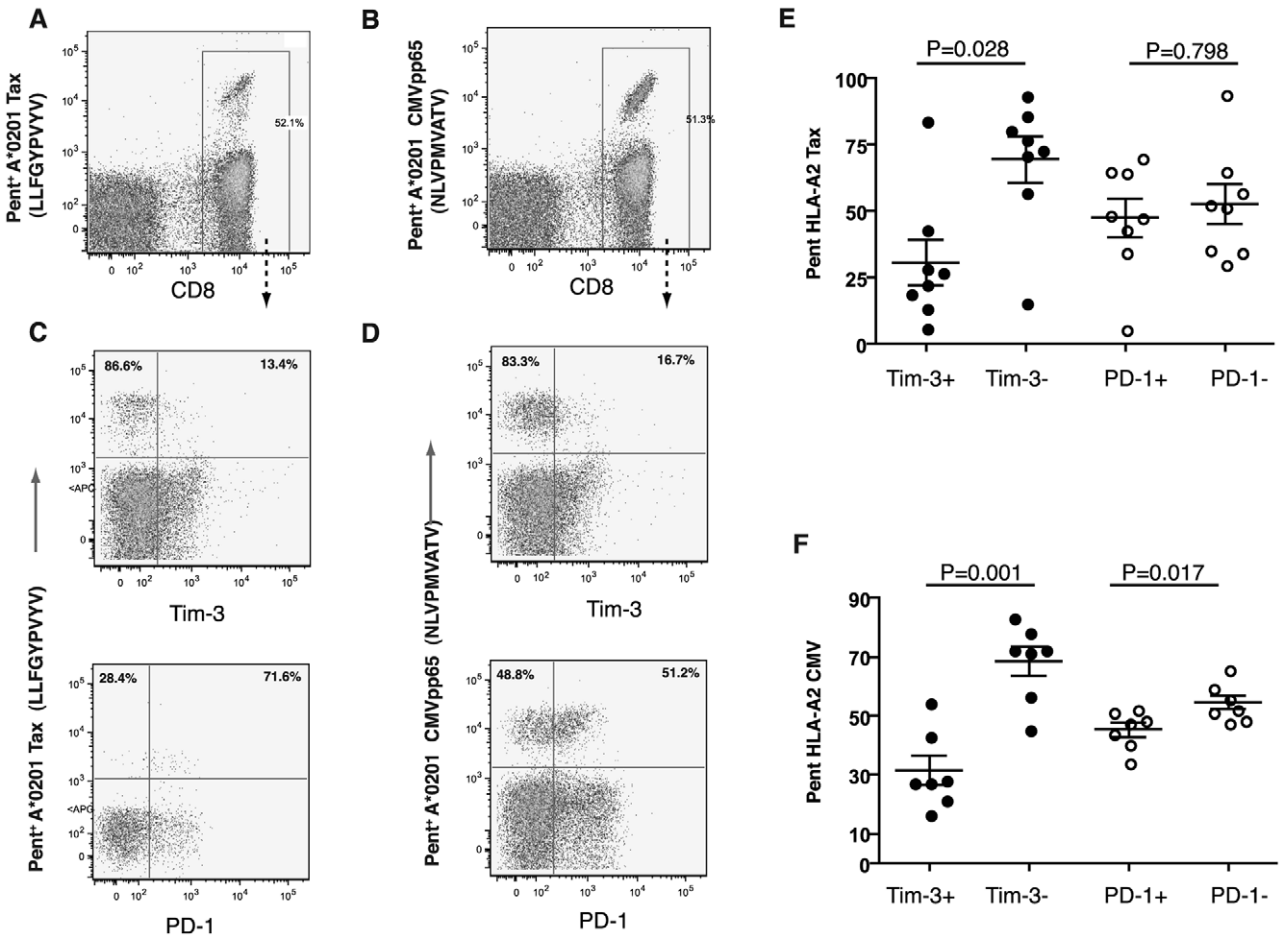

Figure 2. Tim-3 expression on HTLV-1-specific CD8+ T cells in HTLV-1 infection. PBMC from HLA-A*02+ chronically HTLV-1 infected individuals were stained with matched HLA pentamers presenting CMV and HTLV-1 epitopes, and with an anti-Tim-3 antibody. Shown are representative flow cytometry data from one 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

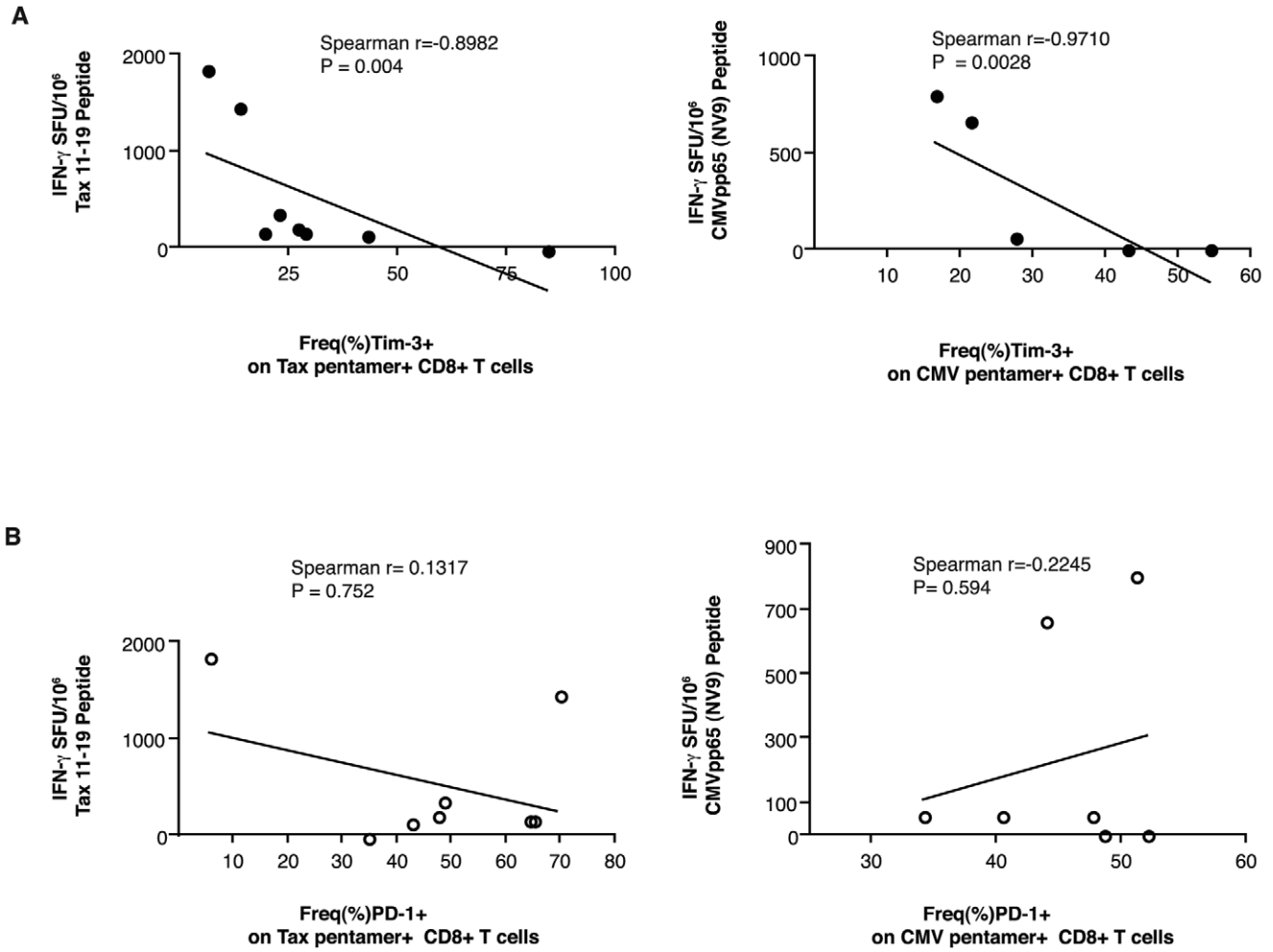

Figure 3. Association of Tax specific CD8+ T cells with effector responses. The graphs show the association between the frequency of Tim-3 (A) and PD-1 (B) expression on HLA-A*02 restricted Tax11-19 or CMV pp65 specific CD8+ T cells with the number of IFN-γ secreting cells ($SFU/10^6$) in response to 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

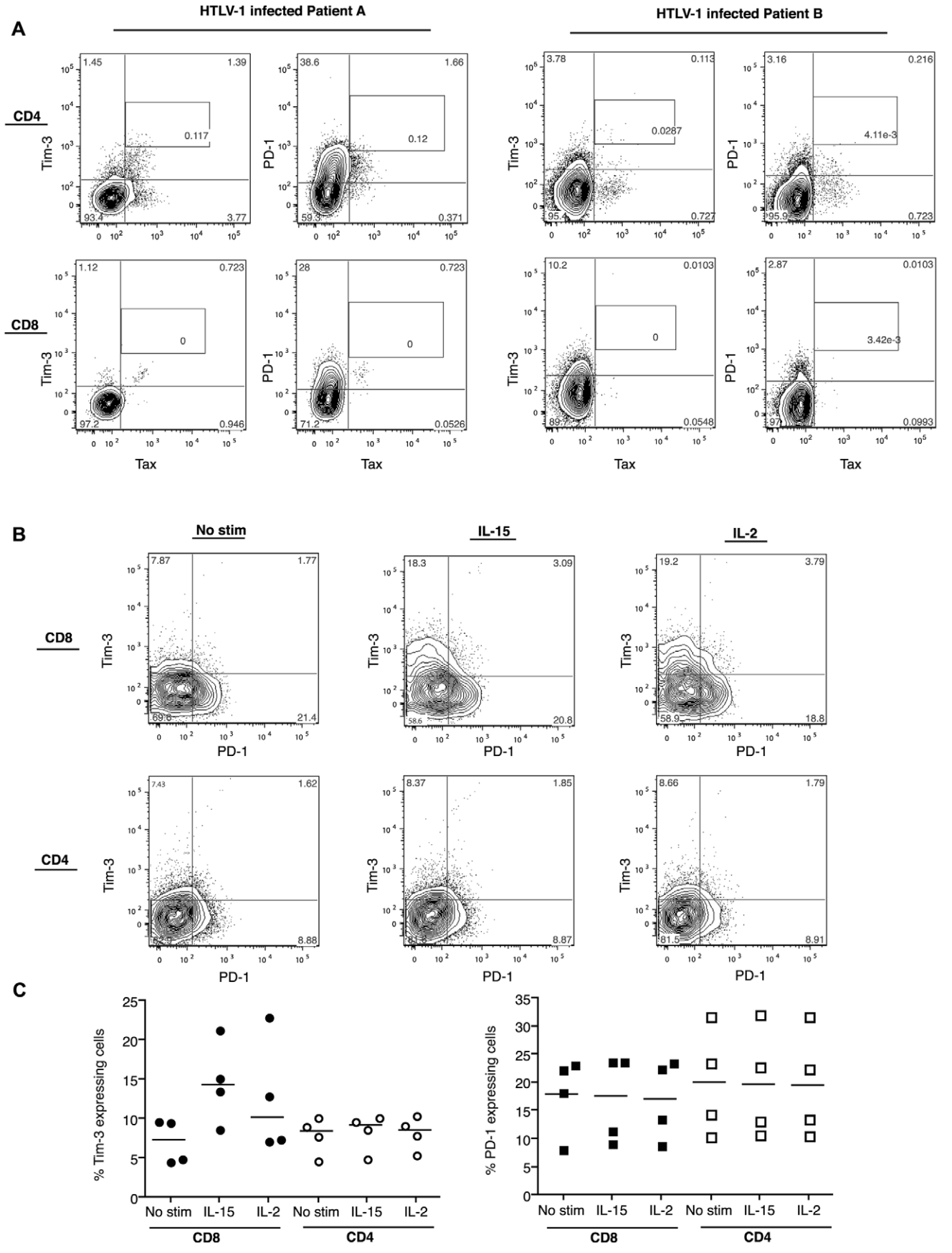
A
HTLV-1 infected Patient A
HTLV-1 infected Patient B
CD4
CD8
Tim-3
PD-1
Tax
1.45
1.39
0.117
93.4
3.77
38.6
1.66
0.12
59.3
0.371
3.78
0.113
0.0287
95
0.727
3.16
0.216
4.11e-3
95.9
0.723
1.12
0.723
0
97.2
0.946
28
0.723
0
71.2
0.0526
10.2
0.0103
0
89
0.0548
2.87
0.0103
3.42e-3
97
0.0993
B
No stim
IL-15
IL-2
CD8
CD4
PD-1
Tim-3
7.87
1.77
69.6
21.4
18.3
3.09
58.6
20.8
19.2
3.79
58.9
18.8
7.43
1.62
8.88
8.37
1.85
8.87
8.66
1.79
81.5
8.91
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.

Acknowledgments

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Allergy and Infectious Diseases or the National Institutes of Health.

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.

References

1. Etoh K, Yamaguchi K, Tokudome S, Watanabe T, Okayama A, et al. (1999) Rapid quantification of HTLV-I provirus load: detection of monoclonal proliferation of HTLV-I-infected cells among blood donors. Int J Cancer 81: 859–864.
2. Orland JR, Engstrom J, Fridey J, Sacher RA, Smith JW, et al. (2003) Prevalence and clinical features of HTLV neurologic disease in the HTLV Outcomes Study. Neurology 61: 1588–1594.
3. Osame M, Usuku K, Izumo S, Ijichi N, Amitani H, et al. (1986) HTLV-I associated myelopathy, a new clinical entity. Lancet 1: 1031–1032.
4. Gessain A, Barin F, Vernant JC, Gout O, Maurs L, et al. (1985) Antibodies to human T-lymphotropic virus type-I in patients with tropical spastic paraparesis. Lancet 2: 407–410.
5. Hinuma Y, Nagata K, Hanaoka M, Nakai M, Matsumoto T, et al. (1981) Adult T-cell leukemia: antigen in an ATL cell line and detection of antibodies to the antigen in human sera. Proc Natl Acad Sci U S A 78: 6476–6480.
6. Uchiyama T, Yodoi J, Sagawa K, Takatsuki K, Uchino H (1977) Adult T-cell leukemia: clinical and hematologic features of 16 cases. Blood 50: 481–492.
7. Yoshida M, Seiki M, Yamaguchi K, Takatsuki K (1984) Monoclonal integration of human T-cell leukemia provirus in all primary tumors of adult T-cell leukemia suggests causative role of human T-cell leukemia virus in the disease. Proc Natl Acad Sci U S A 81: 2534–2537.
8. Nagai M, Kubota R, Greten TF, Schneck JP, Leist TP, et al. (2001) Increased activated human T cell lymphotropic virus type I (HTLV-I) Tax11-19-specific memory and effector CD8+ cells in patients with HTLV-I-associated myelopathy/tropical spastic paraparesis: correlation with HTLV-I provirus load. J Infect Dis 183: 197–205.
9. Hanon E, Stinchcombe JC, Saito M, Asquith BE, Taylor GP, et al. (2000) Fratricide among CD8(+) T lymphocytes naturally infected with human T cell lymphotropic virus type I. Immunity 13: 657–664.
10. Vine AM, Heaps AG, Kaftantzi L, Mosley A, Asquith B, et al. (2004) The role of CTLs in persistent viral infection: cytolytic gene expression in CD8+ lymphocytes distinguishes between individuals with a high or low proviral load of human T cell lymphotropic virus type 1. J Immunol 173: 5121–5129.
11. Hanon E, Hall S, Taylor GP, Saito M, Davis R, et al. (2000) Abundant tax protein expression in CD4+ T cells infected with human T-cell lymphotropic virus type I (HTLV-I) is prevented by cytotoxic T lymphocytes. Blood 95: 1386–1392.
12. Jacobson S (2002) Immunopathogenesis of human T cell lymphotropic virus type I-associated neurologic disease. J Infect Dis 186(Suppl 2): S187–192.
13. Arnulf B, Thorel M, Poirot Y, Tamouza R, Boulanger E, et al. (2004) Loss of the ex vivo but not the reinducible CD8+ T-cell response to Tax in human T-cell leukemia virus type 1-infected patients with adult T-cell leukemia/lymphoma. Leukemia 18: 126–132.
14. Sabouri AH, Usuku K, Hayashi D, Izumo S, Ohara Y, et al. (2008) Impaired function of human T-lymphotropic virus type 1 (HTLV-1)-specific CD8+ T cells in HTLV-1-associated neurologic disease. Blood 112: 2411–2420.
15. Greten TF, Slansky JE, Kubota R, Soldan SS, Jaffee EM, et al. (1998) Direct visualization of antigen-specific T cells: HTLV-1 Tax11-19- specific CD8(+) T cells are activated in peripheral blood and accumulate in cerebrospinal fluid from HAM/TSP patients. Proc Natl Acad Sci U S A 95: 7568–7573.
16. Jeffery KJ, Siddiqui AA, Bunce M, Lloyd AL, Vine AM, et al. (2000) The influence of HLA class I alleles and heterozygosity on the outcome of human T cell lymphotropic virus type I infection. J Immunol 165: 7278–7284.
17. Jeffery KJ, Usuku K, Hall SE, Matsumoto W, Taylor GP, et al. (1999) HLA alleles determine human T-lymphotropic virus-I (HTLV-I) proviral load and the risk of HTLV-I-associated myelopathy. Proc Natl Acad Sci U S A 96: 3848–3853.
18. Matsuura E, Yamano Y, Jacobson S Neuroimmunity of HTLV-I Infection. J Neuroimmune Pharmacol 5: 310–325.
19. Levin MC, Jacobson S (1997) HTLV-I associated myelopathy/tropical spastic paraparesis (HAM/TSP): a chronic progressive neurologic disease associated with immunologically mediated damage to the central nervous system. J Neurovirol 3: 126–140.
20. Elovaara I, Koenig S, Brewah AY, Woods RM, Lehky T, et al. (1993) High human T cell lymphotropic virus type 1 (HTLV-1)-specific precursor cytotoxic T lymphocyte frequencies in patients with HTLV-1-associated neurological disease. J Exp Med 177: 1567–1573.
21. Umehara F, Nakamura A, Izumo S, Kubota R, Ijichi S, et al. (1994) Apoptosis of T lymphocytes in the spinal cord lesions in HTLV-I-associated myelopathy: a possible mechanism to control viral infection in the central nervous system. J Neuropathol Exp Neurol 53: 617–624.
22. Umehara F, Izumo S, Nakagawa M, Ronquillo AT, Takahashi K, et al. (1993) Immunocytochemical analysis of the cellular infiltrate in the spinal cord lesions in HTLV-I-associated myelopathy. J Neuropathol Exp Neurol 52: 424–430.
23. Kubota R, Kawanishi T, Matsubara H, Manns A, Jacobson S (1998) Demonstration of human T lymphotropic virus type I (HTLV-I) tax-specific CD8+ lymphocytes directly in peripheral blood of HTLV-I-associated myelopathy/tropical spastic paraparesis patients by intracellular cytokine detection. J Immunol 161: 482–488.
24. Biddison WE, Kubota R, Kawanishi T, Taub DD, Cruikshank WW, et al. (1997) Human T cell leukemia virus type I (HTLV-I)-specific CD8+ CTL clones from patients with HTLV-I-associated neurologic disease secrete proinflammatory cytokines, chemokines, and matrix metalloproteinase. J Immunol 159: 2018–2025.
25. Zajac AJ, Blattman JN, Murali-Krishna K, Sourdive DJ, Suresh M, et al. (1998) Viral immune evasion due to persistence of activated T cells without effector function. J Exp Med 188: 2205–2213.
26. Gallimore A, Glithero A, Godkin A, Tissot AC, Pluckthun A, et al. (1998) Induction and exhaustion of lymphocytic choriomeningitis virus-specific cytotoxic T lymphocytes visualized using soluble tetrameric major histocompatibility complex class I-peptide complexes. J Exp Med 187: 1383–1393.
27. Wherry EJ, Ha SJ, Kaech SM, Haining WN, Sarkar S, et al. (2007) Molecular signature of CD8+ T cell exhaustion during chronic viral infection. Immunity 27: 670–684.
28. Day CL, Kaufmann DE, Kiepiela P, Brown JA, Moodley ES, et al. (2006) PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. Nature 443: 350–354.
29. Jones RB, Ndhlovu LC, Barbour JD, Sheth PM, Jha AR, et al. (2008) Tim-3 expression defines a novel population of dysfunctional T cells with highly elevated frequencies in progressive HIV-1 infection. J Exp Med 205: 2763–2779.
30. Takamura S, Tsuji-Kawahara S, Yagita H, Akiba H, Sakamoto M, et al. (2010) Premature terminal exhaustion of Friend virus-specific effector CD8+ T cells by rapid induction of multiple inhibitory receptors. J Immunol 184: 4696–4707.
31. Hafler DA, Kuchroo V (2008) TIMs: central regulators of immune responses. J Exp Med 205: 2699–2701.
32. Sehrawat S, Suryawanshi A, Hirashima M, Rouse BT (2009) Role of Tim-3/galectin-9 inhibitory interaction in viral-induced immunopathology: shifting the balance toward regulators. J Immunol 182: 3191–3201.
33. Jin HT, Anderson AC, Tan WG, West EE, Ha SJ, et al. (2010) Cooperation of Tim-3 and PD-1 in CD8 T-cell exhaustion during chronic viral infection. Proc Natl Acad Sci U S A.
34. Vali B, Jones RB, Sakhdari A, Sheth PM, Clayton K, et al. (2010) HCV-specific T cells in HCV/HIV co-infection show elevated frequencies of dual Tim-3/PD-1 expression that correlate with liver disease progression. Eur J Immunol.
35. Sehrawat S, Reddy PB, Rajasagi N, Suryawanshi A, Hirashima M, et al. (2010) Galectin-9/TIM-3 interaction regulates virus-specific primary and memory CD8 T cell response. PLoS Pathog 6: e1000882.
36. Golden-Mason L, Palmer BE, Kassam N, Townshend-Bulson L, Livingston S, et al. (2009) Negative immune regulator Tim-3 is overexpressed on T cells in hepatitis C virus infection and its blockade rescues dysfunctional CD4+ and CD8+ T cells. J Virol 83: 9122–9130.
37. Urbani S, Amadei B, Tola D, Massari M, Schivazappa S, et al. (2006) PD-1 expression in acute hepatitis C virus (HCV) infection is associated with HCV-specific CD8 exhaustion. J Virol 80: 11398–11403.
38. Radziewicz H, Ibegbu CC, Fernandez ML, Workowski KA, Obideen K, et al. (2007) Liver-infiltrating lymphocytes in chronic human hepatitis C virus infection display an exhausted phenotype with high levels of PD-1 and low levels of CD127 expression. J Virol 81: 2545–2553.
39. Petrovas C, Casazza JP, Brenchley JM, Price DA, Gostick E, et al. (2006) PD-1 is a regulator of virus-specific CD8+ T cell survival in HIV infection. J Exp Med 203: 2281–2292.
40. Trautmann L, Janbazian L, Chomont N, Said EA, Gimmig S, et al. (2006) Upregulation of PD-1 expression on HIV-specific CD8+ T cells leads to reversible immune dysfunction. Nat Med 12: 1198–1202.
41. Barber DL, Wherry EJ, Masopust D, Zhu B, Allison JP, et al. (2006) Restoring function in exhausted CD8 T cells during chronic viral infection. Nature 439: 682–687.
42. Golden-Mason L, Palmer B, Klarquist J, Mengshol JA, Castelblanco N, et al. (2007) Upregulation of PD-1 expression on circulating and intrahepatic hepatitis C virus-specific CD8+ T cells associated with reversible immune dysfunction. J Virol 81: 9249–9258.

43. Peng G, Li S, Wu W, Tan X, Chen Y, et al. (2008) PD-1 upregulation is associated with HBV-specific T cell dysfunction in chronic hepatitis B patients. Mol Immunol 45: 963–970.
44. Petrovas C, Price DA, Mattapallil J, Ambrozak DR, Geldmacher C, et al. (2007) SIV-specific CD8+ T cells express high levels of PD1 and cytokines but have impaired proliferative capacity in acute and chronic SIVmac251 infection. Blood 110: 928–936.
45. (March1989) Report of World Health Organization Scientific Group on HTLV-1 Infection and Associated Diseases. Kagoshima, Japan: Manila.
46. Lee B, Tanaka Y, Tozawa H (1989) Monoclonal antibody defining tax protein of human T-cell leukemia virus type-I. Tohoku J Exp Med 157: 1–11.
47. Michaelsson J, Barbosa HM, Jordan KA, Chapman JM, Brunialti MK, et al. (2008) The frequency of CD127low expressing CD4+CD25high T regulatory cells is inversely correlated with human T lymphotrophic virus type-1 (HTLV-1) proviral load in HTLV-1-infection and HTLV-1-associated myelopathy/tropical spastic paraparesis. BMC Immunol 9: 41.
48. Iannone R, Sherman MP, Rodgers-Johnson PE, Beilke MA, Mora CA, et al. (1992) HTLV-I DNA sequences in CNS tissue of a patient with tropical spastic paraparesis and HTLV-I-associated myelopathy. J Acquir Immune Defic Syndr 5: 810–816.
49. Koenig S, Woods RM, Brewah YA, Newell AJ, Jones GM, et al. (1993) Characterization of MHC class I restricted cytotoxic T cell responses to tax in HTLV-1 infected patients with neurologic disease. J Immunol 151: 3874–3883.
50. Sakai JA, Nagai M, Brennan MB, Mora CA, Jacobson S (2001) In vitro spontaneous lymphoproliferation in patients with human T-cell lymphotropic virus type I-associated neurologic disease: predominant expansion of CD8+ T cells. Blood 98: 1506–1511.
51. Blackburn SD, Shin H, Haining WN, Zou T, Workman CJ, et al. (2008) Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. Nat Immunol 10: 29–37.
52. Kozako T, Yoshimitsu M, Fujiwara H, Masamoto I, Horai S, et al. (2008) PD-1/PD-L1 expression in human T-cell leukemia virus type 1 carriers and adult T-cell leukemia/lymphoma patients. Leukemia.
53. Yamano Y, Nagai M, Brennan M, Mora CA, Soldan SS, et al. (2002) Correlation of human T-cell lymphotropic virus type 1 (HTLV-1) mRNA with proviral DNA load, virus-specific CD8(+) T cells, and disease severity in HTLV-1-associated myelopathy (HAM/TSP). Blood 99: 88–94.
54. Kubota R, Kawanishi T, Matsubara H, Manns A, Jacobson S (2000) HTLV-I specific IFN-gamma+ CD8+ lymphocytes correlate with the proviral load in peripheral blood of infected individuals. J Neuroimmunol 102: 208–215.
55. Enose-Akahata Y, Matsuura E, Oh U, Jacobson S (2009) High expression of CD244 and SAP regulated CD8 T cell responses of patients with HTLV-I associated neurologic disease. PLoS Pathog 5: e1000682.
56. Anderson AC, Anderson DE (2006) TIM-3 in autoimmunity. Curr Opin Immunol 18: 665–669.