High frequency of CD4⁺FoxP3⁺ cells in HTLV-1 infection:
inverse correlation with HTLV-1-specific CTL response

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Running Title:
FoxP3⁺ cells and the anti-HTLV-1 CTL response
Abstract:

Evidence from population genetics, gene expression microarrays and assays of ex vivo T cell function indicates that the cytotoxic T lymphocyte (CTL) response to human T lymphotropic virus Type 1 (HTLV-1) controls the level of HTLV-1 expression and the proviral load. The rate at which CTLs kill autologous HTLV-1-infected lymphocytes differs significantly among infected people, but the reasons for such variation are unknown. Here, we demonstrate a strong negative correlation between the frequency of CD4⁺ FoxP3⁺ Tax⁻ Tregs in the circulation and the rate of CTL-mediated lysis of autologous HTLV-1-infected cells ex vivo. We propose that the frequency of CD4⁺ FoxP3⁺ Tax⁻ Tregs is one of the chief determinants of the efficiency of T cell mediated immune control of HTLV-1.
Introduction:

Human T-Lymphotropic Virus Type 1 (HTLV-1) is a persistent retrovirus that infects 10 to 20 million people worldwide. The majority of infected individuals remain lifelong asymptomatic carriers (ACs) of the virus. However, 2-3% of infected individuals develop a progressive inflammation of the central nervous system called HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). HTLV-1 causes leukaemia or lymphoma in about 4% of seropositive individuals.

HTLV-1 encodes a number of regulatory proteins, the most studied of which is Tax. Tax is a pleiotropic transcriptional transactivator and is central to the HTLV-1 life cycle. Tax increases the expression of many cellular genes, including several genes involved in T cell activation and proliferation. Tax expression is thus promitotic and drives CD4 T cell proliferation to increase HTLV-1 proviral load. Tax is also the immunodominant HTLV-1 antigen recognised by the strong CD8 cytotoxic T cell (CTL) response. The host cellular immune response counteracts this Tax-mediated proliferation and increase in proviral load. The level of spontaneous HTLV-1 gene expression in naturally infected PBMCs during short term culture can be increased if CD8 cells are removed. In this study we used Tax protein as a marker of HTLV-1 expression in naturally infected PBMCs.

The steady state HTLV-1 proviral load in vivo is therefore likely to be determined by proliferation of infected cells balanced by the clearance of productively infected cells by CD8 cells.
Because the immune response plays a major role in the control of HTLV-1 infection it is important to study the possible role of immune regulation in the control of HTLV-1 infection. One of the major components of immune regulation is the regulatory T cell (T<sub>reg</sub>)<sup>15,16</sup>. T<sub>reg</sub> cells are specialized subsets of CD<sup>4+</sup> T cells which suppress effector T cell responses in chronic disease, including retroviral infection<sup>17,18</sup>. T<sub>reg</sub> cells can suppress the function of APCs, CD<sup>4+</sup> and CD<sup>8+</sup> effector T cells<sup>16</sup> by cell-cell contact or by the secretion of cytokines such as IL-10 and TGF-β<sub>1</sub>.

Identification of T<sub>reg</sub> cells by flow cytometry remains ambiguous, because more than one subpopulation of T cell is capable of suppressive function<sup>19</sup>, and because most markers of T<sub>reg</sub> proposed until now are also expressed by activated CD<sup>4+</sup> T lymphocytes: CD25, CTLA-4, GITR and CD103<sup>20,21</sup>. The significance of CD25<sup>hi</sup> expression, which has been widely used as part of a phenotypic definition of T<sub>reg</sub> cells (CD<sup>4+</sup> CD25<sup>hi</sup> FoxP3<sup>+</sup>), is especially uncertain in HTLV-1 infection because CD25 expression is strongly induced by the HTLV-1 Tax protein<sup>22,23</sup>. Recently it was reported that the absence of expression of CD127 on CD<sup>4+</sup> cells can be used as a marker of T<sub>reg</sub><sup>24</sup>. It appears that the best current single marker of T<sub>reg</sub> in CD4<sup>+</sup> cells in the human, as in the mouse, is the forkhead transcription factor FoxP3, and the phenotype CD4<sup>+</sup> FoxP3<sup>+</sup> is now increasingly used to identify a major population of T<sub>reg</sub><sup>15</sup>.

In this study, we tested the hypothesis that the efficiency of the CTL response to HTLV-1-infected cells<sup>25</sup> correlates with the frequency of CD4<sup>+</sup> FoxP3<sup>+</sup> cells. We conclude that the CD4<sup>+</sup> FoxP3<sup>+</sup> Tax<sup>-</sup> cell population is a major determinant of the efficiency of immune control of HTLV-1 infection.
**Materials and Methods**

**Cells, Cultures.** PBMCs were isolated by density centrifugation on Histopaque (Sigma, UK) from EDTA-anticoagulated blood samples taken from HTLV-1 infected individuals. All individuals attended the HTLV-1 clinic at St Mary’s Hospital, London and gave informed consent. This study was approved by St. Mary’s NHS Trust Local Research Ethics committee. PBMC were cryopreserved and thawed when required. Cells were cultured in complete medium (RPMI-1640, 10% FCS, pen/strep L-glutamine at 37°C, 5% CO2 for 18 hours. If CD8+ cell-depleted PBMCs were required, CD8+ cells were removed by positive selection using magnetic microbeads following the manufacturer’s instructions (Miltenyi Biotec, Surrey, UK). The median CD8+ cell depletion achieved was 93% (range 84 to 98%).

**Flow cytometry** To detect Tax expression and FoxP3 in HTLV-1 infected cells, whole PBMCs or CD8+ cell-depleted PBMCs were incubated for 18 hours. The cells were then surface-stained with monoclonal antibodies to CD4 and CD8 (each at 15μg/ml; Beckman Coulter, Marseille, France). Cells were then fixed and permeabilised with a commercial kit (Insight Biotechnology, Wembley, UK), following the manufacturer’s protocol. Finally, cells were stained intracellularly with the FITC conjugated antibody anti-Tax protein Lt-426 diluted 1/100 and anti-human FoxP3-PE antibody (clone 236A/E7; Insight Biotechnology) in Permeabilization buffer (Insight Biotechnology) following the manufacturer’s protocol. Following staining, cells were analysed on a Coulter Epics XL flow cytometer. Thirty thousand events were routinely collected. Viable lymphocytes were gated for closer analysis using Expo32 analysis software (Beckman Coulter).
**Proviral Load measurement.** DNA was extracted from PBMCs following the manufacturer’s protocol (Qiagen, DNeasy Tissue Kit, Crawley, UK) and eluted in PCR grade H₂O. Three dilutions of eluted DNA (1:4, 1:8, 1:16) were amplified for HTLV-1 DNA (Tax specific primers as in²⁷) and β-actin by real time quantitative PCR in a Roche light cycler using SYBR®Green 1 Dye incorporation. Incorporation was detected at 85°C at the end of each of 45 amplification cycles. Standard curves were generated using DNA from the TARL-2 cell line which carries a single HTLV-1 provirus copy per cell²⁸. The sample copy number was estimated by interpolation from the standard curve, calculated as an average of the three dilutions and expressed as the proportion of PBMCs infected.

**FoxP3 mRNA measurement.** CD4⁺ cells were positively selected from PBMCs on a MACS column (Miltenyi Biotec) using magnetic microbeads according to the manufacturer’s instructions. Purity was confirmed by flow cytometry to be >90% in most cases. Total RNA was extracted from freshly isolated cells using the RNeasy Method (Qiagen). RNA was reverse-transcribed using an HPLC-purified oligo(dT) primer with a T7 RNA pol site attached to the 5' end (Transgenomic Bioconsumables, Glasgow, UK), with the SuperScript Double-Stranded cDNA Synthesis kit (Invitrogen Life Technologies, Paisley, UK). BIRC2 was chosen as a housekeeping gene because it exhibited the most uniform expression across all patient samples in the microarray data. HTLV-1 tax mRNA expression was assayed using the same method, except that cDNA from the tax⁺ cell line MT2 was used to generate the standard curve²⁹. Gene-specific primers for the constitutively expressed (“housekeeping”) gene BIRC₂ were designed using sequences deposited in GenBank and obtained from Invitrogen Life Technologies. The following primer sets used were: FoxP3-for, 5'- TCC CAG
AGT TCC TCC ACA AC -3’ and FoxP3-rev, 5’- ATT GAG TGG TGT CCG CTG CTT CT -3’. Real-time kinetic PCR was performed on a LightCycler (Roche Diagnostics, Burgess Hill, UK) using the LightCycler FastStart DNA Master SYBR Green I kit as recommended by the manufacturer.

**CD8⁺ Cell Lytic Efficiency Assay.** The rate (or “efficiency”) of CD8⁺ cell-mediated lysis of HTLV-1-infected cells was estimated as recently described²⁵. PBMC were thawed, washed, and then CD8⁺ cells were positively selected (as described above) and titrated back into the CD8⁻-depleted fraction at CD8⁺:CD8⁻ ratios above, below and including the physiological ratio for that individual. Cells were then co-cultured at 37°C for 18 hours, harvested, and stained for Tax, FoxP3, CD4 and CD8 as described above. The proportion of Tax⁺CD4⁺ cells surviving co-culture was plotted against the proportion of CD8⁺ cells present and a mathematical model²⁵ was then fitted to the data. CD8⁺ cell lytic efficiency (expressed as the proportion of Tax expressing CD4⁺ cells killed per CD8⁺ cell per day) was calculated for each HTLV-1 infected individual tested. All assays were done in duplicate and the results are presented as the mean CD8⁺ cell lytic efficiency (% CD4⁺ Tax⁺ cells killed/CD8⁺ cell/day). The results of a typical assay are shown in supplemental Figure 1A.

**Statistical analysis.** Non-parametric statistical tests were used as appropriate taking the null hypothesis and the sample size into account. The Spearman rank-order correlation coefficient was calculated when the significance of observed changes in two parameters across all HTLV-1 infected individuals was tested. The measure of rate of lysis was calculate with the software SPSS 12-0 for Windows.
**Quantification of cytokine concentrations.** Using Luminex multi-bead antibody kit technology (Millipore, Southampton, UK), we quantified the concentration of cytokines in supernatant of PBMC after 18h of incubation at the physiological ratio of CD4⁺:CD8⁺ cells point (with no depletion of CD8). The supernatant was centrifuged at 10,000 rpm for 3 min to remove all debris and cells, and frozen at -80°C. For detection of TGF-β1 the supernatant was first acidified, according to the manufacturer’s indications.

For each patient we also quantified the concentration of the same cytokines in plasma, taken at the time of purification of PBMC. The detection limits of these cytokines were 12-14 pg/ml.
**Results**

**FoxP3 expression in HTLV-1 Patients**

Flow cytometry analysis of samples from 8 patients with HAM/TSP confirmed that CD4$^+$ Tax$^+$ cells were also CD25$^+$ (Supplemental Figure 1C). This result has been previously described$^{30}$, and suggests that it is not appropriate to use high CD25 expression as a marker of T$_{regs}$ in HTLV-1 infection. For this reason we chose to characterize T$_{regs}$ by the expression of FoxP3 in CD4$^+$ T cells.

In each HTLV-1 seropositive patient we measured the frequency of FoxP3 expression in CD4$^+$ cells after 18h of incubation in vitro at the physiological ratio of CD4$^+$:CD8$^+$ cells. In Figure 1A we show the expression of FoxP3 in total CD4$^+$ T cells from 7 uninfected patients and 58 HTLV-1 seropositive patients (28 ACs and 30 patients with HAM/TSP). The results show a significant difference in the frequency of expression of FoxP3 between HTLV-1-infected individuals (ACs and HAM/TSP patients combined) and uninfected patients. But there was no significant difference in the frequency of FoxP3 expression between AC and HAM/TSP patients (P=0.158).

Expression of the IL-7 receptor (CD127) was recently reported to be downregulated in CD25$^{hi}$ FoxP3$^+$ regulatory T cells$^{24}$. Flow cytometry analysis confirmed that all CD4$^+$ FoxP3$^+$ were also CD127$^{low}$ (Figure 1B).

In PBMCs from 8 HTLV-1-infected individuals (4 ACs and 4 HAM/TSP) we measured the expression of FoxP3 in CD4$^+$ T cells during in vitro incubation for 18h (Figure 1C). The results showed no significant variation in mean FoxP3 expression between 0h and 18h, although FoxP3 rose between 0 and 2h in two patients with HAM/TSP.
**HTLV-1 infection in FoxP3⁺ T cells**

A typical expression of FoxP3 and Tax in CD4⁺ T cells from an HTLV-1 infected patient is shown in Figure 2A.

FoxP3 was expressed in both Tax⁺ and Tax⁻ populations. The frequency of FoxP3 expression was higher in Tax⁺ CD4⁺ T cells than in Tax⁻ CD4⁺ T cells (supplemental Figure 2), but the population of FoxP3⁺ Tax⁺ cells was small (<2% of CD4⁺ T cells) compared with the site of FoxP3⁺ Tax⁻ population (≤ 14% of CD4⁺ T cells) (Figure 2B).

Finally, we also separated the different populations expressing FoxP3 by flow sorting and isolated the Tax⁻ FoxP3⁺ population (Figure 2C, gate “b”). By Q-PCR we then measured the proviral load in 3 independent experiments in the Tax⁻ FoxP3⁺ population and in the total CD4⁺ T cell population (Figure 2C, gate “a”). The results (Figure 2C) show that the proviral load was lower in the population of CD4⁺ FoxP3⁺ Tax⁻ cells than in the total CD4⁺ population. This result suggests that HTLV-1 does not preferentially infect FoxP3⁺ T cells, and that the majority of FoxP3⁺ Tax⁻ CD4⁺ T cells do not carry the provirus of HTLV-1.

**Correlation between the rate of lysis and percentage of CD4⁺ FoxP3⁺ cells**

We previously described a method²₅ to quantify the capacity of CD8⁺ cells to kill autologous CD4⁺ Tax⁺ cells in vitro. Supplemental Figure 1A represents the results of typical experiments showing Tax and FoxP3 expression in samples from two HAM/TSP patients (codes TAT and TBS). Each quadrant shows the percentage of Tax⁺ and FoxP3⁺ cells in the CD4⁺ population, for an increasing frequency of CD8⁺ cells. The point marked “Normal” corresponds to the physiological frequency of CD8⁺ cells for that patient. The regression curve is calculated using a mathematical
model, and this permits the determination of the per-cell rate or “efficiency” of lysis of CD4+ Tax+ cells by CD8+ cells, denoted epsilon (ε). In this example the patient with HAM/TSP, coded TAT, had a lower rate of lysis (ε = 0.035) than the patient TBS (ε = 0.184). Note that the patient with a low rate of lysis had a high percentage of CD4+ FoxP3+ cells, and conversely the patient with a high rate of lysis had a low percentage of CD4+ FoxP3+ cells.

We then wished to test the hypothesis that the percentage of CD4+ FoxP3+ cells correlates with the rate of lysis of CD4+ Tax+ cells in vitro. The results (Figure 3A) revealed a significant negative correlation between the rate of lysis and the percentage of CD4+ FoxP3+ cells in patients infected with HTLV-1 (ACs alone, p<0.001; HAM/TSP patients alone, p<0.002; ACs and HAM/TSP patients combined, p<0.001). As previously observed25 the range of variation of lysis rate was similar in ACs and HAM/TSP patients.

To evaluate the influence of FoxP3 expression in Tax+ cells and in Tax- cells respectively in determining the efficiency of lysis, we compared the correlation between the efficiency of lysis and the percentage of CD4+ FoxP3+Tax- cells (Figure 3B) and CD4+ FoxP3+Tax+ cells (Figure 3C). As described above, the percentage of CD4+ FoxP3+ Tax+ cells was consistently lower than the percentage of CD4+ FoxP3+ Tax- cells.

The results showed that the percentage of CD4+ FoxP3+ Tax+ population did not correlate with the rate of lysis (ACs alone: p<0.2; HAM/TSP patients alone: p<0.2; ACs and HAM/TSP patients combined: p<0.5 figure 3C). However, there was a strong negative correlation between the rate of lysis and the percentage of CD4+ FoxP3+ in the Tax- population. Furthermore, the rate of lysis in HAM/TSP patients correlated more strongly with the frequency of FoxP3 expression in Tax- cells (R² =
0.747; \( P < 0.001 \) than with the frequency of FoxP3 expression in all CD4\(^+\) cells (Tax\(^+\) and Tax\(^-\) combined) (\( R^2 = 0.658; P < 0.002 \) ) (Figure 3A, 3C). There was an even stronger correlation between the rate of lysis and \( \log_{10} (\% \text{FoxP3}^+\text{CD4}^+\text{Tax}^-) \) with the rate of lysis (\( R^2 = 0.911 \)).

**CD4\(^+\) FoxP3\(^+\) correlate with Tax expression and proviral load**

In previous work we concluded that the rate of CD8\(^+\) cell-mediated lysis controls the rate of Tax expression and so the proviral load of HTLV-1\(^8,25\). Because the expression of FoxP3 correlates with the rate of lysis, we wished to determine the impact of FoxP3 expression on both Tax expression and the proviral load. We observed (Figure 4A) a correlation between the percentage of CD4\(^+\) FoxP3\(^+\) Tax\(^-\) cells and the percentage of CD4\(^+\) cells that expressed Tax. This correlation was statistically significant in both ACs and HAM/TSP patients. However, the two respective correlations were distinct (Figure 4A). That is, at a given frequency of FoxP3 expression, the level of Tax expression was systematically higher in HAM/TSP patients than in ACs (see supplemental Figure 1B). This is similar to our previous observation that Tax expression at a given proviral load was systematically greater in HAM/TSP patients than in ACs\(^8\). These results confirm the importance of CD4\(^+\) FoxP3\(^+\) cells in the control of Tax expression and in particular the importance of CD4\(^+\) FoxP3\(^+\) Tax\(^-\) cells.

By quantitative PCR, we measured the level of expression of FoxP3 in CD4\(^+\) cells and correlated it with the proviral load (Figure 4B). The results show a significant positive correlation between FoxP3 expression and the proviral load and, as observed with the level of Tax expression (Figure 4A), two distinct correlations were seen (one for ACs and one for HAM/TSP patients).
**Quantification of cytokines in lysis assay supernatant**

Using fluorescent microbead technology we measured the concentration of the cytokines TNF-α, IL2, IL-6, IL-10, IL12(p70), IL-17, INF-γ and TGF-β1 in the supernatant of PBMCs after in vitro incubation for 18h (Supplemental Figure 3). Neither IL-12(p70) nor IL-17 was detected in any sample.

The PBMCs from some HAM/TSP patients had a high IL-10 secretion, but the median level of IL-10 expression in the 15 HAM/TSP patients was not significantly different from the median level in samples from uninfected control subjects. In contrast, there was a significantly lower rate of spontaneous secretion of TGF-β1 in both ACs and HAM/TSP patients than in samples from uninfected controls (Supplemental Figure 3A). For both IL-10 and TGF-β1, we did not observe a correlation between the level of cytokine expression and either the frequency of FoxP3 expression in CD4⁺ cells or the rate of lysis (results not shown). As a further test of the possible effects of IL-10, in two separate experiments we added a high concentration of IL-10 during the lysis assay. The results showed that the addition of the IL-10 did not alter the rate of CD8⁺ cell-mediated lysis (Supplemental Figure 3B).
Discussion:

We report here two principal findings. First, HTLV-1 infection was associated with an abnormally high frequency of expression of FoxP3 in circulating CD4+ cells. Second, the frequency of CD4+ FoxP3+ cells in the circulation showed a strong negative correlation with the rate of CD8+ cell-mediated lysis of autologous HTLV-1-infected cells.

The high frequency of FoxP3 expression in HTLV-1-infected subjects suggests that HTLV-1 directly or indirectly induces FoxP3 expression in the CD4+ T-cell population. In a recent study of Tregs in HTLV-1 infection31, it was reported that the fraction of cell that expressed FoxP3 in the CD4+ CD25+ cells was lower in HTLV-1-infected subjects, in apparent conflict with the results reported here. The authors concluded that the frequency of Tregs was abnormally low in HTLV-1 infection. However, HTLV-1 Tax protein is known to induce strong expression of CD2523,30,32 (see Supplemental Figure 1C). It is therefore likely that the frequency of FoxP3+ cells in the CD4+ CD25+ population is reduced, in HTLV-1 infection, by Tax-induced expression of CD25. This produces an apparent reduction in the frequency of Tregs – i.e. when they are defined as CD4+CD25+FoxP3+ – in HTLV-1 infection. This reduction is especially marked in HAM/TSP patients because Tax expression is typically greater in HAM/TSP patients than in ACs at a given proviral load8,33 (Supplemental Figure 1B). We confirmed this hypothesis by flow cytometric analysis of PBMCs from 4 patients with HAM/TSP, 2 ACs and 2 uninfected controls. The results (Table 1) showed that in patients with HAM/TSP there was indeed an increase in the percentage of FoxP3+ cells in the total CD4+ population compared with
that in uninfected individuals, but at the same time the percentage of FoxP3$^+$ cells in
the CD4$^+$CD25$^+$ population was lower than in uninfected individuals. We conclude
that it is inappropriate to use CD25 as a marker of T$_{regs}$ in HTLV-1 infection. In the
present study we avoided the use of CD25 in the working phenotypic definition of
T$_{regs}$ and instead quantified the frequency of CD4$^+$ FoxP3$^+$ cells.

The percentage of CD4$^+$ FoxP3$^+$ cells in the circulation was proportional to the
level of HTLV-1 expression (Figure 4A). However, there was significant variation
between individuals in the percentage of Tax-expressing cells at a given percentage of
CD4$^+$FoxP3$^+$ cells. Further, the finding that the percentage of Tax-expressing cells
was systematically greater in patients with HAM/TSP than in ACs at a given
percentage of CD4$^+$ FoxP3$^+$ cells (Figure 4A) implies that additional factors act in
patients with HAM/TSP to increase the Tax expression at a given percentage of
FoxP3$^+$ cells. This conclusion is consistent with previous studies$^{8,33}$ which showed
that the level of Tax expression was greater in patients with HAM/TSP than in
asymptomatic carriers at a given proviral load. The factors that determine the rate of
Tax expression at a given proviral load remain unknown: possible factors include
epigenetic modifications and the genomic integration site of HTLV-1 (K. N.
Meekings, GPT, CRMB, submitted).

A small proportion of CD4$^+$ FoxP3$^+$ cells were also found to express HTLV-1
Tax protein themselves (Figure 2A). The fraction of FoxP3$^+$ cells that expressed Tax
typically exceeded the fraction of FoxP3$^+$ cells that expressed Tax (Supplemental 2A).
There are two possible explanations for this observation, which are not mutually
exclusive. First, HTLV-1 might preferentially (though not exclusively) infect FoxP3$^+$
cells. This possibility cannot be excluded on the basis of the present data. However, the majority of FoxP3$^+$ cells were Tax$^-$ (Figure 2B); and cell sorting and quantitative PCR demonstrated that the majority of these FoxP3$^+$Tax$^-$ cells did not carry the HTLV-1 provirus (Figure 2C). Second, HTLV-1 expression might induce FoxP3 expression in the infected cell. Human T cells transiently express FoxP3 upon activation $^{34,35}$; therefore, FoxP3 expression in these cells might result from Tax-induced T cell activation. However, it was recently reported that Tax transfection of CD4$^+$CD25$^+$ cells purified from uninfected PBMCs reduced FoxP3 expression at the mRNA level$^{36}$. Finally Tao et al.$^{37}$ have suggested that histone deacetylases (HDACs) may control FoxP3 expression, and we have previously demonstrated that HDACs also play a role in HTLV-1 infection$^{38}$. But whatever the interaction between FoxP3 and Tax in the infected cell, the present data show that, in contrast with the CD4$^+$ FoxP3$^+$ Tax$^-$ population, the size of the CD4$^+$FoxP3$^+$Tax$^-$ population was small (<0.2% vs. $\leq$4% of circulating CD4$^+$FoxP3$^+$ T cells respectively; Figures 2B and 3), and was unrelated to the CD8$^+$ cell-mediated lysis rate (compare Figures 3B and 3C).

The data reported here revealed a strong negative correlation between the percentage of CD4$^+$ FoxP3$^+$ Tax$^-$ cells and the rate of lysis of naturally-infected CD4$^+$ T cells by autologous CD8$^+$ cells isolated from the fresh blood of HTLV-1-infected individuals. This correlation might result either from suppression of CTL activity by FoxP3$^+$ cells, or conversely from suppression of FoxP3$^+$ cell proliferation by CTLs, presumably by CD8$^+$ T cell-mediated lysis of HTLV-1-infected FoxP3$^+$ cells. We propose that the first possibility is true, for three reasons. First, the present data show a strong (negative) correlation between the CD8$^+$ T cell-mediated lysis rate (epsilon) and the frequency of FoxP3$^+$ cells that are Tax$^-$, i.e. that do not express HTLV-1
antigens and therefore cannot serve as targets for HTLV-1-specific CTLs. Second, there was no significant difference between HAM/TSP patients and ACs in the mean (or median) frequency of FoxP3+ cells in the CD4+ population (Figure 1A), whereas the cells from HAM/TSP patients consistently expressed greater levels of HTLV-1 antigens than those from ACs at the same proviral load8,33. Third, evidence from in vitro experiments demonstrates that FoxP3+ cells can suppress both the replication and the activity of CTLs16,39. Finally, recent reports from other laboratories have demonstrated suppression of CTLs by FoxP3 T cells40,41.

Quantification of cytokines in the PBMC culture supernatant confirmed a high rate of secretion of IFN-γ, TNF-α and IL-2 in cells from HTLV-1 seropositive patients (Supplemental Figure 3A) consistent with T cells activation and with previous results42. The measurement of concentration of IL-10 and TGF-β1 in the supernatant of the lysis assays in this study did not suggest a role of these cytokines in the control of the rate of CD8+ cell-mediated lysis (Supplemental Figure 3B). IL-10 is well-known to be associated with the control of the immune response16,43. In two experiments, addition of recombinant IL-10 to the culture medium had no effect on the efficiency of lysis. However, we cannot exclude such a role in vivo, where CTL activity might be influenced by the relative concentrations of cytokines in the local milieu. It is possible that CD4+ FoxP3+ cells exert their regulatory effect on HTLV-1-specific CD8+ T cells by a mechanism that depends on cell contact, with or without the involvement of cytokines19. But at present no means exist of testing this possibility in our model, because there is no known appropriate surface marker of the CD4+ FoxP3+ cells which might allow their selection and separation. As noted above,
CD25 is not a good marker, because its expression is strongly induced by HTLV-1 Tax protein.

We previously reported a significant negative correlation between the rate of CD8\(^+\) cell-mediated lysis of HTLV-1-infected cells in vitro and the proviral load in vivo\(^\text{25}\). We hypothesized that CTL surveillance limits HTLV-1 replication in vivo. This implies that Tax\(^+\) T cells turn over abnormally rapidly in vivo as a result of CTL-mediated lysis. Recently, measurement of the turnover rate of CD4\(^+\) T cell populations in vivo, by labelling proliferating cells with deuterated glucose, demonstrated that the lifespan of a Tax\(^+\) CD4\(^+\) T cell in vivo was indeed reduced to ~1d from the normal ~30d\(^\text{14}\). The conclusion that CTLs limit proviral load in vivo by killing HTLV-1-expressing cells is consistent with data from host population immunogenetics\(^\text{44-46}\), gene expression microarrays\(^\text{29}\), evidence of positive selection\(^\text{47,48}\) and CTL escape mutations\(^\text{49}\) in the \(\text{tax}\) gene, which encodes the immunodominant antigen recognized by HTLV-1-specific CTLs\(^\text{9,10}\).

We conclude that HTLV-1 infection is associated with abnormal expression of FoxP3 in circulating CD4\(^+\) cells and that the frequency of CD4\(^+\) FoxP3\(^+\) Tax\(^-\) T cells is an important determinant of the rate of CD8\(^+\) cell-mediated immune surveillance of HTLV-1-infected cells in vivo.
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Authorship:

Frederic Toulza: designed and carried experiments, analysed data and wrote the paper
Adrian Heaps: carried out assays of HTLV-1 proviral load
Yuetsu Tanaka: supplied anti-Tax antibody (Lt-4) and provided technical advice
Graham P. Taylor: carried out clinical work and supplied blood samples
Charles RM Bangham: designed experiments and wrote the paper
Reference:

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Table 1 The effect of HTLV-1 infection on the frequency of Tregs depends on the phenotypic definition of Tregs

| Patients   | % FoxP3⁺ in CD4⁺ cells | % FoxP3⁺ in the CD4⁺CD25⁺ fraction |
|------------|------------------------|----------------------------------|
| Uninfected 1 | 1.1                    | 33.1                             |
| Uninfected 2 | 0.7                    | 33.8                             |
| AC 1        | 1.2                    | 22.6                             |
| AC 2        | 2.2                    | 35.6                             |
| HAM/TSP 1   | 4.6                    | 13.4                             |
| HAM/TSP 2   | 6.1                    | 11                               |
| HAM/TSP 3   | 5.6                    | 5                                |
| HAM/TSP 4   | 3.4                    | 15.3                             |

HTLV-1 infection was associated with an increase in the proportion of CD4⁺ T cells that express FoxP3 (column 1). This increase was especially marked in patients with HAM/TSP, who have a high proviral load of HTLV-1 and a high rate of proviral expression. However, the proportion of CD4⁺CD25⁺ cells that express FoxP3 was lower in patients with HAM/TSP than in uninfected individuals. The explanation for this is that HTLV-1 infection of CD4⁺ T cells strongly induces CD25 expression, thereby increasing the denominator of the fraction FoxP3⁺/CD4⁺CD25⁺. Because of the confounding effect of HTLV-1-induced expression of CD25, in the present study we avoided the use of CD25 in the working definition of the phenotype of Tregs, and used instead the simpler definition CD4⁺FoxP3⁺.
**Figure Legends:**

**Figure 1** FoxP3 expression in HTLV-1 seropositive subjects

(A) FoxP3 expression in CD4⁺ cells in 7 uninfected subjects, 28 ACs and 30 HAM/TSP patients gated on the CD4⁺. The P value was calculated by an unpaired t-test (two-tailed). (B) Dot plot showing FoxP3 and CD127 expression in PBMCs, gated on the CD4⁺ population, from a representative HAM/TSP patient. (C) Time course of FoxP3 expression in CD4⁺ cells, for 4 different ACs and 4 HAM/TSP patients.

**Figure 2** FoxP3 expression and Tax expression.

(A) Density plot showing Tax and FoxP3 expression in CD4⁺ cells from a representative AC patient after 18h incubation. The dot plot was divided in 2 gates: ‘a’ total CD4⁺ cells; ‘b’ FoxP3⁺Tax⁻ CD4⁺ cells. (B) Percentage of CD4⁺ cells that express FoxP3 alone (gate ‘α’), FoxP3 and Tax (gate ‘β’) and Tax alone (gate ‘γ’). The P value was calculated by an unpaired t-test (two-tailed). This percentage was measured on 28 ACs and 30 HAM/TSP patients. (C) Classical PCR for one AC and quantification of proviral load in 3 independent HTLV-1-seropositive patients. FoxP3⁺Tax⁻ cells were isolated by cell sorting of the CD4⁺ population (gate ‘c’) and compared with the total CD4⁺ cell population (gate ‘a’). The data were normalized to the level of beta-actin cDNA, and expressed as Tax copy number per 100 PBMC.

**Figure 3** Correlation between FoxP3 expression and the rate of CD8⁺ cell-mediated lysis

The percentage of FoxP3⁺ cells in all CD4⁺ cells (A), the percentage of CD4⁺ FoxP3⁺ Tax⁻ cells (B), and the percentage of CD4⁺ FoxP3⁺ Tax⁺ cells, were plotted against the
efficiency of lysis. The data represent the result obtained with samples from 15 ACs and 19 HAM/TSP patients. The P values were determined by a two-tailed Spearman test. For the percentage of CD4⁺ FoxP3⁺ Tax⁺ cells we have also represented the correlation with the efficiency of lysis on a smaller scale, to clarify the lack of correlation here (C).

**Figure 4** Correlation between FoxP3 expression, Tax expression and proviral load

(A) The percentage of FoxP3 expression in CD4⁺Tax⁻ cells was correlated with the percentage of CD4⁺Tax⁺ cells both in ACs (N = 23) and in HAM/TSP patients (N = 22). The P values were calculated by a two-tailed Spearman test. (B) The proviral load was correlated with the level of FoxP3 mRNA expression in CD4⁺ cells (measured by Q-PCR) both in ACs (N = 11) and in patients with HAM/TSP (N = 13).
Figure 1

A

\[
\begin{align*}
\% \text{ CD4}^+ \text{ Foxp3}^+ \text{ cells} \\
\text{NI} & (n = 7) \\
\text{AC} & (n = 28) \\
\text{HAM/TSP} & (n = 30)
\end{align*}
\]

\[p < 0.0001\]
\[p = 0.158\]
\[p = 0.03\]

B

Gated on CD4^+ cells

C

\[
\begin{align*}
\% \text{ CD4}^+ \text{ Foxp3}^+ \text{ cells} \\
\text{Time in hours}
\end{align*}
\]

• HAM/TSP

□ AC
Figure 2

A. Flow cytometry scatterplot showing gated CD4+ cells.

B. Bar graph showing % CD4+ cells with different conditions.

C. Table showing Proviral load (copy of Tax/100 PBMC) across different experiments:

| Exp | AC (Tax) | HAM/TSP (Tax) |
|-----|----------|---------------|
| Exp 1 | 4.35     | 1.79          |
| Exp 2 | 22.09    | 8.82          |
| Exp 3 | 32.16    | 18.90         |

Annotations:
- FOX3: FoxP3
- Tax: Tax
- HTLV-1
- β-Actin
- Proviral load (copy of Tax/100 PBMC)
Figure 3

A

Rate of lysis

% CD4⁺ Foxp3⁺ cells

AC (□)  HAM/TSP (◆)
R² = 0.684  R² = 0.614
p < 0.001  p < 0.002

All plots  p < 0.001
R² = 0.658

B

Rate of lysis

% CD4⁺ Foxp3⁺ Tax⁻ cells

AC (□)  HAM/TSP (◆)
R² = 0.727  R² = 0.808
p < 0.001  p < 0.001

All plots  p < 0.001
R² = 0.747

C

Rate of lysis

% CD4⁺ Foxp3⁺ Tax⁺ cells

AC (□)  HAM/TSP (◆)
R² = 0.035  R² = 0.146
p < 0.2  p < 0.2

All plots  p < 0.5
R² = 0.041
Figure 4

A

% Tax+ CD4+ vs % Foxp3+ CD4+ Tax− cells

HAM/TSP (●)  ACs (○)
R² = 0.719  R² = 0.536
p = 0.001  p = 0.02

All points
R² = 0.558
p = 0.001

B

Provirial load / βActin vs FoxP3 / BIRC₂

HAM/TSP (●)  ACs (○)
R² = 0.538  R² = 0.195
p = 0.01  p = 0.02

All points
R² = 0.431
p = 0.01
High frequency of CD4⁺FoxP3⁺ cells in HTLV-1 infection: inverse correlation with HTLV-1-specific CTL response

Frederic Toulza, Adrian Heaps, Yuetsu Tanaka, Graham P Taylor and Charles R M Bangham