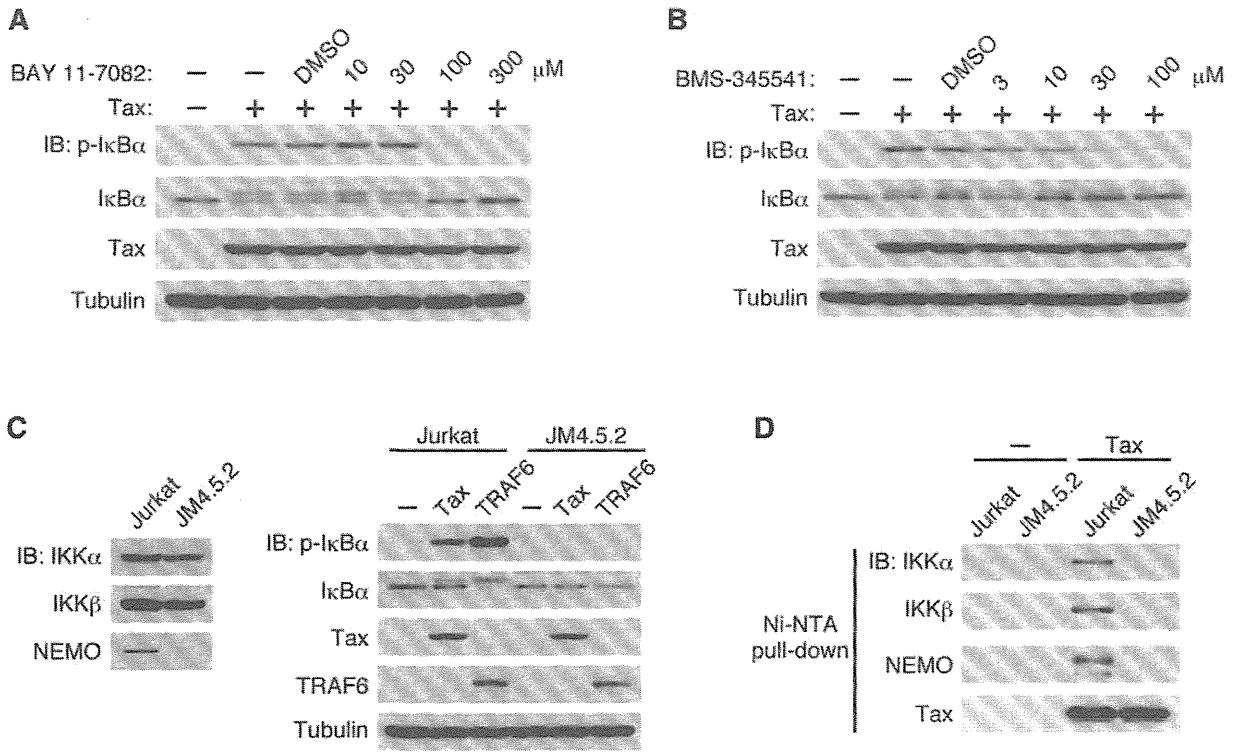

Fig. 2 Phosphorylation of IκBα induced by recombinant Tax is dependent on IKK activation. (A and B) IKK inhibitors inhibit IκBα phosphorylation induced by recombinant Tax in a dose-dependent manner. The Jurkat cytosolic extract (10 mg/ml) was incubated with recombinant Tax (0.5 μM) and ATP (2 mM) and either the IKK inhibitor BAY 11-7082 (A) or BMS-345541 (B) at 30°C for 1 h. Phosphorylation of IκBα was detected by immunoblot with an anti-p-IκBα antibody. (C) NEMO is essential for Tax-induced phosphorylation of IκBα. The expression levels of IKKα, IKKβ and NEMO were analysed by immunoblot (left). Either the Jurkat or JM4.5.2 cytosolic extract (10 mg/ml) was incubated with either recombinant TRAF6 (0.5 μM) or Tax (0.5 μM) and ATP (2 mM) at 30°C for 1 h. Phosphorylation of IκBα was detected by immunoblot with an anti-p-IκBα antibody (right). (D) Tax binding to the IKK complex depends on NEMO. The cell-free assay was performed as described in (C), and the reaction mixtures were then subjected to a Ni-NTA pull-down assay to analyse interaction of Tax with the IKK complex subunits by immunoblot with anti-IKKα, anti-IKKβ and anti-NEMO antibodies.

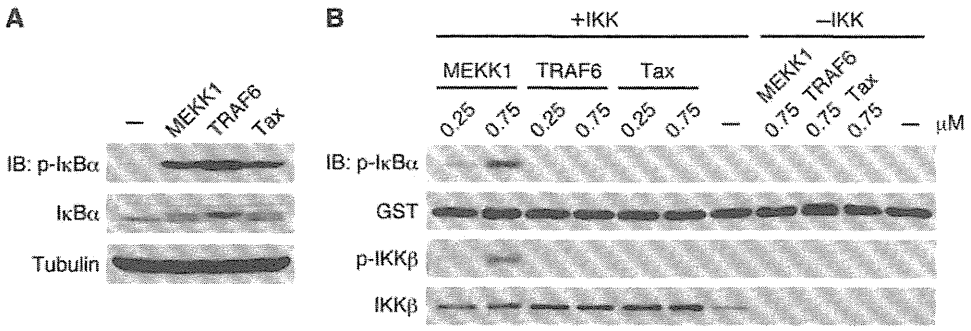

Fig. 3 Tax requires intermediary factors for IKK activation. (A) Recombinant MEKK1, TRAF6 and Tax activated the IKK complex in the Jurkat cytosolic extract. The Jurkat cytosolic extract (10 mg/ml) was incubated with either recombinant MEKK1 (0.75 μM), TRAF6 (0.75 μM) or Tax (0.75 μM) and ATP (2 mM) at 30°C for 1 h. Phosphorylated IκBα was detected by immunoblot with an anti-p-IκBα antibody. (B) Recombinant Tax did not activate the purified IKK complex. Recombinant MEKK1, TRAF6 or Tax was incubated with ATP (2 mM) and GST-IκBα (100 ng) at 30°C for 1 h either without (−IKK) or with (+IKK) the IKK complex purified from unstimulated Jurkat cells. The reaction mixtures were analysed by immunoblot with anti-p-IκBα, anti-GST, anti-p-IKKβ and anti-IKKβ antibodies.

MEKK1 was unable to induce phosphorylation of IκBα without the IKK complex (Fig. 3B, −IKK), indicating that MEKK1 does not directly phosphorylate IκBα. Similar to TRAF6, Tax alone did not activate the purified IKK complex (Fig. 3B), indicating that Tax requires intermediary factors for IKK activation, which are in the Jurkat cell cytosol.

Polyubiquitination is involved in Tax-induced IKK activation but not in the interaction between Tax and NEMO

We next sought to determine that cytosolic intermediary factor(s) required for Tax-induced IKK activation. It has been well established that various polyubiquitin chains are crucial in IL-1- and TNF-α-induced IKK

activation (*7*). IL-1 induces the polyubiquitinated forms of TRAF6 and TAK1, and TNF-α induces those of RIP1 (*8*, *9*, *11*, *12*). Moreover, it has been reported that unanchored polyubiquitin chains are generated in response to these stimulating events (*27*). These polyubiquitin chains act as a platform for the formation of an active TAK1-containing signalling complex, which activates IKK. In addition to TAK1 activation, the activation of IKK requires stimulation-induced Lys63-linked or linear polyubiquitin chain conjugation to NEMO (*28–32*), which may induce either oligomer formation or a conformational change in NEMO to activate the IKK complex. The former conjugation is catalysed by TRAF6 with Ubc13 and the latter by the linear ubiquitin chain assembly complex (LUBAC), which is composed of HOIL-1, HOIP and Sharpin (*33–35*). We have previously shown that Ubc13 is dispensable to Tax-induced IKK activation, and expression of CYLD does not affect Tax-induced NF-κB activation (*15*). However, we cannot exclude the possibility that Lys63-linked polyubiquitination is involved in Tax-induced IKK activation because E2 ubiquitin-conjugating enzymes other than Ubc13 could be involved and that the Lys63-linked polyubiquitin chains could be somehow blocked from CYLD-mediated deubiquitination. Because our cell-free assay system is not affected by the Tax-induced secretion of various cytokines that trigger generation of polyubiquitin chains, we next investigated whether polyubiquitination was required in Tax-mediated IKK activation using the cell-free system. To inhibit polyubiquitin chain formation, we used two ubiquitin mutants. We used a lysine-free ubiquitin mutant, in which all the lysine residues were mutated to arginine (K0-Ub). We also used a methylated ubiquitin, in which the amino groups, including the ε-amino group, on all the lysine residues and the N-terminal Met α-amino group were blocked by methylation (Me-Ub). The addition of these ubiquitin mutants suppressed TRAF6-induced IKK activation, whereas MEKK1-induced IKK activation was not affected (Fig. 4A). This result is consistent with the notion that polyubiquitination is involved in TRAF6-induced IKK activation but not in MEKK1-induced IKK activation (*9*). More importantly, both ubiquitin mutants suppressed Tax-induced IKK activation (Fig. 4A). This result indicates that polyubiquitination is involved in Tax-induced IKK complex activation.

As Tax binding to NEMO is essential for Tax-induced IKK activation (*22*, *23*), we next examined whether the ubiquitin mutants inhibited the interaction between Tax and the IKK complex. The Jurkat cytosolic extract was incubated with recombinant Tax with and without either wild-type ubiquitin or the mutants. An aliquot of the reaction mixture was immunoblotted directly to examine IKK activation, and the remaining mixture was used for Ni-NTA pull-down assays to analyse the interaction between Tax and the IKK complex. Although K0-Ub and Me-Ub inhibited Tax-induced IKK activation as expected (Fig. 4B, left), the addition of these mutants had no effect on the association between Tax and the IKK complex (Fig. 4B, right). These results suggest that the interaction between Tax and the IKK complex is insufficient to induce IKK activation, and there may be additional steps, in which polyubiquitination is involved, for Tax-induced activation of the IKK complex.

As the addition of K0-Ub does not inhibit the generation of linear polyubiquitin chains (*36*), K0-Ub-mediated inhibition of Tax-induced IKK activation suggests that linear polyubiquitination is

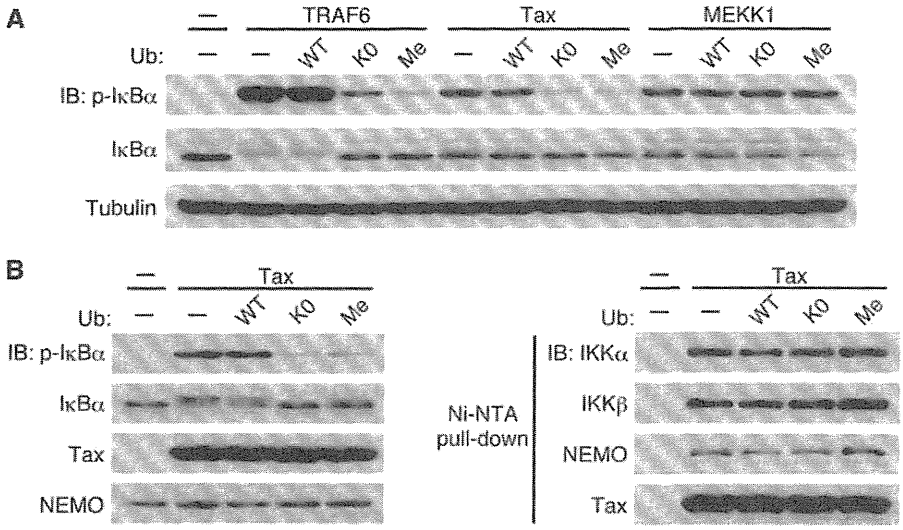

Fig. 4 Polyubiquitination is involved in Tax-induced IKK activation. (A) The Jurkat cytosolic extract (10 mg/ml) was mixed with wild-type (WT, 100 μM), lysine-free (K0, 100 μM), or methylated (Me, 100 μM) ubiquitin and then incubated with recombinant TRAF6 (0.5 μM), Tax (0.5 μM) or MEKK1 (0.5 μM) and ATP (2 mM) at 30°C for 1 h. Phosphorylation of IκBα was detected by immunoblot with an anti-p-IκBα antibody. (B) The Jurkat cytosolic extract (10 mg/ml) was mixed with either WT ubiquitin or the ubiquitin mutants (100 μM), as well as recombinant Tax (0.5 μM) and ATP (2 mM) at 30°C for 1 h. After incubation, aliquots of the reaction mixtures were immunoblotted directly with an anti-p-IκBα antibody to examine IKK activation, and the remaining mixture was used for a Ni-NTA pull down to analyse the interaction between Tax and the IKK complex by immunoblot with anti-IKKα, anti-IKKβ, anti-NEMO and anti-Tax antibodies.

dispensable or insufficient for IKK activation by Tax. These results led us to test whether Lys63-linked polyubiquitination is involved. We first examined whether Tax induces polyubiquitination in the cell-free system. Immunoprecipitation and subsequent immunoblot with an anti-ubiquitin antibody revealed that Tax induces polyubiquitination of Tax and NEMO in the cell-free system (Fig. 5A). The cell-free assay was then performed with the ubiquitin mutant, either K48R or K63R, in which the Lys-48 or Lys-63 of ubiquitin was mutated to Arg, respectively. K48R did not have an effect, but K63R completely inhibited Tax-induced IKK activation (Fig. 5B), which indicates that Lys-63-linked polyubiquitination is required for Tax-induced IKK activation. In the IL-1R and TLR pathways, TRAF6 acts as an E3 ubiquitin ligase to generate K63-linked polyubiquitin chains upon stimulation (*8*, *9*). Therefore, cytosolic extracts were prepared from wild-type MEF cells and *Traf6*$^{-/-}$ MEF cells and then tested for Tax-induced IKK activation. Similar levels of IκBα phosphorylation were observed in the WT and *Traf6*$^{-/-}$ extracts (Fig. 5C), which indicates that TRAF6 is dispensable for Tax-induced IKK activation.

In this study, using a cell-free assay system, we demonstrated that activation of the IKK complex by HTLV-1 Tax requires cytosolic factor(s) that are involved in Tax-induced polyubiquitination. As polyubiquitination is catalysed by three enzymes, E1, E2 and E3 (*37*), it is important to identify these enzymes to further elucidate the molecular mechanisms of Tax-induced IKK activation. Although our results strongly suggest that K63-linked polyubiquitin chains are involved in the Tax-induced IKK activation, TRAF6 does not act as an E3 and the involvement of Ubc13 as an E2 is still controversial (*15*, *16*). It is well known that polyubiquitin chains with different lysine linkages have distinct biological roles (*38*, *39*). Lys48-linked polyubiquitin chains are a signal for proteasomal degradation. On the other hand, Lys63-linked and linear polyubiquitin chains play an important role in the cytokine signal transduction pathway that leads to NF-κB activation. Further studies are required to determine which types of polyubiquitin chain are involved in Tax-induced IKK complex activation. We also found that polyubiquitination was not required for the interaction between Tax and the IKK complex. This result raises the possibility that polyubiquitination might be required either to recruit IKK kinase (IKKK) or to induce *trans*-autophosphorylation of the IKK complex, which induces IKK activation. It has been reported that ubiquitination of Tax is required for the interaction of Tax with NEMO (*16*). Although the reason for the discrepancy

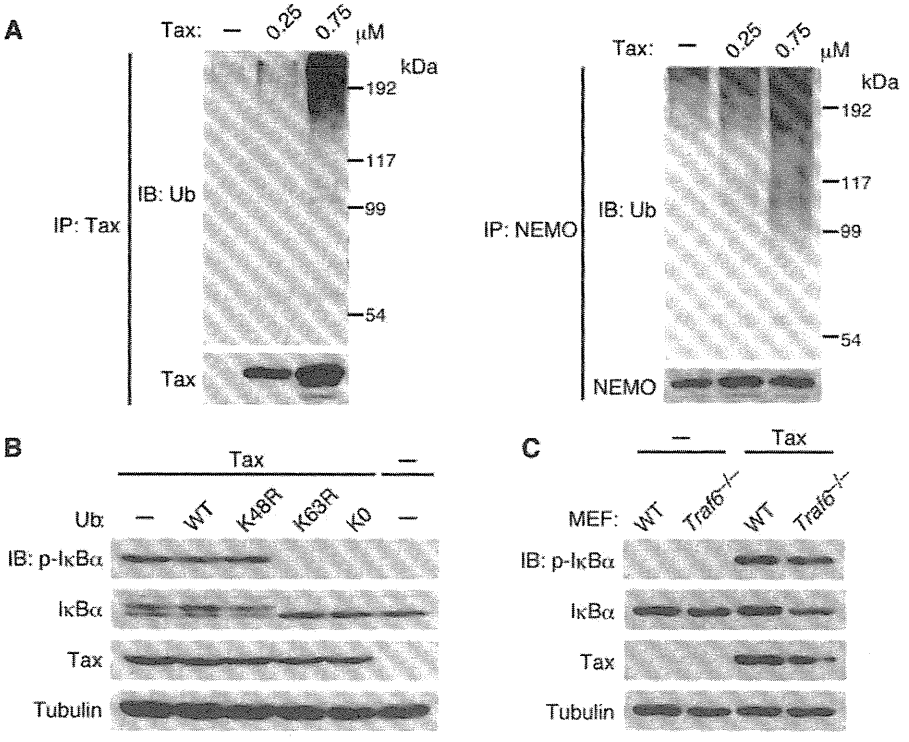

Fig. 5 Potential involvement of Lys-63-linked polyubiquitination in Tax-induced IKK activation. (A) Recombinant Tax induces polyubiquitination of Tax and NEMO in the cell-free system. The Jurkat cytosolic extract (10 mg/ml) was incubated with recombinant Tax with ATP (2 mM) at 30°C for 1 h. After incubation, the reaction mixture was first boiled to remove the non-covalently attached proteins and then immunoprecipitated with either an anti-Tax (left) or anti-NEMO (right) antibody. Ubiquitination of Tax and NEMO was detected by immunoblot with an anti-ubiquitin antibody. (B) The Lys63-linked polyubiquitin chain is involved in Tax-induced IKK activation. The Jurkat cytosolic extract (10 mg/ml) was incubated with recombinant Tax (0.5 μM) and either the WT or ubiquitin mutants (100 μM) with ATP (2 mM) at 30°C for 1 h. IκBα phosphorylation was detected by immunoblot with an anti-p-IκBα antibody. (C) TRAF6 is not involved in Tax-induced IKK activation. Recombinant Tax (0.5 μM) was incubated with the cytosolic extract from either WT or *Traf6*$^{-/-}$ MEFs (10 mg/ml) with (2 mM) at 30°C for 1 h. Phosphorylation of IκBα was detected by immunoblot with an anti-p-IκBα antibody.

is not clear, identification of the polyubiquitinated proteins and IKK-interacting proteins using our cell-free system may provide clues to understanding the molecular mechanisms of Tax-induced IKK activation.

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Conflict of interest

None declared.

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Molecular and Clinical Effects of Betamethasone in Human T-Cell Lymphotropic Virus Type-I-Associated Myelopathy/Tropical Spastic Paraparesis Patients

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There is no effective therapy for human T-cell lymphotropic virus type I (HTLV-I)-associated myelopathy/tropical spastic paraparesis (HAM/TSP). Glucocorticoids are effective to reduce the motor disability in these patients, but its role as anti-spastic drugs is unknown. Here it is reported the use of corticosteroids in HAM/TSP. The goal was to find reliable molecular markers linked to treatment effectiveness. The clinical efficacy of corticosteroids was studied in 22 HAM/TSP. The treatment was a single dose of 7.0 mg of systemic betamethasone. Pre-treatment samples were obtained immediately before steroid administration and post-treatment samples were collected after 5 days. Neurological disability was evaluated by the Osame's Motor Disability Scales. Relative levels of Tax, Foxp3, IL-10, TGF-β, CTLA-4, and GITR mRNA were measured and the percentage of $CD4^+Foxp3^+$ and $CD4^+Tax^+$ populations was quantified in PBMCs by real-time PCR and flow cytometry, respectively. The same parameters were studied in eight untreated carriers. Betamethasone treatment showed neurological improvement in 21 HAM/TSP patients, with one patient without response to treatment. This therapy was associated with a decrease in Tax mRNA load and $CD4^+Tax^+$ T cells in HAM/TSP. Simultaneously, an increase in Foxp3 mRNA and $CD4^+Foxp3^+$ T cell was detected in these patients. The other markers studied had no significant changes after treatment. Clinical improvement in betamethasone-treated HAM/TSP was associated with an inverse relationship between a decrease in Tax and an increase in Foxp3 at the mRNA and protein levels. These results suggest that both Tax and Foxp3 may represent potential biomarkers for drug treatment assessments in HAM/TSP.
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KEY WORDS: HTLV-I; betamethasone therapy; HAM/TSP

INTRODUCTION

Human T-cell lymphotropic virus type I (HTLV-I) is the etiologic agent of HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [Osame et al., 1986]. This progressive spastic paraparesis without remittances is interpreted as an inflammatory disease [Nakagawa et al., 1995; Uchiyama, 1997], and also as a neurodegenerative disease [Cartier et al., 1997, 2007; Liberski et al., 1999]. Nevertheless, the progression of the disease is still unknown [Oh et al., 2006]. Worldwide the majority of infected individuals remain asymptomatic carriers while approximately 0.25–3.0% of those infected develop HAM/TSP [Kaplan et al.,

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1990]. There are no effective ways to prevent the development of HAM/TSP. Glucocorticoids are effective to reduce the motor disability in these patients, probably because of their anti-inflammatory properties [Gotuzzo et al., 2004; Verdonck et al., 2007].

The anti-inflammatory and immunosuppressive effects of the corticoids are widely known, but the anti-spastic action of these drugs is less mentioned [Cartier et al., 1977; Cartier and Verdugo, 1988]. Based on the last effect, corticoids have been used to obtain an effective decrease of spasticity in HAM/TSP patients. The repeated use of corticosteroids allowed the selection of 7.0 mg of rapid and slow action betamethasone in a single-dose monthly as a suitable treatment. This small dose of the drug produces defined improvements, increasing the step-length, speed, gait, and balance in HAM/TSP patients; effects on the control of spastic bladder are seen as well. Additionally, patients in long-term treatment show a slower functional deterioration compared with untreated patients [Cartier, 1998]. As a result of these findings, factors that could be involved in this functional improvement were studied.

In HAM/TSP, natural regulatory T cells (nTreg) characterized by the markers $CD4^{+}CD25^{hi}Foxp3^{+}$ are the main reservoir of the virus in vivo [Yamano et al., 2004, 2005]. These cells have the function to suppress, through cell–cell contact, the response of effector T cells and antigen-presenting cells in chronic diseases, including those caused by retroviruses [Grant et al., 2006; Roncarolo and Gregori, 2008]. Tax is a viral and cellular transcriptional regulator associated with the progression of HAM/TSP in most cases [Uchiyama, 1997; Nagai and Jacobson, 2001]. In vitro studies have demonstrated a decrease in Foxp3 levels in the presence of Tax protein [Yamano et al., 2004]. The mechanism of this regulation and its implication in the progression of HAM/TSP are still unknown [Yamano et al., 2005; Toulza et al., 2008].

The aim of this work was to find reliable molecular markers for the effectiveness of betamethasone treatment. In order to characterize the immunological and neurological effects of betamethasone in HAM/TSP patients, this study was directed to evaluate the variation of cytokine levels and receptors involved in down-regulate the immune-response as well as the neurological improvement reflected in gait commitment. In this study, a clinical improvement of HAM/TSP patients treated with betamethasone is reported. This improvement is associated with a characteristic molecular pattern of immunological and viral markers. A clear inverse relationship between a decrease in Tax viral protein and an increase in nTreg marker Foxp3 in response to treatment was found. Other immunological markers such as IL-10, TGF-β, CTLA-4, and GITR were also studied to estimate the involvement in immuno-modulator processes, but no correlation was found either between markers or between markers with gait improvement.

MATERIALS AND METHODS

Patients and Healthy Control Subjects

All the experiments were performed in compliance with relevant laws and the University of Chile Ethics Committee guidelines and in accordance with the ethical standards of the Declaration of Helsinki. Informed consent was obtained from all individuals. HAM/TSP patients fulfilled criteria of gait commitment according to The World Health Organization. Patients were evaluated clinically before and after therapy. Motor disability was evaluated by neurologists in each patient visit. Motor dysfunction was evaluated on the basis of the Osame's Motor Disability Score, in which motor dysfunction is graded on the scale from 0 (normal gait and running) to 13 (completely bedridden). EDTA-treated blood was obtained from 22 HAM/TSP patients, 8 asymptomatic carriers (Carriers), and 8 healthy non-infected subjects. All patients were treated with systemic betamethasone in a single dose of 3.0 mg of rapid action betamethasone plus 4.0 mg of slow action betamethasone. Pre-treatment samples were obtained immediately before steroid administration and post-treatment samples were collected after 5 days. Table I shows clinical data of HAM/TSP patients before and after treatment. Carriers were used as a reliable infected control group without neurological manifestations, and healthy non-infected donors were used as non-infected controls. Both control groups were not treated with betamethasone. The Carriers group was characterized by absence of motor disability, a male:female ratio of 1:1 and a mean age of 37.3 ± 10.2. The healthy non-infected group was similar but with a mean age of 32.7 ± 13.6.

PBMCs Isolation

Peripheral blood mononuclear cells (PBMCs) were obtained from 10 ml of EDTA-treated blood by Ficoll–Hypaque density gradient centrifugation; they were washed three times with phosphate-buffered saline (PBS). The number of PBMCs collected varied between 7×10^6 and 10×10^6.

Nucleic Acid Isolation and cDNA Synthesis

RNA was isolated with RNeasy kit (Qiagen, Valencia, CA) from PBMCs according to the manufacturer's protocol. Reverse transcription was performed with Taq-Man reverse transcription in a one-step PCR.

Relative Real-Time PCR

cDNA was synthesized for relative quantitation of Tax, CTLA-4, GITR, IL-10, TGF-β, and Foxp3 transcripts, after and before betamethasone treatment of HAM/TSP patients. Samples from Carriers and non-infected healthy donors were also analyzed. Hypoxanthine ribosyltransferase (HPRT) was used as housekeeping gene. We designed Tax, Foxp3, HPRT,

TABLE I. Clinical Data of HAM/TSP Patients Treated With Betamethasone

Patients	Age	Sex	Disease evolution (years)	OMDS before treatment	OMDS after treatment
1	40	F	3	4	3
2	46	F	3	5	4
3	68	F	14	4	3
4	74	F	9	5	4
5	63	F	12	5	4
6	69	F	12	5	4
7	61	M	17	5	4
8	56	M	7	6	5
9	53	M	5	4	3
10	71	F	17	7	6
11	74	F	20	5	4
12	79	M	25	4	2
13	54	M	12	9	8
14	61	F	16	7	7
15	36	F	11	6	4
16	50	F	10	5	4
17	57	F	10	3	2
18	50	M	18	4	3
19	72	F	12	5	4
20	49	F	24	7	6
21	63	F	14	4	3
22	65	F	15	5	4

Each column shows different parameters related with age, sex, and years of disease evolution. Motor disability stage measured as gait commitment was evaluated before treatment and 5 days after betamethasone administration.
OMDS, Osame's Motor Disability Scales.

and GITR primers using AmplifiX 1.4 software based on sequences reported in GeneBank; Tax: forward primer (5′-ATC CCG TGG AGA CTC CTC AA-3′), reverse primer (5′-CCA AAC ACG TAG ACT GGG TAT CC-3′); GITR: forward primer (5′-CGA GGA GTG CTG TTC CGA GT-3′), reverse primer (5′-TGG AAT TCA GGC TGG ACA CAC-3′); Foxp3: forward primer (5′-AAT GGC ACT GAC CAA GGC TTC ATC T-3′), reverse primer (5′-GTG CCT CCG GAC AGC AAA CA-3′); and HPRT: forward primer (5′-TGC TGA GGA TTT GGA AAG GGT GTT-3′), reverse primer (5′-AGC ACA CAG AGG GCT ACA ATG TGA-3′). Primers for CTLA-4, IL-10, and TGF-β were described previously [Boeuf et al., 2005; Schaub et al., 2006]. PCR products were spanning exon–intron borders in order to avoid amplification of contaminant genomic DNA. cDNA was amplified using Brilliant® II SYBR® Green master mix (Stratagene, Agilent Technologies, Wilmington, DE). When possible, amplifications were carried out in duplicates. Analysis of melting curves showed a single pick for each marker amplified, coincident with the size of PCR products analyzed in agarose gels. Relative quantitation was made with the comparative threshold cycle ($\Delta\Delta C_T$) formula with HPRT as endogeneous housekeeping gene. Carriers were used as control group for calculation of $\Delta\Delta C_T$, having previously compared them with healthy non-infected individuals. It was not possible to compare Tax mRNA levels in both groups because of the lack of Tax gene in healthy non-infected individuals. Data were normalized to the average value of Carriers.

Flow Cytometry

PBMCs for flow cytometry were cultured 14 h in RPMI 1640 (Gibco, Paisley, UK) supplemented with 10% fetal bovine serum (Gibco) and 20 nM Concanamycin A (Sigma–Aldrich, St Louis, MO) in order to inhibit the action of $CD8^+$ cytotoxic T lymphocytes. Cells were harvested and stained with fluorophore-conjugated antibodies against the following antigens: CD4-FITC (BD Biosciences, San Jose, CA), Foxp3-PE (BD Biosciences) and Tax-APC, kindly provided by Dr. Yuetsu Tanaka. For nuclear Foxp3 and Tax staining, cells were permeabilized with fixation and permeabilization reagents (eBiosciences, San Diego, CA). Matched isotype controls were used at the same concentration as the respective antibodies. A three-color flow cytometry in a FACS-CANTO instrument (Beckton Dickinson) was performed; WinMDI 2.9 software was used for data analysis. To calculate mean fluorescence intensity (MFI) the MFI software (University of Massachusetts) was used. Mean fluorescence of each marker over $CD4^+$ subpopulation was used to determine the level of Foxp3 and Tax protein. Values of MFI plotted were divided by 100 to keep the same scale range of the mRNA data.

Statistics

Statistical analysis was made with Graph Pad Prisma 5.0. Data were verified for Gaussian distribution. For evaluating betamethasone effects in HAM/TSP patients, data before and after treatment were compared using Wilcoxon-singed rank test. Kruskal–Wallis test was used for calculation of differences between independent groups. Data are shown as mean ± SD. The Pearson's correlation was used to evaluate relationship between mRNA and protein levels and between cell populations percentage. Differences in *P*-values of 0.05 or less were considered significant.

RESULTS

Effects of Betamethasone in Relative Expression of Foxp3 and Tax mRNA in PBMCs of HAM/TSP Patients

A significant increase in the relative amounts of Foxp3 mRNA in HAM/TSP patients treated with systemic betamethasone was observed, compared with pre-treatment samples from the same individuals. There was also a statistical difference between amounts of Foxp3 mRNA from pre-treatment patients and Carriers, but not between healthy non-infected individuals and Carriers, or post-treatment samples (Fig. 1A). Samples from 21 HAM/TSP patients treated with betamethasone showed significant decreases in Tax mRNA compared with those from pre-treatment controls (Fig. 1B). Pre-treatment samples of HAM/

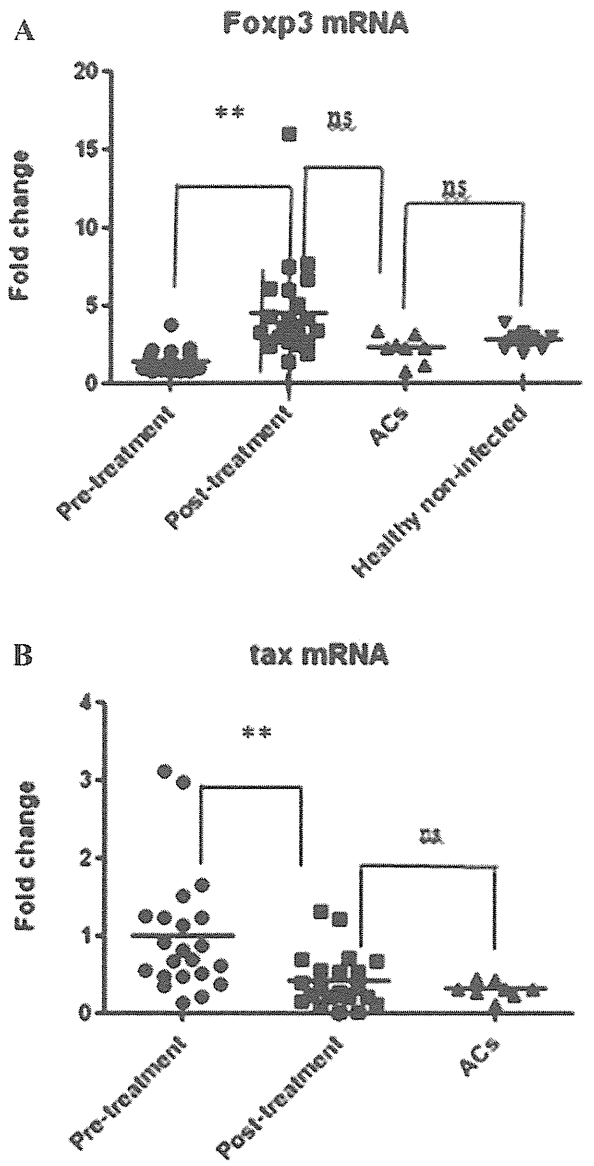

Fig. 1. Fold change of Foxp3 and tax mRNA in HAM/TSP patients treated with betamethasone. **A**: Patients treated with betamethasone showed significant differences in Foxp3 mRNA amounts (4.315 ± 0.73) compared to their pre-treatment condition (1.368 ± 0.16; $^{**}P < 0.0032$). Carriers did not reveal significant differences compared to healthy non-infected controls, as well as with post-treatment samples. **B**: Betamethasone produced a decrease in relative amounts of tax mRNA in HAM/TSP patients (0.469 ± 0.09), in relation to pre-treatment samples (1.039 ± 0.18; $^{**}P < 0.0045$). Relative quantitation of mRNA in Carriers (0.308 ± 0.038) did not show significant differences with post-treatment samples.

TSP showed significant increases compared with those from Carriers. Furthermore, post-treatment HAM/TSP did not show differences from the carriers group. Thus, betamethasone decreased Tax mRNA close to the amounts seen in Carriers. Only patient 14 did not show changes in Tax mRNA levels before and after treatment. Tax mRNA levels were low and similar with those from Carriers.

No significant differences were found in the amounts of mRNA of the immunological markers CTLA-4, GITR, IL-10, and TGF-β in PBMCs from HAM/TSP patients before and after betamethasone treatment, compared with Carriers. There were no statistical differences between these two groups in all analyzed markers (data not shown).

Effect of Betamethasone Treatment in $CD4^+Foxp3^+$ and $CD4^+Tax^+$ Cell Population in HAM/TSP Patients

Betamethasone treatment led to a significant increase in the $CD4^+Foxp3^+$ cell population in 21 HAM/TSP patients compared with pre-treatment controls (Fig. 2A and B). Patient 14 showed a decrease from 2.5 to 1.9% in CD4 $^+Foxp3^+$ cells in pre-treatment and post-treatment samples, respectively. However, the relative amount of Foxp3 mRNA remained similar before and after treatment. No significant differences between the percentages of $CD4^+Foxp3^+$ in carriers, healthy non-infected and HAM/TSP patients post-treatment were observed. Inverse results in the $CD4^+Tax^+$ cell population in response to betamethasone compared to those of $CD4^+Foxp3^+$ cells were found. There was a significant decrease in the percentage of $CD4^+Tax^+$in 21 HAM/TSP-treated patients compared with non-treated controls (Fig. 3A and B). Only patient 14 did not show changes in the percentage of $CD4^+Tax^+$ cells, with similar levels as those of asymptomatic carriers characterized by very low levels of $CD4^+Tax^+$. A comparison of this cell population between Carriers and HAM/TSP-treated patients did not show statistically significant differences. A correlation between $CD4^+Foxp3^+$ and $CD4^+Tax^+$ population before treatment did not show a statistically significant result (data not shown). When data of both cell populations after betamethasone treatment were plotted together, the result was a negative and statistically significant correlation (Pearson's $r = -0.56$; Fig. 4A).

Correlation of mRNA Expression and Protein Levels of Foxp3 and Tax

To support the findings at the mRNA level related with Foxp3 and Tax levels, a correlation between protein and mRNA for each marker was made. Foxp3 and Tax showed both a positive and statistically significant correlation between mRNA expression and protein levels in HAM/TSP patients before and after treatment (Fig. 4B and C). Tax and Foxp3 mRNA of PBMCs from patients before treatment did not show a statistically significant correlation. Instead, the evaluation of Tax and Foxp3 mRNA after the treatment revealed a significant negative correlation with a Pearson's $r = -0.55$ (data not shown).

DISCUSSION

Effective treatment strategies for HAM/TSP are still a challenge and clinical studies available are not enough to support their therapeutic effects. Most

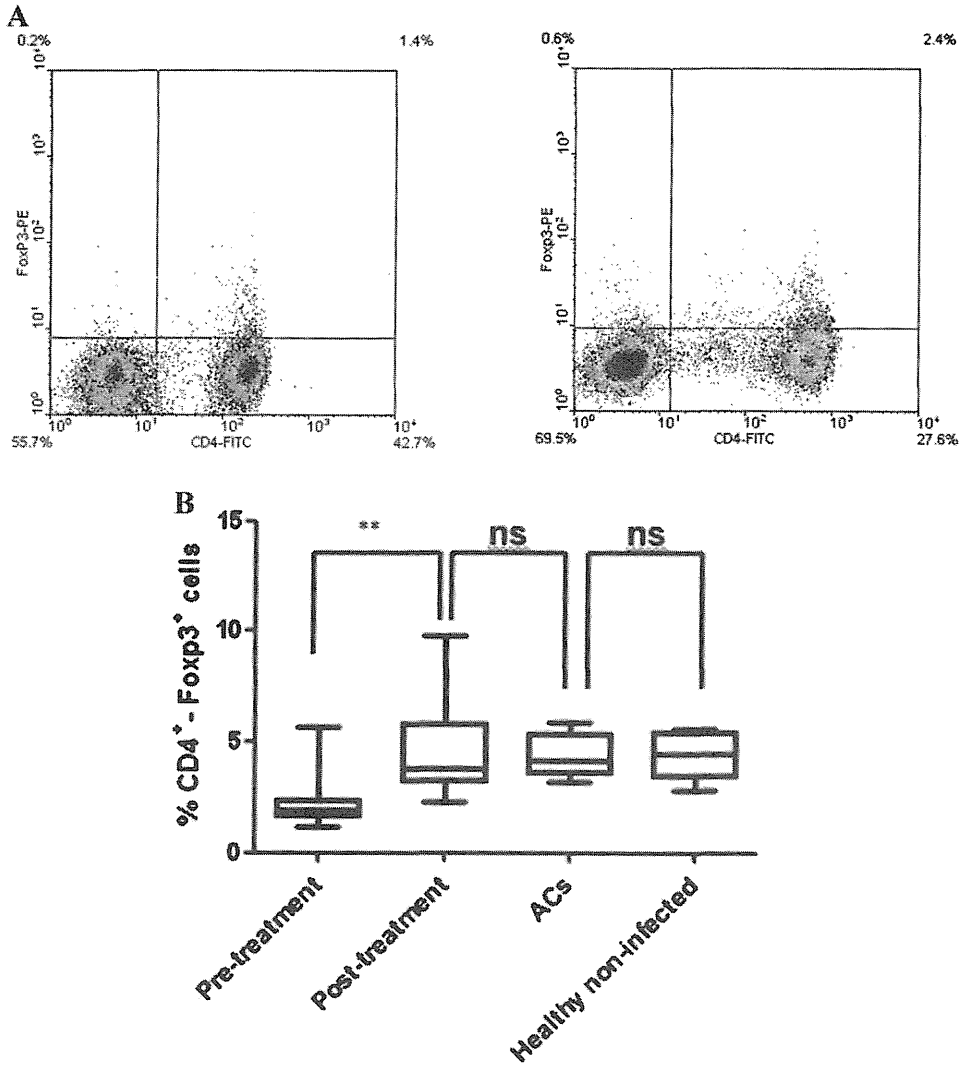

Fig. 2. Flow cytometry analysis of $CD4^+Foxp3^+$ population in HAM/TSP patients treated with betamethasone. Percentage of Treg population was obtained from PBMCs collected from HAM/TSP patients before treatment and 5 days after betamethasone administration. Data were also compared with PBMCs from Carriers and healthy non-infected controls. **A**: Dot-plot representation of $CD4^+Foxp3^+$ population obtained from a pre-treatment sample (*left*) and post-treatment sample (*right*). **B**: Comparison of results from different groups analyzed. Significant differences were found between pre-treatment (2.33 ± 1.24) and post-treatment (5.01 ± 2.24) conditions ($^{**}P < 0.0036$). Post-treatment patients did not show significant differences compared to Carriers (4.34 ± 1.01) and healthy non-infected controls (4.44 ± 1.03).

therapies are symptomatic, being focused on reducing the inflammatory response in affected tissues. Interferon-α (IFN-α), which has both cytostatic and antiviral activity has been used as a potential therapy for HAM/TSP, but with modest results in reducing HTLV-I proviral load [Izumo et al., 1996; Nakagawa et al., 1996; Feng et al., 2003, 2004]. This decrease would be associated with changes in the number of $CD8^+$ T cells [Saito et al., 2004]. Alternative treatments such as oral prednisolone, intrathecal hydrocortisone [Kira et al., 1991; Araujo et al., 1995; Nakagawa et al., 1996], plasmapheresis, and intravenous gammaglobulin have been used [Matsuo et al., 1988; Kuroda et al., 1991; Gold et al., 2007]. Nevertheless, they have not shown clear beneficial effects. The use of antiretroviral drugs including zidovudine and lamivudine has not reported clinically significant changes in 16 patients in a randomized, double-blind study [Taylor et al., 2006]. Even though other kinds of glucocorticoids have been used in HAM/TSP, the advantages of the treatment presented in this work are based on the following characteristics: (a) betamethasone has no interaction with mineralocorticoid receptors, thus there is a higher concentration interacting with glucocorticoid receptors compared with other drugs [Habib and Safia, 2009]; (b) the equivalent dosage compared with other glucocorticoids is eight times less; (c) patients treated with

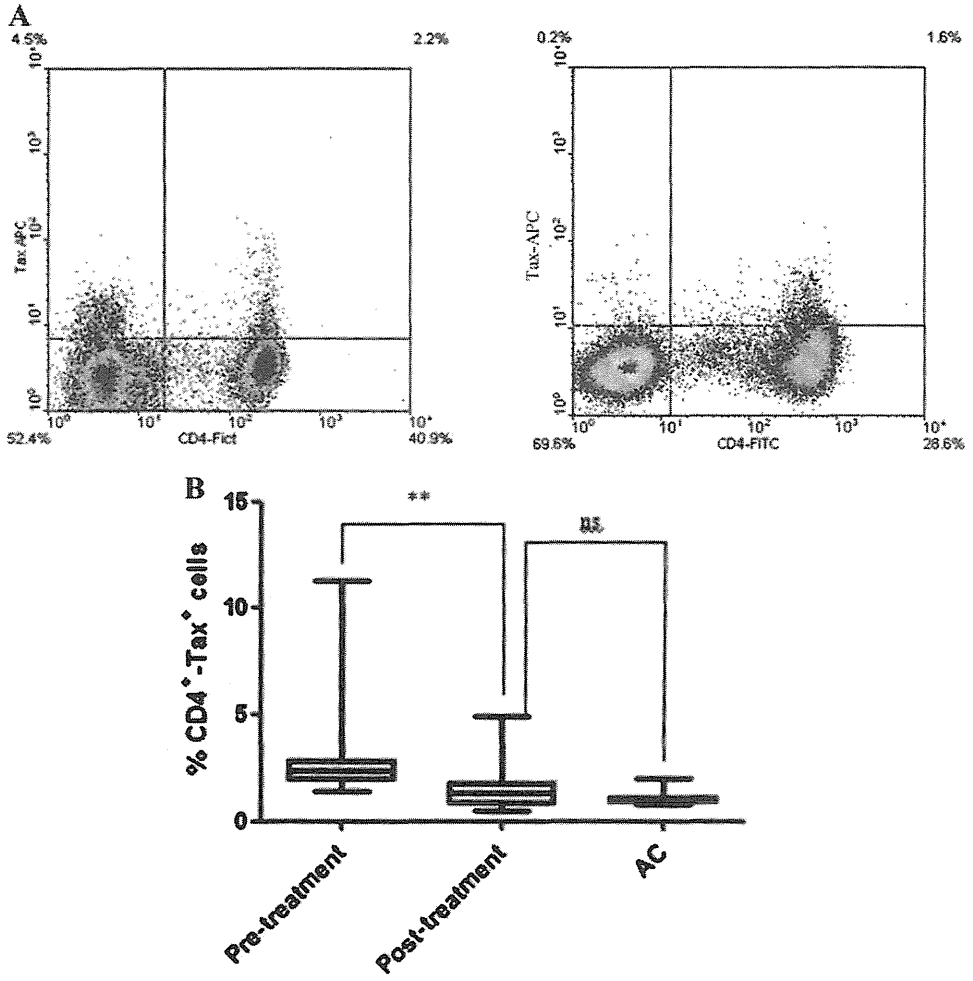

Fig. 3. Quantitation of $CD4^+Tax^+$ population in HAM/TSP patients under betamethasone treatment. PBMCs obtained from HAM/TSP patients before and after treatment as well as from Carriers, were used to quantify $CD4^+Tax^+$ cells and to evaluate the effect of systemic betamethasone in this population. We used Carriers as control group, considering that their $CD4^+Tax^+$ levels are very low and manifestation of motor disability are absent in this individuals. **A**: Dot-plot analysis of a pre-treatment sample (*left*) and post-treatment sample (*right*) highlighting the percentage of $CD4^+Tax^+$ cells. **B**: Results show a statistical difference between pre-treatment (3.03 ± 2.79) and post-treatment (1.43 ± 1.23) conditions ($^{**}P < 0.0038$). No statistical differences were observed when post-treatment and Carriers individuals were compared.

betamethasone do not develop hypertension and features related with this condition; (d) betamethasone has the longest half-life compared with other glucocorticoids; (e) the drug formulation with phosphate and acetate salts increases its bioavailability; (f) a single unique dose is effective for ameliorate disease symptoms; (g) patients do not show secondary effects related with corticoid usage like polyuria, polydipsia, and polyphagia (NIH Clinical Trials). These data were obtained from experimental database (http://www.cancer.gov/Search/ClinicalTrialsLink.aspx?id=39273&idtype=1" active clinical trials) and from clinical observation of patients in this study.

The action of glucocorticoids as immune-modulators in chronic diseases by increasing Foxp3 is well documented [Karagiannidis et al., 2004; Braitch et al., 2009]. It has been suggested that HAM/TSP patients present a reduced Foxp3-dependent suppression capacity [Grant et al., 2008]. This study showed that HAM/TSP patients treated with betamethasone exhibit an increase in Foxp3 mRNA and $CD4^+Foxp3^+$ T cell population at levels comparable with those of non-infected individuals and Carriers. It remains to establish if glucocorticoids-dependent Foxp3 up-regulation leads to a recovery in Treg function compared to Treg obtained from pre-treated HAM/TSP patients. Foxp3 regulatory properties only become active if the immunological environment lacks of danger signals or immune responses are exhausted [Karagiannidis et al., 2004]. Thus, the relatively high levels of pro-inflammatory cytokines or inflammatory-signals present in HAM/TSP might explain the Treg response observed in these patients. A positive correlation was observed between the mRNA expression

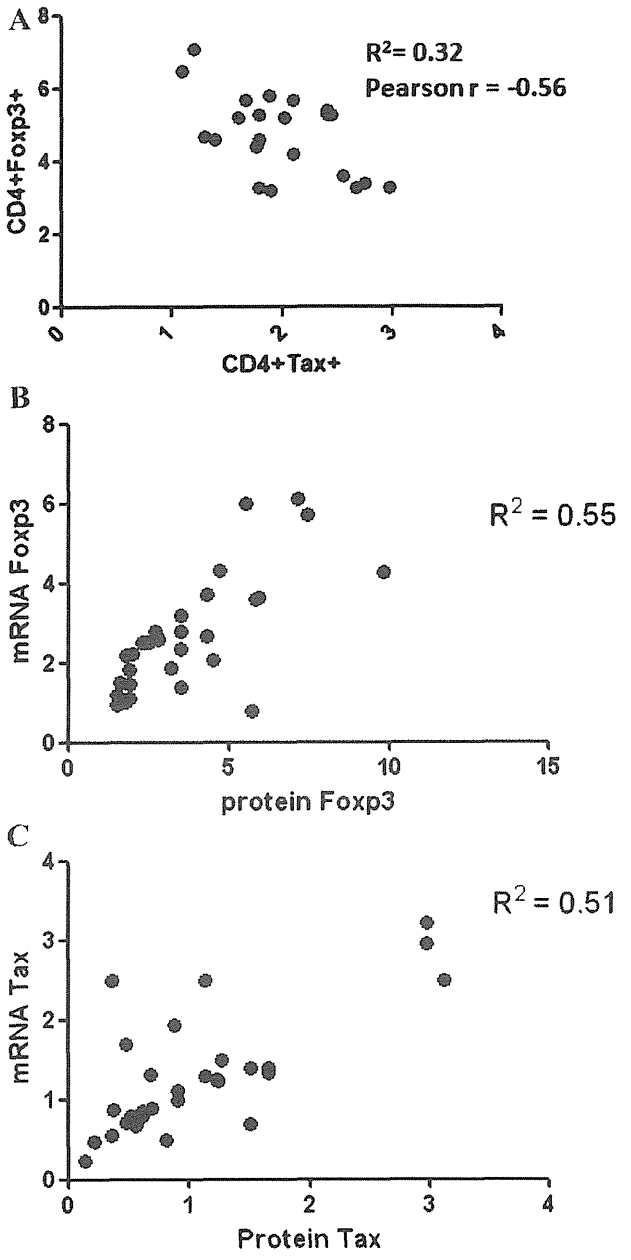

Fig. 4. Correlation between Foxp3 and Tax in HAM/TSP patients. A correlation between $CD4^+Tax^+$ and $CD4^+Foxp3^+$ post-treatment percentage is shown. Data obtained from pre-treatment and post-treatment samples were plotted together in a correlation graph of mRNA and protein levels as well. **A**: 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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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	Clinical Presentation	PBMC	HLA-A*02
		(years)		(cps/1000)	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)

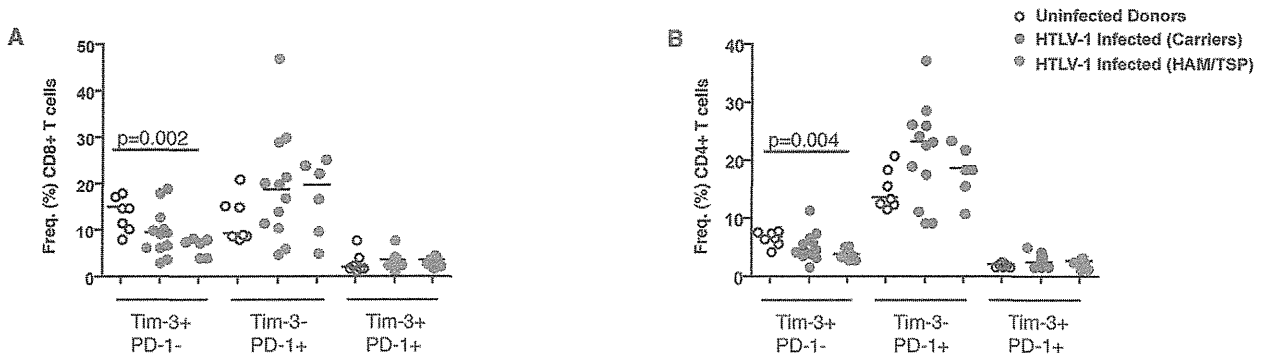


Figure 1. Tim-3 expression on T cells in HTLV-1 infection. Graphs show the frequencies of co-expression of Tim-3 and PD-1 on (A) CD8+ (left), and (B) CD4+ (right), T cells as assessed by multiparametric flow cytometry from PBMCs derived 18 HTLV-1 seropositive (12 asymptomatic and 6 with diagnosis of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

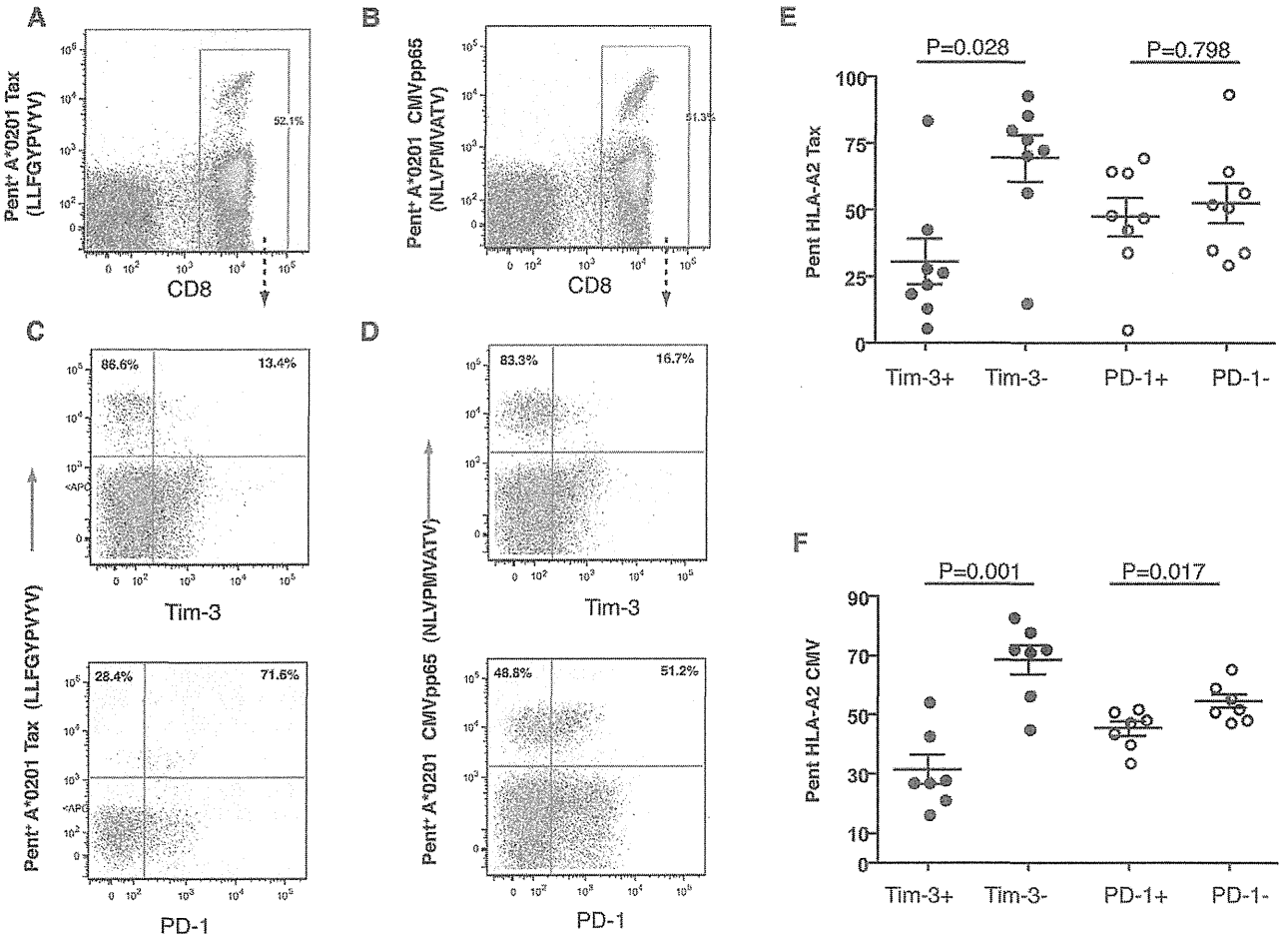


Figure 2. Tim-3 expression on HTLV-1-specific CD8+ T cells in HTLV-1 infection. PBMC from HLA-A*02+ chronically HTLV-1 infected individuals were stained with matched HLA pentamers presenting CMV and HTLV-1 epitopes, and with an anti-Tim-3 antibody. Shown are representative flow cytometry data from one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

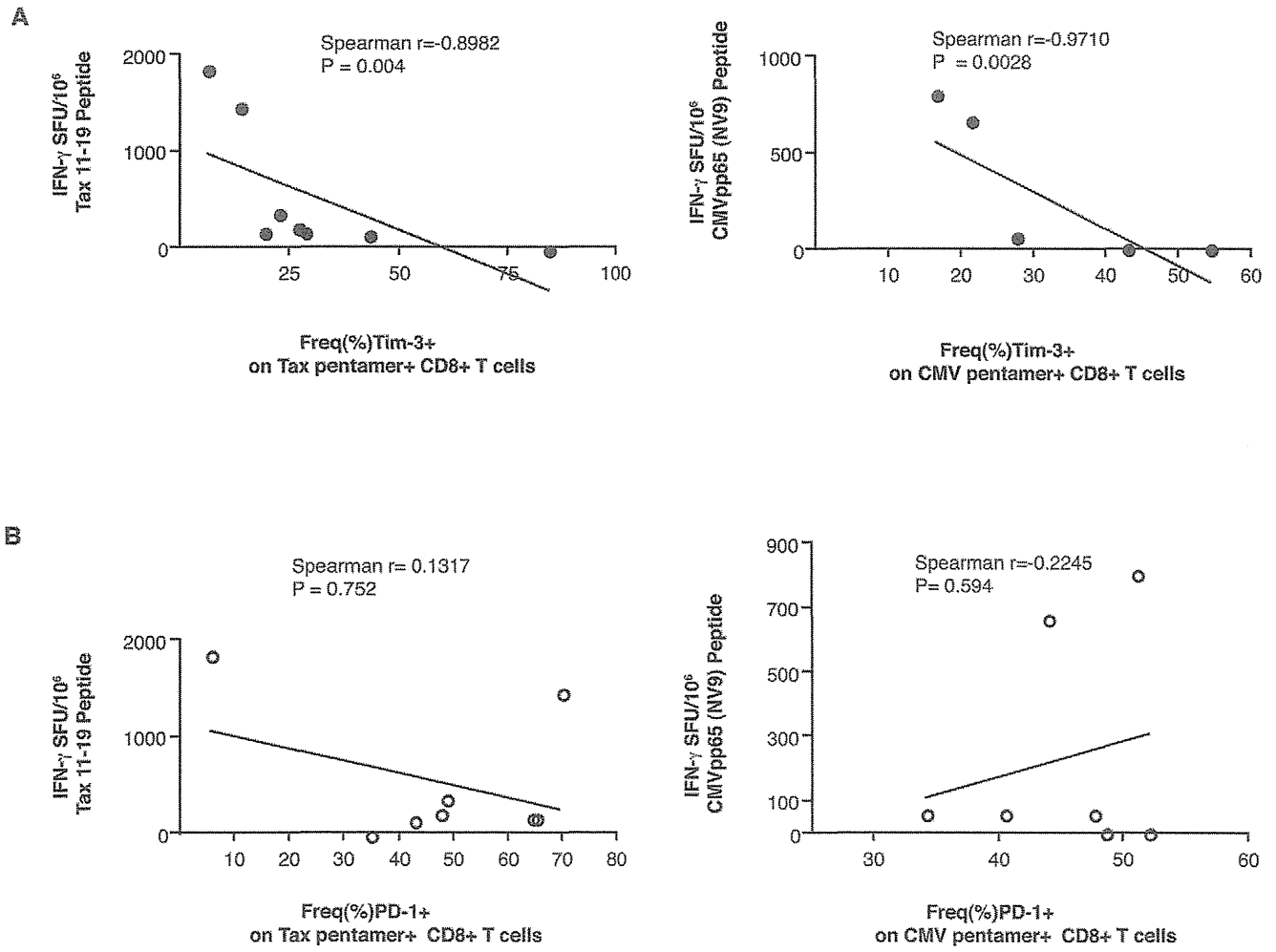
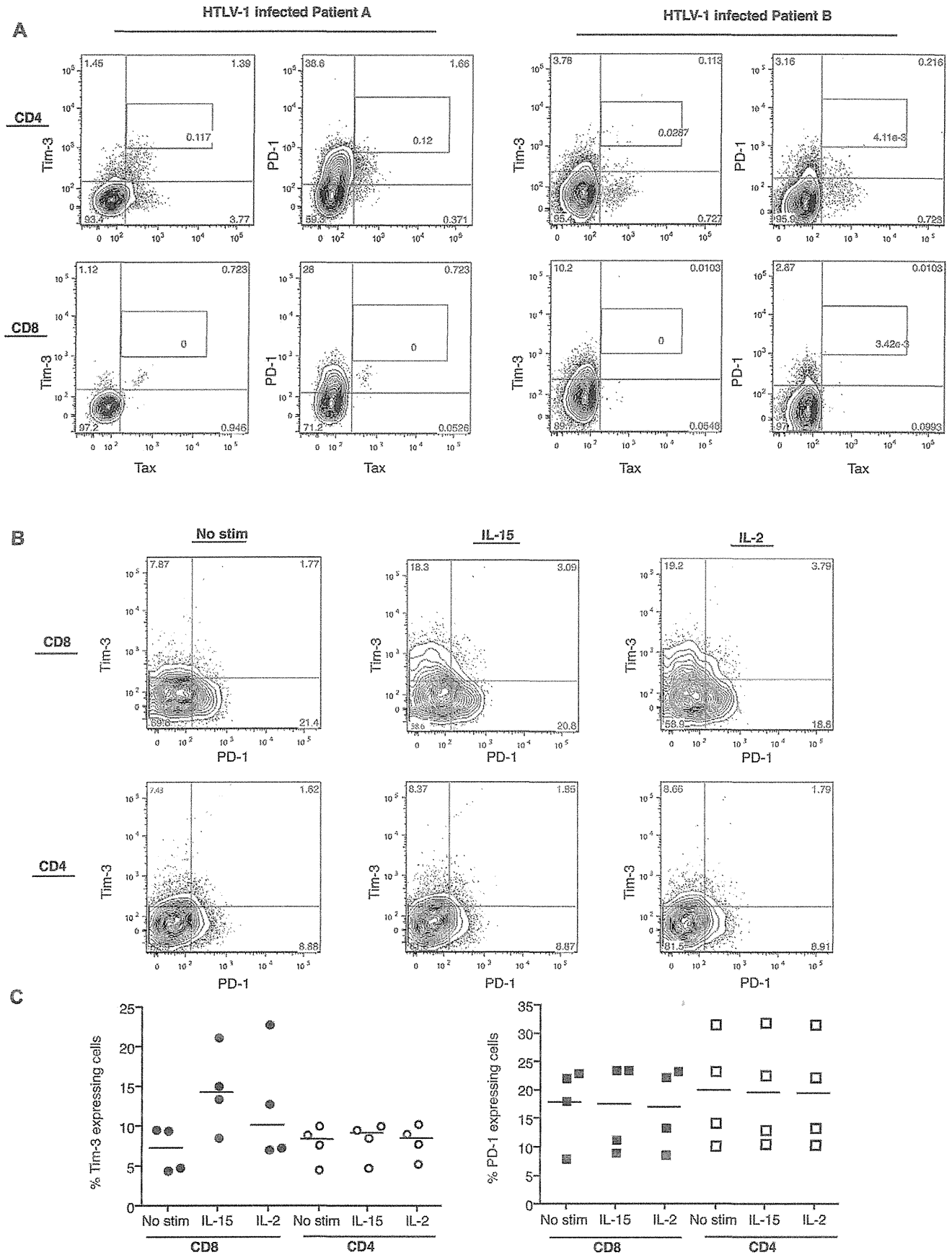


Figure 3. Association of Tax specific CD8+ T cells with effector responses. The graphs show the association between the frequency of Tim-3 (A) and PD-1 (B) expression on HLA-A*02 restricted Tax11-19 or CMV pp65 specific CD8+ T cells with the number of IFN-γ secreting cells (SFU/10^6) in response to Tax 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
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
95.4
0.727
3.16
0.216
4.11e-3
95.9
0.728
1.12
0.723
0
97.2
0.946
28
0.723
0
71.2
0.0526
10.2
0.0103
0
89
0.0646
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