CD4+ T cell subsets and Tax expression in HTLV-1 associated diseases

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Human T lymphotropic virus type 1 (HTLV-1) infection displays variable clinical manifestations. These include inflammatory diseases such as HTLV-1 associated myelopathy (HAM) or immunosuppressive conditions such as *Strongyloides stercoralis* hyperinfection. The viral protein, Tax causes activation and proliferation of T cells. We hypothesize that the expression of Tax in T cell subsets characterizes the clinical manifestations of HTLV-1. To test this hypothesis, we measured T helper 1 effector cells and regulatory T cells (Tregs) among Tax expressing lymphocytes from peripheral blood mononuclear cells (PBMCs) of 32 HTLV-1 infected patients with HAM, with *S. stercoralis* co-infection or with asymptomatic infection. We observed increased ratios of Th1/Treg among Tax expressing lymphocytes in HAM patients. These data suggest that the expression of Tax among the different target cells may explain the variable presentation of HTLV-1.

Keywords: HTLV-1, Tax, Immunology, Regulatory T cells, Th1, Foxp3

Introduction

Human T lymphotropic virus type 1 (HTLV-1) was the first retrovirus isolated from humans.¹ The global prevalence of infection is uncertain due to insufficient data and high heterogeneity amongst regions. In Latin America, the prevalence of infection is estimated to be 0.8%.² Most infected people remain asymptomatic throughout their lives, but approximately 10% develop diverse clinical manifestations. The clinical manifestations can be grouped into inflammatory diseases (e.g., HTLV-1 associated myelopathy [HAM], thyroiditis, uveitis, Sjögren syndrome, and arthritis), neoplastic diseases (e.g., adult T cell leukemia/lymphoma), and opportunistic infections (e.g., *Strongyloides stercoralis* hyperinfection and crusted scabies).³

HTLV-1 mainly infects CD4+ T cells including T helper 1 (Th1), T helper 2 (Th2), and regulatory T cells (Tregs).^{4–6} Regulatory T cells are a CD4+ T lymphocyte subset that actively suppresses immune activation and controls the expansion of self-reactive lymphocytes. Thus, they play an important role in

maintaining peripheral tolerance.^{7,8} The expression of the transcription factor, Forkhead box P3 (Foxp3) controls Tregs function and was touted as a marker to discriminate Tregs from conventional effector T cells (Th1, Th2, and Th17).^{9–12} While FoxP3 is also transiently expressed at low levels by activated effector T cells, it appears to be the best marker of Tregs during HTLV-1 infection.^{13–16}

HTLV-1 encodes the viral protein Tax, which plays a pivotal role in the activation, proliferation, and differentiation of T cell lineages.^{17–19} Thus, Tax expression could eventually lead to alterations in the T cell subsets. Our group has recently noted a dramatic increase in Tregs in patients with HTLV-1 and *S. stercoralis* co-infection. This inversely correlates with circulating eosinophil counts and antigen-driven IL-5 production, suggesting that augmented Treg counts might confer increased susceptibility to *S. stercoralis* infection.²⁰

Compared to asymptomatic HTLV-1 carriers, HAM patients have a higher proportion of Th1 and cytotoxic T cells.²¹ A previous report in HAM patients showed that the proportion of Tregs inversely correlated with the HTLV-1 specific cytotoxic T lymphocytes response.¹⁶ Altogether, these data suggest that a pro-inflammatory state could be responsible for spinal

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cord damage. One study performed on cryopreserved samples found decreased Treg counts in HAM patients.²² However, subsequent reports were unable to replicate those findings using fresh samples.^{14–16,20,23}

As Tax is involved in lymphocyte activation and proliferation, we hypothesized that the clinical manifestations can be characterized by different ratios of Th1 and Tregs among Tax expressing lymphocytes.

Materials and Methods

Subjects and peripheral blood mononuclear cell (PBMC) separation

The 'Instituto de Medicina Tropical Alexander von Humboldt' serves as a national referral center for HTLV-1 infection in Peru. Human T lymphotropic virus type 1 patients are periodically evaluated to determine the presence of clinical manifestations. During follow up, PBMCs are obtained by density gradient centrifugation with Ficoll-Hypaque following manufacturers' instructions (GE HealthCare Life Sciences, USA) and are then cryopreserved in DMSO and stored in liquid nitrogen.

We used the cryopreserved PBMCs of 11 HAM patients, 11 *S. stercoralis* and HTLV-1 (SS/HTLV-1) co-infected patients, and 10 HTLV-1 asymptomatic carriers. In addition, we isolated PBMCs from fresh blood drawn from six HAM patients, seven SS/HTLV-1 patients, and eight asymptomatic carriers who were included in the first phase of the study.

Flow cytometry staining

PBMCs were cultured in RPMI-1640 (GIBCO, Invitrogen Corporation, Carlsbad CA, USA), 10% fetal bovine serum antibiotic supplemented media (1×10^6 /mL, 37°C, 5% CO₂, 12 hour.). After adding Golgi Stop (BD Bioscience, San José, CA, USA) PBMCs were incubated for 6 hours without stimulation. Cells were then washed with PBS 2% FBS (GIBCO, Invitrogen corporation, Carlsbad CA, USA) and stained with anti-human CD4 PE mAb (BD Pharmingen, San Diego, CA, USA) and anti-human CD45 PERCP mAb (BD, Franklin Lakes, NJ, USA) for 30 minutes at 4°C. After permeabilization with Perm/Fix Kit (eBioscience, San Diego CA, USA), cells were stained for Tax protein with primary anti-Tax mAb (LT-4)²⁴ (20 minutes, 4°C) and secondary goat F(ab')₂ anti-mouse IgG3 FITC (BD Bioscience, San José, CA, USA, 15 minutes, 4°C). After washing with Perm buffer 5% goat serum, cells were stained with anti-human IFN-gamma AlexaFluor 647 mAb or with anti-human FoxP3 APC mAb (eBioscience, San Diego CA, USA, 30 minutes, 4°C). Peripheral blood mononuclear cells were resuspended in PBS 2% FBS, then acquired with a FACScalibur flow cytometer (Beckton Dickinson, Franklin Lakes,

NJ, USA), and analyzed with Flow Jo software (Ver.8.8.4 Tree Star, Ashland, Oregon, USA).

Lymphocytes were acquired using CD45 and SSC lymphocyte gating. Regulatory T cells were defined by the phenotype CD4+Foxp3+. Th1 cells were defined by the phenotype CD4+ IFN-gamma+.

Statistical analyses

Analyses were performed using Prism 4 (Graph Pad Software, Inc.). Kruskal–Wallis and Mann–Whitney tests were used to explore the differences between lymphocytes subsets among the three and two groups respectively. *P* values <0.05 were considered significant.

Ethical consideration and human subject protection

The institutional review board of Universidad Peruana Cayetano Heredia approved the study. All patients signed a consent form prior to enrollment.

Results

We evaluated the proportion of Th1, Tregs, and Tax expressing lymphocytes among the CD4+ T cells. No differences were found in Tax expression among the CD4+ T cells of patients from the three clinical groups. The proportion of Th1 cells was significantly increased in HAM patients compared to the other groups (Fig. 1A, Table 1). No differences were found in the proportion of Treg cells.

Since Foxp3 expression can be transiently increased in activated T cells, we compared the proportions of cells expressing FoxP3 from fresh samples with those after 12 hours of culture.¹³ No differences were found between these time points (data not shown). As previously described in the literature, most of the Foxp3 expressing lymphocytes were CD25^{high}CD127^{low/neg} (Fig. 2).^{25,26}

We found an increased proportion of Th1 among the Tax expressing lymphocytes in HAM patients (Fig. 1B). Although not statistically significant, the proportion of Tregs among the Tax expressing lymphocytes was found increased in *S. stercoralis* co-infected patients.

We then examined the Th1/Tregs ratio among the Tax expressing lymphocytes. We found this ratio increased in HAM patients (Fig. 1C, Table 1). When assessing fresh samples, we obtained similar results regarding the ratios of Th1 to Tregs in the HAM patients.

Discussion

In this study, we show that HAM patients present higher ratios of Th1/Tregs among Tax expressing lymphocytes compared to HTLV-1 asymptomatic carriers and SS/HTLV-1 patients.

HTLV-1 encodes several key proteins, of which Tax is thought to be the most relevant to host

Figure 1 Comparative median proportions of CD4+ T cells subsets and ratios among the clinical manifestations of human T lymphotropic virus type 1 (HTLV-1) infection (A) HAM patients presented a larger proportion of Th1 when compared to SS/HTLV-1 and asymptomatic carriers (Kruskal–Wallis: $P=0.04$; Mann–Whitney test: *SS/HTLV-1 vs HAM: $P=0.04$; **asymptomatic carriers vs HAM: $P=0.04$) (B) HAM patients presented higher median proportions of Th1 among Tax expressing CD4+ T cells when compared to asymptomatic carriers or SS/HTLV-1 patients (Kruskal–Wallis $P=0.01$; *Mann–Whitney test: $P=0.03$; **Mann–Whitney test: $P=0.009$) (C) The Th1/Tregs ratio among Tax expressing CD4+ was increased in HAM compared to asymptomatic infection and strongyloidiasis patients (Kruskal–Wallis $P=0.02$; *Mann–Whitney test: $P=0.01$; **Mann–Whitney test: $P=0.04$). SS/HTLV-1: *Strongyloides stercoralis* and HTLV-1 co-infection, ACs: asymptomatic carriers, HAM: HTLV-1 associated myelopathy.

immune function.³ During infection, Tax interacts with multiple cellular pathways affecting secondary messenger and cytokine expression. This leads to the

activation and proliferation of different T cell lineages which, in turn, alter the T cell function.^{17–19} A recent report showed that HTLV-1 persistence is driven by

Table 1 T lymphocytes subsets and ratios among the clinical manifestations of HTLV-1 infection

T cell subset	<i>S. stercoralis</i> /HTLV-1	Asymptomatic carriers	HTLV-1 Associated myelopathy
Th1	0.08 (0.04–0.15) *	0.07 (0.03–0.17) *	0.17 (0.09–0.28) *
Treg	3.67 (2.38–6.41)	3.85 (1.55–7.77)	4.20 (3.00–5.24)
Tax % CD4+	3.59 (1.38–7.70)	3.82 (1.94–7.50)	2.89 (2.22–7.03)
Th1 % Tax	1.53 (0.54–3.43) †	0.96 (0.72–2.02) †	2.30 (1.80–5.01) †
Treg % Tax	28.57 (17.61–37.98)	23.38 (12.71–46.42)	23.05 (14.63–29.47)
Th1/Treg	0.02 (0.01–0.08)	0.02 (0.01–0.05)	0.04 (0.03–0.07)
Th1/Treg % Tax	0.06 (0.02–0.11) ‡	0.04 (0.01–0.11) ‡	0.15 (0.08–0.22) ‡

Data represent median and interquartile range. Lymphocytes were gated using CD45 and SSC plot. Th1 and Tregs are characterized by CD4+ and IFN- γ or Foxp3 expression respectively. Tax % CD4+ represents the percentage of Tax expressing lymphocytes among CD4+ T cells. Th1 % Tax and Tregs % Tax represents Th1 and Tregs lymphocytes among Tax expressing CD4+ T cells. Th1/Tregs and Th1/Tregs%Tax represent ratios among CD4+ T cells and Tax expressing CD4+ T cells. SS/HTLV-1: *S. stercoralis* and HTLV-1 co-infection; HAM: HTLV-1 associated myelopathy.

*: Kruskal–Wallis: $P=0.04$.

†: Kruskal–Wallis: $P=0.02$.

‡: Kruskal–Wallis: $P=0.02$.

Figure 2 Regulatory T cell (Tregs) characterization by flow cytometry. Regulatory T cells are characterized by the expression of the transcription factor Forkhead Box p3 (Foxp3). In addition, they present a high expression of the IL-2R (CD25) and low or no expression of IL-7R (CD127). Here we show that the majority of Foxp3 cells were also CD25^{high}/CD127^{low/neg}, making Foxp3 a reliable marker for Tregs during HTLV-1 infection.

the clonal proliferation of infected T lymphocytes.²⁷ This may imply that the infection of different T cell subsets may drive their proliferation and characterize the clinical manifestations.

A higher proportion of Tax expressing lymphocytes has been described in HAM patients. However, we did not observe differences in the proportion of Tax expressing lymphocytes among the three clinical groups.²¹ Although Tax expression is an important factor in HTLV-1 pathophysiology, its expression is highly variable and alone cannot account for the diverse clinical manifestations.

Th1 cells produce the pro-inflammatory cytokine IFN- γ , which is crucial in establishing cellular immune response.²⁸ During HTLV-1 infection, Th1 cells are the predominant T cell subsets infiltrating the spinal cord of HAM patients.^{29–32} In agreement with prior reports, we found higher proportions of Th1 cells in HAM patients. These findings support the theoretical model of a pro-inflammatory state in HAM.^{3,18}

In contrast, Tregs play a central role in protecting an individual from autoimmunity.³³ They limit the collateral damage of an immune response and can render an individual susceptible to infections.^{7,8} Although statistically not significant, SS/HTLV-1 patients had a higher Treg proportion among Tax expressing T cells when compared to the other clinical groups.

In conclusion, our data suggest that the differences in Th1 and Treg ratios may be implicated in the different clinical manifestations of HTLV-1. These findings further support the hypothesis that HTLV-1 may generate a preferential expansion of varying infected CD4+ T cells subsets.^{17–19} In inflammatory diseases such as HAM, the predominant effector lymphocyte population of Th1 cells could be responsible for the pro-inflammatory state, and autoimmune manifestations. On the other hand, Treg predominance could be responsible for the susceptibility to opportunistic infections such as strongyloidiasis. These data

suggest that infection of different target cells may explain the variable presentation of HTLV-1.

Funding

The experiments reported in this article have been supported by the funds of the National Institute of Health (NIH) grant (1R01 TW007642-02), a Fogarty Center Training grant (D43TW006569) and internal funds from Universidad Peruana Cayetano Heredia. No funding body contributed in any way to study the design or the collection, analysis, and interpretation of data, or the writing of the manuscript, and/or the decision to submit the manuscript for publication.

Competing Interests

None declared.

Ethical Approval

The institutional review board of Universidad Peruana Cayetano Heredia approved the study. All patients signed a consent form prior to enrollment.

Acknowledgements

We thank the UPCH/IMT HTLV-1 team for coordinating clinical procedures and providing the samples and Dr Angélica Terashima and the IMT parasitology laboratory for *S. stercoralis* microscopic analysis.

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