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E. Ramirez^1,2, L. Cartier^3, L. Rodriguez^4, C. Alberti^1,2,5 and M.A. Valenzuela^5

^1Departamento de Virología, Instituto de Salud Pública de Chile, Santiago, Chile
^2Programa de Virología, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Santiago, Chile
^3Departamento de Ciencias Neurológicas, Facultad de Medicina, Universidad de Chile, Santiago, Chile
^4Sección de Bioestadística, Instituto de Salud Pública de Chile, Santiago, Chile
^5Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile, Santiago, Chile

Abstract

HTLV-1 Tax expression exerts an inhibitory effect on the Foxp3 transcription factor in CD4^+CD25^+ T-regulatory cells (Treg). For a better understanding of the role of Tax mRNA in the gene expression of cellular markers we measured Tax, Foxp3, CTLA-4, GITR, TGF-β, and IL-10 mRNA in Treg cells of 50 patients with human T-lymphotropic virus type 1 (HTLV-1)-associated myelopathy/tropical spastic paraparesis (HAM/TSP; 27 women and 23 men; mean age: 56.7 years). The control group consisted of 23 non-infected subjects (12 women and 11 men) with a mean age of 51.3 years. Real-time PCR was used to measure mRNA of Tax proteins and several cellular markers of Treg function. Determinations revealed a high level of Tax mRNA in HAM/TSP (124.35 copies/100 CD4^+CD25^+ T cells). Foxp3, GITR, and CTLA-4 mRNA levels were lower in HAM/TSP patients (mean ± SD, 22.07 ± 0.78, 9.63 ± 0.36, and 4.54 ± 0.39, respectively) than in non-infected controls (47.15 ± 12.94, 22.14 ± 1.91, and 21.07 ± 2.31). Both groups had similar levels of TGF-β and IL-10. An inverse relationship was found between Tax levels and Foxp3, CTLA-4, and GITR levels. Conversely, there was a direct correlation between levels of Foxp3, GITR, and CTLA-4. Disease severity and evolution time did not correlate with Tax or Foxp3 levels. The present results suggest that Tax and Foxp3 mRNA vary with the same degree of disease severity in HAM/TSP patients. Tax fluctuations may affect CTLA-4 and GITR expression via the Foxp3 pathway, causing virus-induced dysfunction of CD4^+CD25^+ T cells in HAM/TSP patients.

Key words: HTLV-1-associated myelopathy/tropical spastic paraparesis; Tax; Foxp3; CTLA-4; GITR mRNA expression

Introduction

Human T-lymphotropic virus type 1 (HTLV-1) is the retrovirus causing adult T-cell leukemia (ATL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (1-3). The latter is a neurological disease defined as a progressive and slow spastic paraparesis without remittance. The demyelination of the pyramidal tract is the main feature of this progressive neurological disease (4).

According to available data, Tax protein may play a major role in the development of HAM/TSP, regulating multiple cellular responses by protein-protein interactions with various host cell factors. HTLV-1-mediated activation of the host T cell is primarily induced by the viral Tax protein, which influences transcriptional activation, signal transduction, cell cycle control, and apoptosis (5,6).

A correlation has been suggested between HTLV-1 Tax mRNA and disease severity (7). However, practically no longitudinal studies have characterized Tax mRNA expression in relation to HTLV-1 proviral DNA load and disease progression in HAM/TSP patients. We found wide temporal variation in the provirus load and Tax mRNA expression in patients, independent of functional damage (8).

The main reservoir of HTLV-1 in HAM/TSP patients is the CD4^+CD25^+ T-regulatory cell population (Treg cells) (9). In addition, Tax-specific inhibition of Foxp3 mRNA expression may be associated with the suppression of CD4^+CD25^+ T cells that in turn might be related to the suppression of Treg...
function (9,10). Foxp3 is critical for the development and function of Treg, with Foxp3 reduction and Treg dysfunction being associated with autoimmune diseases such as multiple sclerosis, myasthenia gravis, and type 1 diabetes (11-13). A TGF-β-mediated signaling has been associated with the induction and maintenance of Foxp3 and Treg differentiation (14,15). Recent studies suggest that the Tax protein could affect TGF-β signaling by inhibiting the gene expression controlled by this factor (16). However, CD4+ T cells of HAM/TSP patients apparently do not respond to the antiproliferative effects of in vitro TGF-β (17). The molecular mechanisms involved in the suppression of HTLV-1-infected Treg cell have not yet been elucidated.

For a better understanding of the mechanisms involved in the suppression of Treg cells by HTLV-1 infection, we measured Tax, Foxp3, CTLA-4, GITR, TGF-β, and IL-10 mRNA in CD4+CD25+ T cells of HAM/TSP patients and compared them with non-infected controls.

Material and Methods

Patients and samples

A total of 73 individuals were studied: 50 HTLV-1 seropositive HAM/TSP patients and 23 non-infected controls (blood donors). All subjects gave written informed consent to participate, and the study was approved by the El Salvador Hospital Ethics Committee. All subjects resided in Santiago de Chile and had Spanish ethnic background. We confirmed HTLV-1 infection by immunofluorescence and PCR assays (18). The diagnosis of HAM/TSP was made according to the World Health Organization guidelines (19). Other known causes (i.e., multiple sclerosis, spinal cord compression) of progressive spastic paraparesis were excluded according to clinical presentation and according to clinical, neurophysiological, radiological, immunological, and cerebrospinal fluid cytological studies (20). We performed hematological analysis to detect leukemic lymphocytes. The functional status of the patients was measured by the expanded disability status scale (EDSS) and the march scale of eight functional levels (21).

The HAM/TSP group was composed of 27 women and 23 men, mean age 56.7 ± 9.9 years, age range 24-73 years, with a mean evolution time of 10.4 ± 7.1 years (range 2-30 years). Sixty percent of the patients had sensory failure, and 42% had a mean evolution time of 10.4 ± 7.1 years (range 2-30 years).

We obtained a blood sample (15 mL) from each subject to study Tax, Foxp3, CTLA-4, GITR, IL-10, and TGF-β mRNA expression. PBMCs were obtained by centrifugation on Ficoll-Hypaque gradients. Cells were cryopreserved in liquid nitrogen until used.

Isolation of CD4+CD25+ T cells

CD4+ T cells were positively isolated from cryopreserved PBMC with the Dynal CD4+ Positive Isolation Kit (Invitrogen Dynal, Norway) according to manufacturer instructions. CD25+ cells were enriched with PE-anti-CD25 and anti-PE magnetic beads (Miltenyi Biotec, Germany) using the Midi-MACS system (Miltenyi Biotec). The purity of the resulting CD4+CD25+ T cells was shown to be 90% by flow cytometry.

Real-time RT-PCR of Tax mRNA

RNA was extracted from Treg cells using the RNeasy Mini Kit (Qiagen, USA) according to manufacturer instructions. RT-PCR was performed using the LightCycler, one-step RNA Amplification Kit SYBR Green I (Roche Molecular Systems, Inc., USA). Primers (Invitrogen, USA) for the amplification of HTLV-1 Tax mRNA have been previously described (7). The forward primer RPX-3 (5096-5115; 5'-ATCCCGTGG AGA CT CCT CCA A-3') and the reverse primer RPX-4+F (7360-7338; 5'-CCAAAGATGAGGTTATC C-3') were located upstream and downstream of the second splice junction site of HTLV-1 pX (tax/ex) mRNA, respectively. Primers (Invitrogen) for the amplification of Foxp3, CTLA-4, GITR, IL-10, and TGF-β mRNA have been previously described (9,22,23). We used primers for the human housekeeping gene hypoxanthine ribosyl transferase (HPRT; BiosChile IGSN, Chile) for internal calibration (24). RT-PCR conditions were as follows: 50 ng RNA was added to a 20-µL reaction mixture containing 1X LightCycler - RT-PCR Reaction Mix SYBR Green I, 1X Resolution solution, and 1X LightCycler - RT-PCR Enzyme Mix. 6.0 mM MgCl2, 0.5 mM of each primer. The thermal cycler conditions were 15 min at 55°C for cDNA synthesis, followed by 45 cycles of 10 s at 95°C (denaturation), 5 s at 55°C (annealing), and 7 s at 72°C (extension).

Standard curves for the calculation of HTLV-1 Tax mRNA and HPRT mRNA were generated using cDNA from MT-2 cells. MT-2 cDNA was 10-fold serially diluted with diethyl pyrocarbonate (DEPC) H2O to a 10-5 dilution, and a sample of cDNA from 100 ng of RNA per reaction was amplified. When a serial dilution of 109 to 10-5 MT-2 cDNA was used as template for the real-time PCR, a specific signal was observed in contrast to negative controls. The threshold cycle (Ct) values were used to plot a standard curve in which Ct decreased in linear proportion to the log of the template copy number.

All assays were carried out in triplicate and the average value was used for calculations. The relative HTLV-1 Tax, Foxp3, IL-10, TGF-β, CTLA-4, and GITR mRNA levels were calculated by the following formulas: Tax = (value of tax) / (value of HPRT) x 10,000, and Foxp3 = 2^(Ct value of HPRT - Ct value of Foxp3), IL-10 = 2^(Ct value of HPRT - Ct value of IL-10), TGF-β = 2^(Ct value of HPRT - Ct value of TGF-β), CTLA-4 = 2^(Ct value of HPRT - Ct value of CTLA-4), GITR = 2^(Ct value of HPRT - Ct value of GITR).

Statistical analysis

A multiple Pearson correlation graph was obtained with
the statistical package Stata version 9.1. We obtained the least-squares regression and three-dimensional graphics with the Statgraphics Centurion XV package, using the regression and graphic modules from the General Linear Model. We used robust estimators (median absolute difference) when some outliers could generate a strong deformation of the regression curves. The original data were sorted in an Excel file and then copied to the relevant statistical packages. The accurate P values were calculated with the WHATIS module from the WINPEPI program.

**Results**

**Levels of Tax, Foxp3, CTLA-4, IL-10, and TGF-β mRNA in samples from HAM/TSP patients and controls**

Table 1 shows the mean values and standard deviation of the levels of Tax, Foxp3, CTLA-4, GITR, IL-10, and TGF-β mRNA in CD4+CD25+ T cells of patients and controls. Tax mRNA was present in the cells of all HAM/TSP patients. The average of Tax in T cells of patients was 124.35 ± 40.17 copies/100 CD4+CD25+ T cells. The average of Foxp3 mRNA was lower in HAM/TSP patients (22.07 ± 0.78 copies/100 CD4+CD25+ T cells) than in T cells of non-infected controls (47.15 ± 12.94 copies/100 CD4+CD25+ T cells). The mean values of GITR and CTLA-4 mRNA were also lower in T cells of HAM/TSP patients (9.63 ± 0.36 and 4.54 ± 0.39, respectively) than in non-infected controls (47.15 ± 12.94 copies/100 CD4+CD25+ T cells). The mean values of TGF-β (1.92 ± 0.12 and 1.83 ± 0.12) and IL-10 (2.38 ± 0.11 and 2.51 ± 0.17) mRNA were not statistically different in both groups.

**Correlation of Tax with Foxp3, CTLA-4 and GITR mRNA in T cells of HAM/TSP patients**

Regression analyses were performed on data from HAM/TSP patients to evaluate the possible correlation between Tax and Foxp3, CTLA-4 and GITR mRNA (Figure 1). A strong negative association was found between Tax and Foxp3, which fitted a linear model equation: Foxp3 mRNA = 32.549 - 0.0888 x mRNA Tax, with P < 0.001 (Figure 1A). Tax had a negative linear regression with CTLA-4 according to the following equation: CTLA-4 mRNA = 6.1954 - 0.0168 x Tax mRNA (Figure 1B). An inverse association between Tax and GITR mRNA fitted the following linear regression equation: GITR mRNA = 12.133 - 0.019 x Tax mRNA (Figure 1C). On the other hand, no correlation was detected between Tax and TGF-β or IL-10 mRNA (data not shown).

**Correlation between patients and controls regarding Foxp3, CTLA-4 and GITR mRNA**

Both groups showed linear correlations between Foxp3 and CTLA-4. Figure 2 shows the positive regression curve.

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**Table 1. Tax, Foxp3, TGF-β, IL-10, GITR, and CTLA-4 mRNA expression in CD4+CD25+ T cells from HAM/TSP patients and non-infected controls.**

<table>
<thead>
<tr>
<th></th>
<th>HAM/TSP (N = 50)</th>
<th>Non-infected control (N = 23)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tax</td>
<td>124.35 ± 40.17</td>
<td>0</td>
</tr>
<tr>
<td>Foxp3</td>
<td>22.07 ± 0.7836*</td>
<td>47.15 ± 12.94</td>
</tr>
<tr>
<td>TGF-β</td>
<td>1.92 ± 0.1231</td>
<td>1.83 ± 0.1205</td>
</tr>
<tr>
<td>IL-10</td>
<td>2.38 ± 0.1132</td>
<td>2.51 ± 0.1703</td>
</tr>
<tr>
<td>GITR</td>
<td>9.63 ± 0.3611*</td>
<td>22.14 ± 1.907</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>4.54 ± 0.3908*</td>
<td>21.07 ± 2.311</td>
</tr>
</tbody>
</table>

Data are reported as means ± SD mRNA levels (copies/100 CD4+CD25+ T cells). The levels of mRNA were determined by real-time PCR. HAM/TSP = human T-lymphotropic virus type 1 (HTLV-1)-associated myelopathy/tropical spastic paraparesis. *P < 0.05 compared to control (Student t-test).
exhibited by HAM/TSP data, with a lower slope than that exhibited by non-infected controls. This means that patients had a smaller CTLA-4 increase with the same Foxp3 variation than controls. For both patients and controls, linear model equations were obtained, illustrating relationships of Foxp3 vs GITR mRNA, and GITR vs CTLA-4 mRNA (data not shown). The regression curves for patients exhibited lower slopes than those for controls. Foxp3, CTLA-4 and GITR mRNA did not correlate with TGF-β or IL-10 mRNA in either group.

**Tax and Foxp3 mRNA levels in T cells of HAM/TSP patients with varying severity of disease**

The level of Tax mRNA did not correlate with the severity of disease in HAM/TSP patients (Figure 3A). No statistically significant difference was detected between Tax level and most degrees of disease severity (Figure 3A). Significant differences were detected between patients

![Figure 2](image2.png)

**Figure 2.** Correlation between Foxp3 and CTLA-4 expression in CD4⁺CD25⁺ T cells from 50 HAM/TSP patients. Correlation was calculated by the Pearson method. HAM/TSP = human T-lymphotropic virus type 1 (HTLV-1)-associated myelopathy/tropical spastic paraparesis. Data are reported as Foxp3 and CTLA-4 RNA copy numbers/100 CD4⁺CD25⁺ T cells.

![Figure 3](image3.png)

**Figure 3.** Severity and evolution of HAM/TSP disease in 50 patients related to Tax and Foxp3 mRNA levels in CD4⁺CD25⁺ T cells. A and B. Box plots of severity of disease and Tax mRNA and Foxp3 RNA levels. The severity of disease or motor involvement was reported as efficient spastic gait without support (2), inefficient spastic gait without support (3), efficient spastic gait with support (4), inefficient spastic gait with support (5), wheelchair (6), bedridden or unable to sustain (7), bedridden with global difficulty (8) (Student t-test and Graph Pad Prism software). C and D, Evolution of disease plotted against Tax mRNA and Foxp3 mRNA levels. The evolution of disease was reported as a period of time: 1-5 years, 6-10 years, 11-15 years, 16-20 years, 21-25 years. Data are reported as Tax and Foxp3 RNA copy numbers/100 CD4⁺CD25⁺ T cells.
with efficient spastic gait with support (stage 4) and those with inefficient spastic gait without support (stage 5). In addition, significant differences were found in T cells of patients with inefficient spastic gait without support (stage 5) and bedridden patients with global difficulty (stage 8). Similarly, Foxp3 mRNA levels did not change in any of the six clinical stages of HAM/TSP patients (Figure 3B). No association between Tax or Foxp3 mRNA levels and disease evolution was detected, since Tax and Foxp3 mRNA expressions were not significantly different in T cells of HAM/TSP patients with 5, 10, or 20 years of disease (Figure 3C and D, respectively).

**Discussion**

The decrease of Foxp3, which plays a key role in Treg deregulation, could cause a loss of Treg suppressive ability (25,26). In T cells of HAM/TSP patients, this phenomenon has been suggested to be an important mechanism of viral pathogenesis (27). Within the context of HTLV-I, the frequency of non-infected CD4+CD25+ Treg lymphocytes can be an important marker of the efficiency of T cell-mediated immune control (10). The transfection of the HTLV-1 tax gene into purified CD4+CD25+ T cells from non-infected healthy donors caused a decrease in Foxp3 expression. Thus, in T cells of HAM/TSP patients, Tax transactivation might be associated with a Foxp3 reduction both in mRNA expression and protein synthesis (9). Despite the cardinal role of lymphocytes in controlling the immune response, the molecular mechanisms involved in the interaction between Foxp3 and Tax remain unknown.

The suppression exerted by Treg lymphocytes might be mediated by soluble factors acting both at long and short distances. Cytokines such as IL-10 and TGF-β have been related to the development of HAM/TSP (28). The levels of TGF-β receptor II (TGF-βRII) as well as Smad7 (a TGF-β-inducible gene) could be reduced in CD4+ T cells of HAM/TSP patients compared with healthy donors (16). Moreover, the TGF-βRII expression would be inversely correlated with HTLV-1 proviral load (16). These observations suggest that dysregulation of TGF-β signaling and silencing of downstream TGF-β-responsive genes, including Foxp3, could be involved in the pathogenesis of HAM/TSP. However, other studies have found no correlation of IL-10 and TGF-β1 mRNA with Foxp3 levels, or their correlation with HTLV-1 proviral load (10). CD4+Foxp3+ cells probably exert their regulatory effect on HTLV-1-specific CD8+ T cells by a cell contact mechanism, with or without the involvement of cytokines. Our findings suggest that IL-10 and TGF-β have little involvement in the progression of HAM/TSP since TGF-β and IL-10 mRNA values are similar in T cells of both HAM/TSP patients and controls (Table 1).

A direct correlation between the progression of HAM/TSP and both proviral load and Tax mRNA levels has been reported (7). High levels of proviral DNA or Tax mRNA have been detected in patients with important neurological involvement when compared to asymptomatic carriers (10,29,30). Tax mRNA has been suggested to be a predictor of HAM/TSP progression (7). However, no correlation was recently found between Tax mRNA and disease severity in Japanese patients (31). In the HAM/TSP patients studied here, we found a variability in Tax mRNA, which prevented us from demonstrating a correlation between this parameter and the progression or duration of disease. Similarly, other researchers have found highly variable Tax RNA levels in the T cells of these patients. Japanese and American investigators have found 39.3 and 100% Tax mRNA in all patients analyzed, respectively (7,31). These discrepancies could be attributed to host genetic and virological factors, such as HLA haplotypes, quantity of soluble suppressive proteins, CD8+ T-cell responses, and Tax genomic sequences (32). In our study, we detected Tax mRNA in all HAM/TSP patients. This suggests that the presence of Tax rather than its amount is required for the maintenance of HAM/TSP. We found a highly significant negative association between Tax and Foxp3 in the T cells of HAM/TSP patients. Foxp3 mRNA in HAM/TSP patients was lower than in non-infected controls (Table 1), in agreement with data reported by other investigators (10,27).

In addition, we detected a direct correlation between the levels of Foxp3, CTLA-4 and GITR. To our knowledge, this finding has not been reported before. These results suggest a probable suppressive mechanism mediated by cell-to-cell interactions. According to a previous report, the expression of inhibitory molecules in the Treg cell membrane, such as CTLA-4, could be significant in the suppressive effect of Tregs (25). CTLA-4 has been shown to trigger the induction of the activation of indoleamine 2,3-dioxygenase (IDO) when it interacts with its ligands in dendritic cells (33). IDO catalyzes the conversion of tryptophan to kynurenine and other metabolites, which have potent immunosuppressive effects on the local environment of dendritic cells.

Undoubtedly, our findings need further in-depth studies, ideally monitoring the patient’s condition with serial samples to assess the temporal dynamics of Foxp3 mRNA. Additionally, we believe that variations in Foxp3 mRNA levels could be an important molecular marker for the assessment of the progression of HAM/TSP.

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