

# HTLV-1 induces a Th1-like state in CD4<sup>+</sup>CCR4<sup>+</sup> T cells

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**Human T-lymphotropic virus type 1 (HTLV-1) is linked to multiple diseases, including the neuroinflammatory disease HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) and adult T cell leukemia/lymphoma. Evidence suggests that HTLV-1, via the viral protein Tax, exploits CD4<sup>+</sup> T cell plasticity and induces transcriptional changes in infected T cells that cause suppressive CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> Tregs to lose expression of the transcription factor FOXP3 and produce IFN- $\gamma$ , thus promoting inflammation. We hypothesized that transformation of HTLV-1-infected CCR4<sup>+</sup> T cells into Th1-like cells plays a key role in the pathogenesis of HAM/TSP. Here, using patient cells and cell lines, we demonstrated that Tax, in cooperation with specificity protein 1 (Sp1), boosts expression of the Th1 master regulator T box transcription factor (T-bet) and consequently promotes production of IFN- $\gamma$ . Evaluation of CSF and spinal cord lesions of HAM/TSP patients revealed the presence of abundant CD4<sup>+</sup>CCR4<sup>+</sup> T cells that coexpressed the Th1 marker CXCR3 and produced T-bet and IFN- $\gamma$ . Finally, treatment of isolated PBMCs and CNS cells from HAM/TSP patients with an antibody that targets CCR4<sup>+</sup> T cells and induces cytotoxicity in these cells reduced both viral load and IFN- $\gamma$  production, which suggests that targeting CCR4<sup>+</sup> T cells may be a viable treatment option for HAM/TSP.**

## Introduction

The flexibility of the CD4<sup>+</sup> T cell differentiation program that underlies the success of the adaptive immune response has recently been implicated in the pathogenesis of numerous inflammatory diseases (1–3). The majority of CD4<sup>+</sup> T lymphocytes belong to a class of cells known as Th cells, so called because they provide help on the metaphorical immune battlefield by stimulating the other soldiers — namely, B cells and cytotoxic T lymphocytes — via secretion of various cytokines. Interestingly, there is also a minority group of CD4<sup>+</sup> T cells with quite the opposite function: Tregs actively block immune responses by suppressing the activities of CD4<sup>+</sup> Th cells as well as many other leukocytes (4). Tregs are credited with maintaining immune tolerance and preventing inflammatory diseases that could otherwise occur as a result of uninhibited immune reactions (5). Thus, the up- or downregulation of certain CD4<sup>+</sup> T cell lineages could disrupt the carefully balanced immune system, threatening bodily homeostasis.

The plasticity of CD4<sup>+</sup> T cells, particularly Tregs, makes CD4<sup>+</sup> T cell lineages less clean-cut than they may originally appear. CD4<sup>+</sup> T cells are subdivided according to various lineage-specific chemokine receptors and transcription factors they express, as well as the cytokines they produce (6). Th1 cells, for example, can be identified by expression of CXC motif receptor 3 (CXCR3) and T box

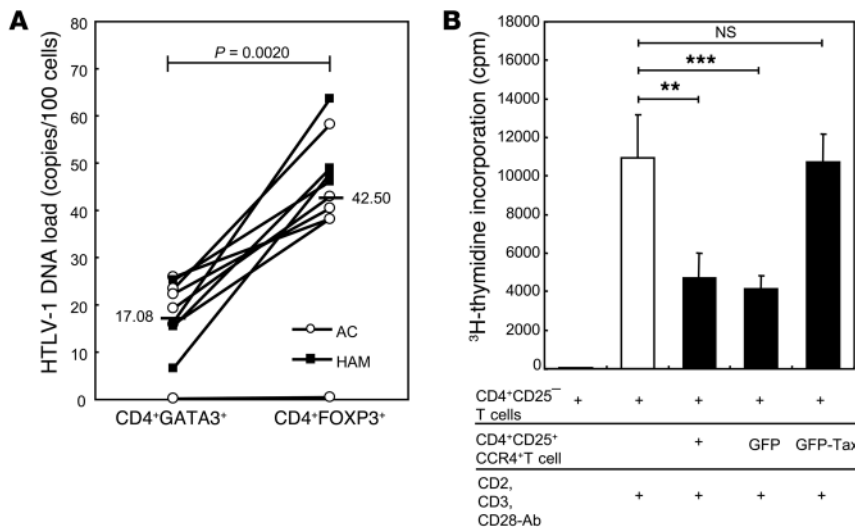
transcription factor (T-bet; encoded by *TBX21*) and are known to secrete the proinflammatory cytokine IFN- $\gamma$  (6). While both have been known to express CC chemokine receptor 4 (CCR4) and CD25, Th2 cells and Tregs can usually be distinguished from each other by their expression of GATA-binding protein 3 (GATA3) and forkhead box p3 (FOXP3), respectively (6, 7). CCR4 is coexpressed in the majority of CD4<sup>+</sup>FOXP3<sup>+</sup> cells and in virtually all CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> cells, making it a useful — albeit not fully specific — marker for Tregs (8, 9). FOXP3 is a particularly noteworthy marker because its expression is said to be required for Treg identity and function (10). In fact, *Foxp3* point mutations are reported to cause fatal multiorgan autoimmune diseases (11). Even partial loss of FOXP3 expression can disrupt the suppressive nature of Tregs, representing one of several pathways by which even fully differentiated Tregs can reprogram into inflammatory cells (12). There have been several reports of Tregs reprogramming in response to proinflammatory cytokines such as IL-1, IL-6, IL-12, and IFN- $\gamma$  (12, 13); it is thought that this reprogramming may have evolved as an adaptive mechanism for dampening immune suppression when protective inflammation is necessary (12). However, this same plasticity can lead to pathologically chronic inflammation, and several autoimmune diseases have been associated with reduced FOXP3 expression and/or Treg function, including multiple sclerosis, myasthenia gravis, and type 1 diabetes (14, 15).

Of the roughly 10–20 million people worldwide infected with human T-lymphotropic virus type 1 (HTLV-1), up to 2%–3% are affected by the neurodegenerative chronic inflammatory dis-

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**Figure 1. HTLV-1 mainly infects Tregs and inhibits their regulatory function. (A)** Higher HTLV-1 proviral DNA load in CD4<sup>+</sup>FOXP3<sup>+</sup> cells (Tregs) compared with CD4<sup>+</sup>GATA3<sup>+</sup> cells ( $P = 0.0020$ , Wilcoxon test) from asymptomatic carriers (AC;  $n = 6$ ) and HAM/TSP patients ( $n = 4$ ). PBMCs were FACS sorted, and proviral load was measured using quantitative PCR. Horizontal bars represent the mean value for each set. **(B)** Loss of regulatory function in Tax-expressing CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> cells (Tregs). CD4<sup>+</sup>CD25<sup>+</sup> T cells from an HD were stimulated with CD2, CD3, and CD28 antibodies and cultured alone or in the presence of equal numbers of CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> T cells, GFP lentivirus-infected HD CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> T cells, or GFP-Tax lentivirus-infected HD CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> T cells. As a control, CD4<sup>+</sup>CD25<sup>+</sup> T cells alone were cultured without any stimulus. Proliferation of T cells was determined using <sup>3</sup>H-thymidine incorporation by adding <sup>3</sup>H-thymidine for 16 hours after 4 days of culture. All tests were performed in triplicate. Data are mean  $\pm$  SD. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , ANOVA followed by Tukey test for multiple comparisons.

ease HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). The main other condition associated with the retrovirus is adult T cell leukemia/lymphoma (ATLL), a rare and aggressive cancer of the T cells. HAM/TSP represents a useful starting point from which to investigate the origins of chronic inflammation, because the primary cause of the disease — viral infection — is so unusually well defined. HAM/TSP patients share many immunological characteristics with FOXP3 mutant mice, including multiorgan lymphocytic infiltrates, overproduction of inflammatory cytokines, and spontaneous lymphoproliferation of cultured CD4<sup>+</sup> T cells (16–18). We and others have proposed that HTLV-1 preferentially infects CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> T cells, a group that includes Tregs (7, 19). Samples of CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> T cells isolated from HAM/TSP patients exhibited low FOXP3 expression as well as reduced production of suppressive cytokines and low overall suppressive ability — in fact, these CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup>FOXP3<sup>+</sup> T cells were shown to produce IFN- $\gamma$  and express Ki67, a marker of cell proliferation (19). The frequency of these IFN- $\gamma$ -producing CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> T cells in HAM/TSP patients was correlated with disease severity (19). Finally, evidence suggests that the HTLV-1 protein product Tax may play a role in this alleged transformation of Tregs into proinflammatory cells in HAM/TSP patients: transfecting Tax into CD4<sup>+</sup>CD25<sup>+</sup> cells from healthy donors (HDs) reduced FOXP3 mRNA expression, and Tax expression in CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> cells was higher in HAM/TSP versus ATLL patients despite similar proviral loads (19, 20). Therefore, we hypothesized that HTLV-1 causes chronic inflammation by infecting

CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> T cells and inducing their transformation into Th1-like, IFN- $\gamma$ -producing proinflammatory cells via intracellular Tax expression and subsequent transcriptional alterations including but not limited to loss of endogenous FOXP3 expression.

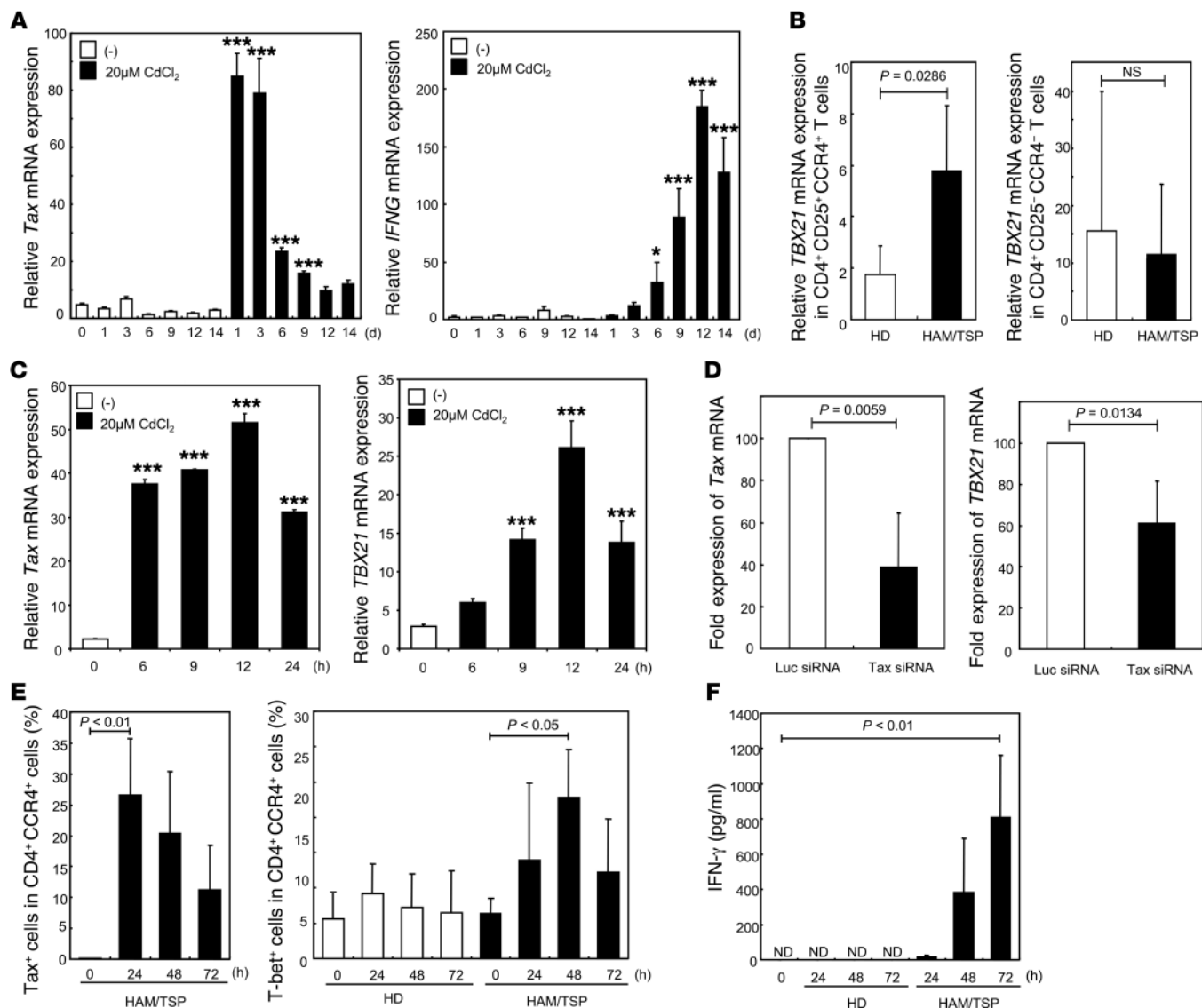
In this study, we first sought to discover the detailed mechanism by which Tax influences the function of CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> T cells. We used DNA microarray analysis of CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> T cells from HAM/TSP patients to identify *TBX21*, known as a master transcription factor for Th1 differentiation, as a key intermediary between Tax expression and IFN- $\gamma$  production. We demonstrated that Tax, in concert with specificity protein 1 (Sp1), amplified *TBX21* transcription and subsequently IFN- $\gamma$  production. Next, we established the presence of Th1-like CD4<sup>+</sup>CCR4<sup>+</sup> T cells in the CSF and spinal cord lesions of HAM/TSP patients. The majority of these CD4<sup>+</sup>CCR4<sup>+</sup> T cells coexpressed CXCR3 as well as T-bet and IFN- $\gamma$ . Finally, we investigated the therapeutic potential of an anti-CCR4 monoclonal antibody with antibody-dependent cellular cytotoxicity (ADCC) (21). Applying this antibody in vitro diminished the proliferative capacity of cultured PBMCs and reduced both proviral DNA load and IFN- $\gamma$  production in cultured CSF cells as well as PBMCs. In conclusion, we

were able to elucidate a more detailed mechanism for the pathogenesis of HAM/TSP and use our findings to suggest a possible therapeutic strategy.

## Results

*HTLV-1 preferentially infects Tregs and alters their behavior via Tax.* Experiments were conducted to determine which among CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> T cells were infected by HTLV-1, and how the infection influenced their functionality. Analysis of fluorescence-activated cell sorting (FACS)-sorted PBMCs obtained from asymptomatic carriers ( $n = 6$ ) as well as HAM/TSP patients ( $n = 4$ ) revealed that Tregs (CD4<sup>+</sup>FOXP3<sup>+</sup>) carried much higher proviral loads than Th2 cells (CD4<sup>+</sup>GATA3<sup>+</sup>) ( $P = 0.0020$ ; Figure 1A). As it is well established that each infected cell contains only 1 copy of the HTLV-1 provirus (22, 23), these results indicate that a larger proportion of FOXP3<sup>+</sup> than GATA3<sup>+</sup> CD4<sup>+</sup> T cells are infected. As expected, proliferation of CD4<sup>+</sup>CD25<sup>+</sup> cells after stimulation, as measured by <sup>3</sup>H-thymidine incorporation, was suppressed upon coculture with CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> cells, including Tregs ( $n = 3$ ,  $P < 0.01$ ; Figure 1B). However, after being transduced with lentiviral vector expressing GFP-Tax, the CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> cells no longer suppressed cell proliferation; conversely, cells transduced with the control vector expressing only GFP retained full suppressive function ( $P < 0.001$ ; Figure 1B).

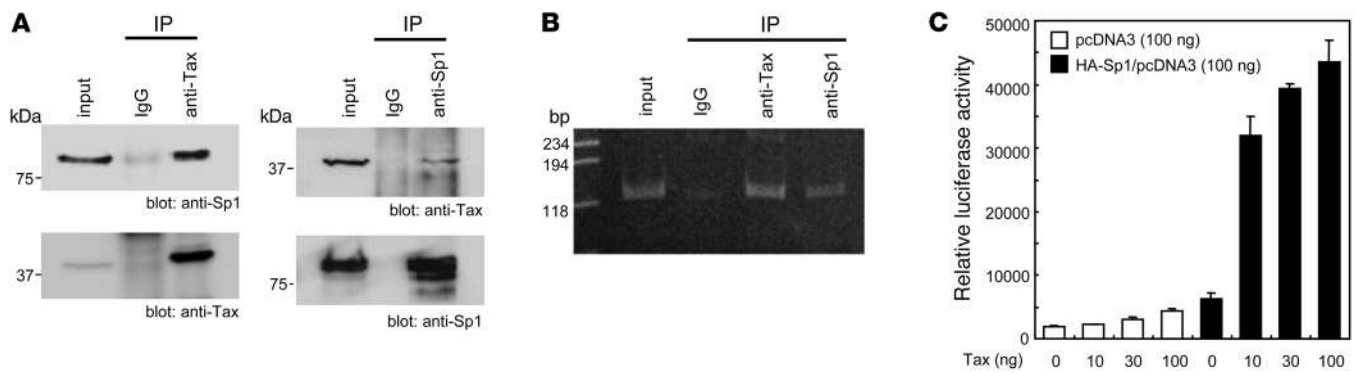
*The HTLV-1 protein product Tax induces IFN- $\gamma$  production via T-bet.* Experiments were conducted to determine if and how Tax affects IFN- $\gamma$  production in infected T cells. First, the existence of a functional link between *Tax* and *IFNG* was established by using the



**Figure 2. Tax induces IFN- $\gamma$  production via T-bet.** (A) Tax-dependent *IFNG* mRNA expression in JPX-9 cells. Experiments were performed in triplicate. (B) Elevated *TBX21* mRNA expression in CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> T cells from HAM/TSP patients relative to HDs ( $n = 4$  per group). (C) Tax-dependent *TBX21* mRNA expression in JPX-9 cells. Experiments were performed in triplicate. (D) Reduced *TBX21* mRNA expression after silencing Tax in CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> T cells from HAM/TSP patients. PBMCs from HAM/TSP patients ( $n = 5$ ) were FACS sorted, transfected with either Luc or Tax siRNA, and incubated for 24 hours. (E and F) Tax expression correlated with T-bet expression and IFN- $\gamma$  production in CD4<sup>+</sup>CCR4<sup>+</sup> T cells from HAM/TSP patients. CD4<sup>+</sup>CCR4<sup>+</sup> T cells isolated from HDs and HAM/TSP patients ( $n = 4$  per group) were cultured before being stained for Tax and T-bet protein and analyzed using FACS. IFN- $\gamma$  production in the culture medium was measured using a CBA assay. ND, not detectable. All data are mean  $\pm$  SD.  $P$  values were calculated using (A and C) 1-way ANOVA followed by Dunnett test for multiple comparisons, (B) Mann-Whitney  $U$  test, (D) paired  $t$  test, or (E and F) Friedman test followed by Dunn test for multiple comparisons. \* $P < 0.05$ , \*\*\* $P < 0.001$  vs. time point 0.

JPX-9 cell line possessing a stably integrated CdCl<sub>2</sub>-inducible *Tax* construct and measuring *IFNG* mRNA expression. Inducing *Tax* expression with CdCl<sub>2</sub> periodically over 2 weeks yielded a steady rise in *IFNG* expression (Figure 2A). Although there was clearly a correlation between *Tax* and IFN- $\gamma$  expression, the *IFNG* expression level was not proportional to that of *Tax*, and the steepest rise in the former was delayed several days after the steepest rise in the latter. Thus, we suspected that expression of 1 or more additional genes may represent an important middle step on the pathway linking *Tax* and IFN- $\gamma$  production. DNA microarray results revealed that expression of *TBX21*, which is known to be associated with IFN- $\gamma$  pro-

duction, was elevated in CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> cells from the HAM/TSP patient, but not the ATLL patient, compared with the HD (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI75250DS1). *TBX21* mRNA expression, measured via real-time RT-PCR, was elevated in CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> cells, but not CD4<sup>+</sup>CD25<sup>-</sup>CCR4<sup>-</sup> cells, from HAM/TSP patients compared with HDs (Figure 2B). A direct correlation between *Tax* and *TBX21* mRNA expression was then established using the JPX-9 cell line, as described above (Figure 2C). Silencing the *Tax* gene with siRNA in CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> cells from HAM/TSP patients reduced *TBX21* as well as *Tax* expression (Figure 2D). Similarly,



**Figure 3. Tax and Sp1 cooperatively enhance *TBX21* promoter activity.** (A) Co-IP of endogenous Tax and Sp1. Nuclear extracts from MT-2 cells were immunoprecipitated with anti-Tax or anti-Sp1 antibodies or with normal IgG as a control, then immunoblotted with anti-Tax or anti-Sp1 antibodies as indicated. (B) Tax bound to the *TBX21* promoter in vivo. ChIP assay using anti-Tax antibody followed by primers encompassing the *TBX21* promoter region (-179 to -59) was performed on genomic DNA isolated from MT-2 cells. DNA (input) and IP with anti-Sp1 served as positive controls, and normal IgG served as a negative control. (C) Coactivation of *TBX21* promoter by Sp1 and Tax. HEK293 cells were transfected with 100 ng of *TBX21*-Luc reporter plasmid or Sp1 expression plasmid, as well as 0–100 ng of Tax expression plasmid as indicated. Values were normalized to  $\beta$ -galactosidase activity as an internal control. Data are mean  $\pm$  SD.

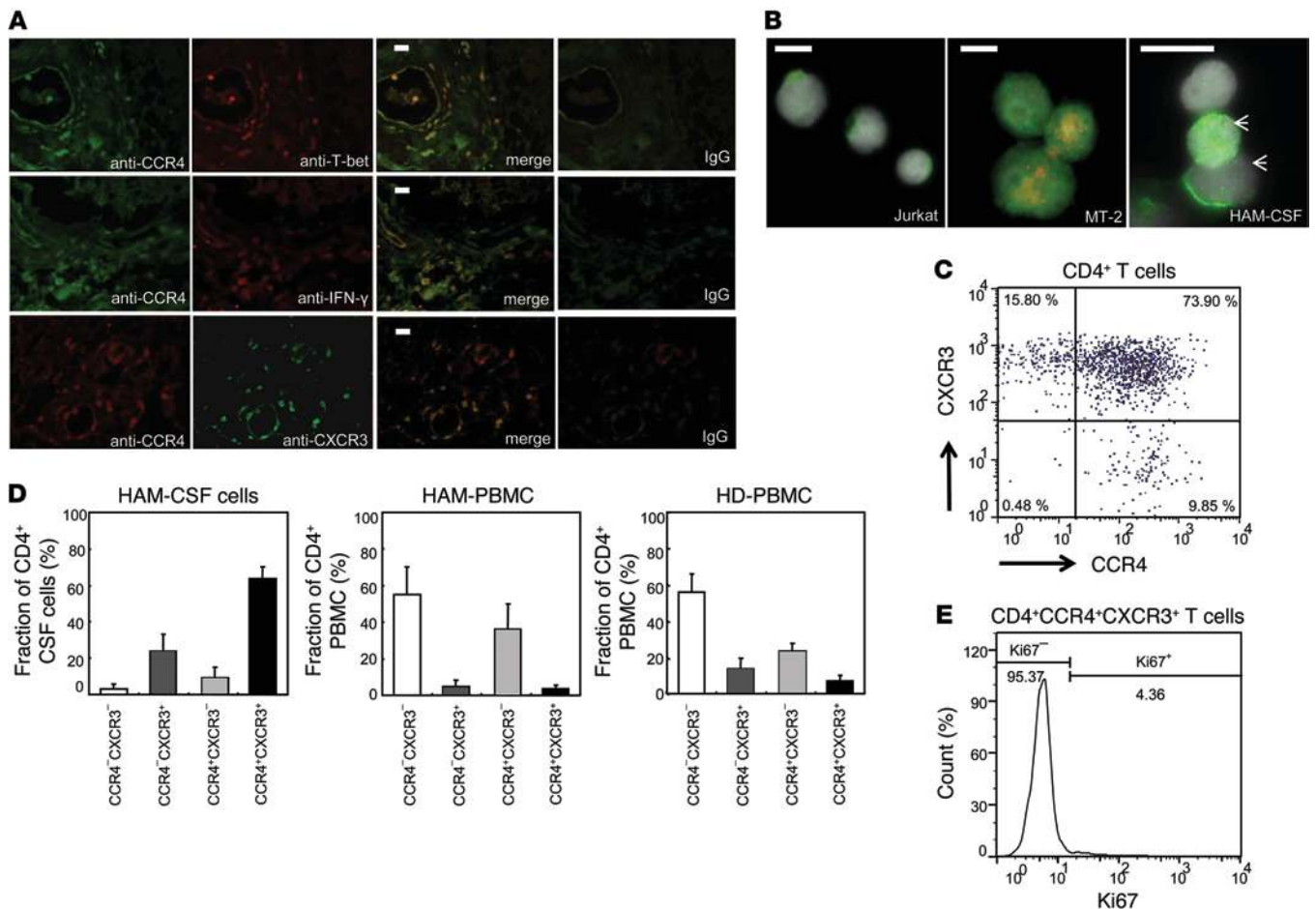
elevation of *Tax* expression via transduction of a GFP-Tax construct into CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> cells from a HD increased expression of *TBX21* as well as *Tax* (Supplemental Figure 2). Thus, a functional relationship between *Tax* and *TBX21* was confirmed. Finally, among CD4<sup>+</sup>CCR4<sup>+</sup> cells from HAM/TSP patients, the appearance of Tax<sup>+</sup> cells was associated with a rise in the percentage of T-bet<sup>+</sup> cells, which was associated with a delayed but roughly proportional rise in the amount of IFN- $\gamma$  protein (Figure 2, E and F). The production of Tax versus T-bet in these CD4<sup>+</sup>CCR4<sup>+</sup> cells from HAM/TSP patients was compared at 0 versus 48 hours of culturing. At 0 hours, the overwhelming majority of the CD4<sup>+</sup>CCR4<sup>+</sup> cells were both Tax<sup>-</sup> and T-bet<sup>-</sup>; by 48 hours, a substantial presence of Tax<sup>+</sup>T-bet<sup>+</sup> cells had emerged, and there were very few T-bet<sup>+</sup> cells that were not also Tax<sup>+</sup> (Supplemental Figure 3).

**Tax in concert with Sp1 induces *TBX21* transcription.** Experiments were conducted to investigate the mechanism by which Tax may be involved in *TBX21* transcription in HTLV-1-infected T cells. First, co-IP reactions were performed using nuclear extracts from the HTLV-1-infected MT-2 T cell line to confirm a suspected interaction between endogenous Tax and Sp1, which is known to both form a complex with Tax and to activate *TBX21* transcription (24, 25). Precipitation with anti-Tax or anti-Sp1 antibodies yielded bands corresponding to both Tax and Sp1, whereas precipitation with the non-specific IgG antibody as the negative control yielded neither band (Figure 3A), thus demonstrating the existence of a Tax-Sp1 complex in HTLV-1-infected T cells. Second, a ChIP assay using primers encompassing the *TBX21* promoter region (-179 to -59) was performed on the MT-2 cells to confirm the suspected interaction between this Tax-Sp1 complex and the *TBX21* promoter. Precipitation with anti-Tax or anti-Sp1, but not IgG, yielded a PCR product corresponding to the *TBX21* promoter (Figure 3B), which suggests that a Tax-Sp1 complex does bind to the *TBX21* promoter site. Finally, a reporter assay was performed using cells transfected with *TBX21*-Luc, a luciferase reporter plasmid containing the *TBX21* promoter region, to confirm a functional relationship among Tax, Sp1, and *TBX21* transcription. Cotransfection with Sp1 resulted in elevated luciferase activity compared with transfection with the reporter

alone, and addition of Tax heightened this effect in a concentration-dependent manner (Figure 3C). These findings suggested that Tax, in concert with Sp1, induces *TBX21* transcription.

**HTLV-1-infected Th1-like CCR4<sup>+</sup> cells are in the CNS of HAM/TSP patients.** We next sought to confirm that HTLV-1-infected CCR4<sup>+</sup> T cells infiltrate the spinal cords of HAM/TSP patients and exhibit Th1-like traits, such as T-bet and IFN- $\gamma$  production. Fluorescent immunohistochemical staining of tissue sections from HAM/TSP patient spinal cord lesions revealed the presence of abundant CCR4<sup>+</sup> cells infiltrating around the small blood vessels and coexpressing T-bet and IFN- $\gamma$  (Figure 4A and Supplemental Figure 4). Further investigation revealed that these CCR4<sup>+</sup> cells also expressed CXCR3, the marker for Th1 cells (6). It should be noted that both IFN- $\gamma$  and CXCR3 expression are reported to be induced by T-bet expression (6). Immunofluorescent staining was also used to demonstrate the existence of HTLV-1-infected CCR4<sup>+</sup> cells in the CSF of HAM/TSP patients (Figure 4B). CCR4<sup>+</sup>CXCR3<sup>+</sup> cells were numerous among cells isolated from the CSF of HAM/TSP patients, representing 73.90% of CD4<sup>+</sup> cells isolated from a representative patient (Figure 4C) and 63.63%  $\pm$  6.73% of CD4<sup>+</sup> cells isolated from all patients ( $n = 8$ ; Figure 4D). However, nearly all of these CD4<sup>+</sup>CCR4<sup>+</sup>CXCR3<sup>+</sup> cells were negative for Ki67, a marker of cell proliferation, in the CSF of the HAM/TSP patients (93.94%  $\pm$  2.07%,  $n = 3$ ; Figure 4E). The majority of these CD4<sup>+</sup>CCR4<sup>+</sup>CXCR3<sup>+</sup> cells were also CD25<sup>+</sup> (70.16%  $\pm$  14.08%,  $n = 3$ , Supplemental Figure 5), confirming the existence of a substantial CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup>CXCR3<sup>+</sup> cell population in the CSF of HAM/TSP patients. Importantly, CD4<sup>+</sup>CCR4<sup>+</sup>CXCR3<sup>+</sup> cells did not make up the majority of PBMCs in HAM/TSP patients nor in HDs; in fact, such cells were very few (HAM/TSP, 3.65%  $\pm$  1.96%,  $n = 8$ ; HD, 6.88%  $\pm$  3.09%,  $n = 4$ ; Figure 4D). PBMCs were also isolated from ATLL patients for comparison, and CD4<sup>+</sup>CCR4<sup>+</sup>CXCR3<sup>-</sup> cells made up the overwhelming majority (83.03%  $\pm$  18.61%,  $n = 5$ ; Supplemental Figure 6).

**CCR4 shows potential as a molecular target for HAM/TSP immunotherapy.** Analysis of HTLV-1 proviral DNA load in subpopulations of CD4<sup>+</sup> PBMCs from HAM/TSP patients confirmed that CCR4<sup>+</sup> cells were heavily infected, compared with less than



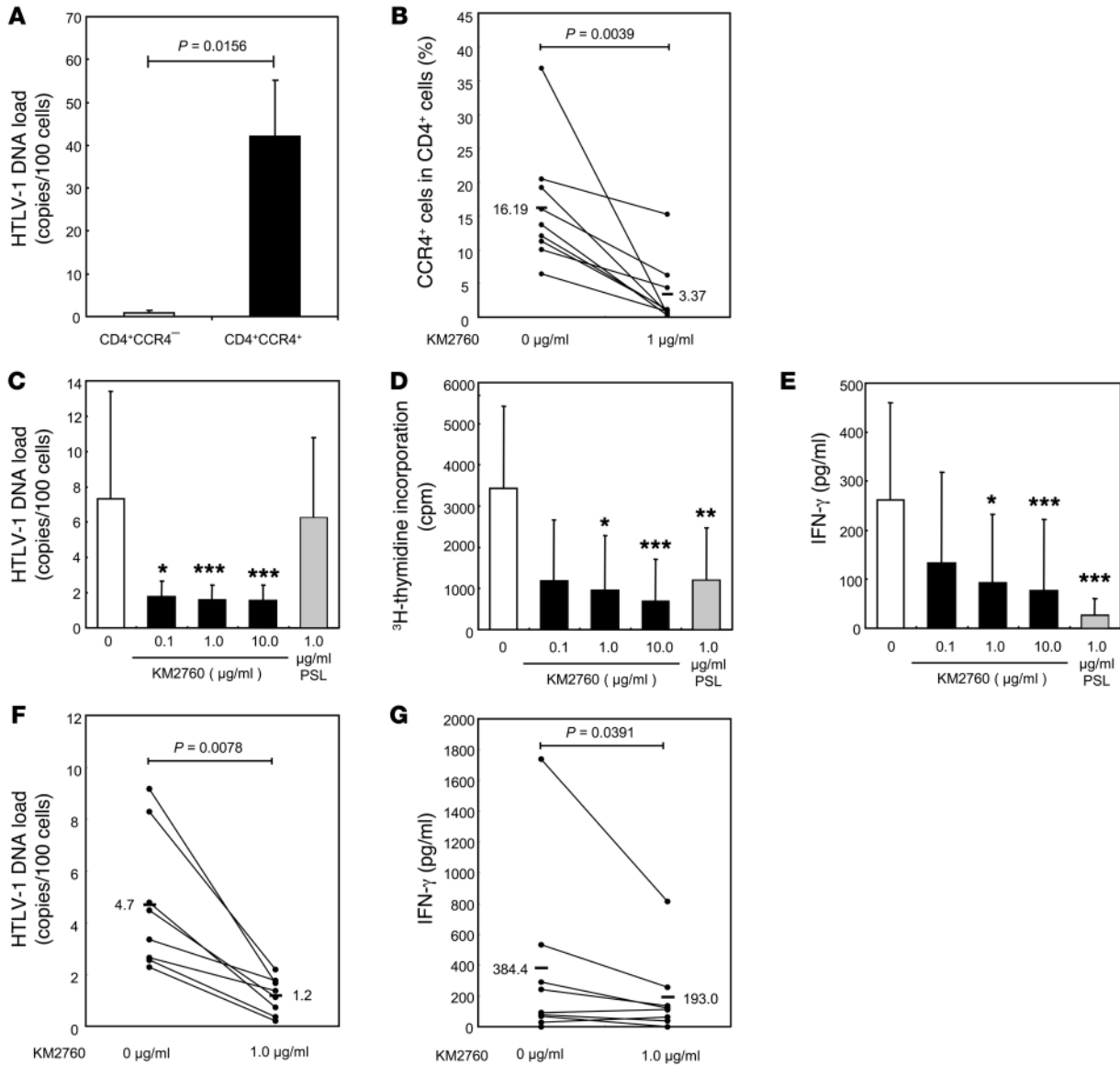
**Figure 4. HTLV-1-infected Th1-like CCR4<sup>+</sup> cells invade the CNS of HAM/TSP patients.** (A) Detection of CCR4<sup>+</sup> cells expressing T-bet, IFN- $\gamma$ , and CXCR3 infiltrating the spinal cord of a HAM/TSP patient. Representative images show immunofluorescent codetection of CCR4 with T-bet, IFN- $\gamma$ , and CXCR3, as well as the merged images, in thoracic spinal cord sections. Rabbit and goat IgG antibody served as a negative control. Scale bars: 20  $\mu$ m. (B) Presence of HTLV-1-infected CCR4<sup>+</sup> cells in HAM/TSP patient CSF. Representative images show immunofluorescence-FISH codetection of CCR4 (green) and HTLV-1 provirus (red) in Jurkat cells (uninfected control), MT-2 cells (infected control), and CSF cells from the patients. Arrows denote red provirus signal in the CSF sample. Scale bars: 20  $\mu$ m. (C) CD4<sup>+</sup> T cells in HAM/TSP patient CSF were mostly CCR4<sup>-</sup>CXCR3<sup>+</sup>. A dot plot of CCR4 and CXCR3 expression in CD4<sup>+</sup> gated cells isolated from the CSF of a representative HAM/TSP patient is shown. (D) CD4<sup>+</sup>CCR4<sup>+</sup>CXCR3<sup>+</sup> cells were numerous in CSF, but not elevated in peripheral blood, of HAM/TSP patients. Graphs show the percentages of CCR4<sup>-</sup>CXCR3<sup>-</sup>, CCR4<sup>-</sup>CXCR3<sup>+</sup>, CCR4<sup>+</sup>CXCR3<sup>-</sup> and CCR4<sup>+</sup>CXCR3<sup>+</sup> T cells among CD4<sup>+</sup> PBMCs and CSF cells from HAM/TSP patients ( $n = 8$ ) and PBMCs from HDs ( $n = 4$ ). Analysis was performed using FACS. Data are mean  $\pm$  SD. (E) Proliferation was not observed in CD4<sup>+</sup>CCR4<sup>+</sup>CXCR3<sup>+</sup> cells from HAM/TSP patient CSF. The rate of Ki67 expression, a marker for cell proliferation, is shown for CD4<sup>+</sup>CCR4<sup>+</sup>CXCR3<sup>+</sup> gated cells from the CSF of a representative HAM/TSP patient.

1% of CCR4<sup>+</sup> cells ( $n = 7$ ; Figure 5A). To predict the efficacy of a CCR4<sup>+</sup> cell-targeting cytotoxic antibody as a treatment for HAM/TSP, PBMCs were isolated from patients ( $n = 9$ ) and analyzed after being cultured with and without the defucosylated chimeric anti-CCR4 monoclonal antibody KM2760 (21) or, for comparison, the steroid therapy prednisolone (PSL). Addition of 1  $\mu$ g/ml KM2760 significantly reduced the percentage of CCR4<sup>+</sup> cells, as measured after 7 days ( $P = 0.0039$ ; Figure 5B). As little as 0.1  $\mu$ g/ml KM2760 was necessary to reduce the HTLV-1 DNA load ( $P < 0.05$ ), whereas PSL had no significant impact (Figure 5C). Use of 1  $\mu$ g/ml of either KM2760 or PSL was sufficient to suppress spontaneous proliferation of the PBMCs, as measured by <sup>3</sup>H-thymidine incorporation ( $P < 0.05$  and  $P < 0.01$ , respectively; Figure 5D) as well as IFN- $\gamma$  production ( $P < 0.05$  and  $P < 0.001$ , respectively; Figure 5E). Similar results were observed in experiments using cells isolated from

the CSF of HAM/TSP patients ( $n = 8$ ): cultures to which 1  $\mu$ g/ml of KM2760 had been added exhibited reduced HTLV-1 DNA load ( $P = 0.0078$ ; Figure 5F) and IFN- $\gamma$  production ( $P = 0.0391$ ; Figure 5G). Certain samples shown in Figure 5G did not exhibit this reduction in IFN- $\gamma$  production; those samples had particularly low cell counts (0.33–2.00 cells/ $\mu$ l), yielding less reliable data. Despite the presence of those lower-quality samples, statistical significance was still established for the sample group as a whole.

## Discussion

Previously, we hypothesized that HTLV-1 gives rise to HAM/TSP by altering the behavior of infected cells via Tax expression to yield a new population of Th1-like proinflammatory cells (26). Evidence indicated that a significant portion of this population might be Tregs, as suggested by the CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> expres-



**Figure 5. CCR4 shows potential as a molecular target for HAM/TSP immunotherapy.** (A–G) Cells isolated from HAM/TSP patients were sorted via FACS (A; *n* = 7) or cultured for 7 days under the following conditions: PBMCs were cultured with various concentrations of KM2760 or 1 μg/ml PSL (B–E; *n* = 9), and CSF cells were cultured with 1 μg/ml KM2760 (F and G; *n* = 8). (A, C, and F) HTLV-1 proviral DNA loads were measured using quantitative PCR. (D) Degree of spontaneous proliferation was assessed by measuring <sup>3</sup>H-thymidine incorporation. (E and G) IFN-γ production in the culture media was evaluated using CBA assays. HTLV-1 resided in CD4<sup>+</sup>CCR4<sup>+</sup> rather than CCR4<sup>-</sup> cells among PBMCs (A), and KM2760 treatment effectively targeted these cells (B). Consequently, KM2760 treatment successfully reduced HTLV-1 proviral DNA load (C), suppressed spontaneous proliferation (D), and decreased IFN-γ production (E) in PBMC cultures as well as reducing HTLV-1 DNA load (F) and IFN-γ production (G) in CSF cell cultures derived from HAM/TSP patients. (A and C–E) Data are mean ± SD. (B, F, and G) Thick horizontal bars represent mean value for all patients; line segments represent individual patients. Statistical analyses were performed using Friedman test followed by Dunn test for multiple comparisons (C–E) or Wilcoxon test (A, B, F, and G). \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001 vs. untreated control.

sion profile (19). We suspected that these infected cells may infiltrate the CNS and trigger an inflammatory positive feedback loop, ultimately leading to chronic spinal cord inflammation (27). In the present study, we provided concrete evidence to support these theories on HAM/TSP pathogenesis, with a particular emphasis on the mechanism by which Tax can induce a proinflammatory phenotype intracellularly via transcriptional regulation.

There is strong evidence to support the conclusion that a substantial portion of the Treg population in HAM/TSP patients is

infected with HTLV-1 (28, 29). In a previous study, we demonstrated that CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> cells were the main reservoir for HTLV-1 in HAM/TSP patients (19), but that expression profile is not exclusive to Tregs. Our present observation that CD4<sup>+</sup> T cells positive for FOXP3, a well-established marker for Tregs (10), were more thoroughly infected than the GATA3<sup>+</sup> subgroup (Figure 1A) strengthens the argument that Tregs may be the main viral reservoir. It remains debatable whether the virus preferentially infects these cells, promotes their survival (30), or even induces the expression of these

markers. One report postulates that HTLV-1 preferentially infects CCR4<sup>+</sup> cells by upregulating CCL22 to encourage cell-to-cell transfer via chemotactic attraction (31). More research is necessary to determine the true mechanism by which infected CCR4<sup>+</sup> and FOXP3<sup>+</sup> cells become so abundant in HAM/TSP patients.

We demonstrated that the suppressive ability of CD4<sup>+</sup>CD25<sup>+</sup> CCR4<sup>+</sup> cells that characterizes Treg function was impaired by expression of the Tax protein, encoded in the pX region of the HTLV-1 genome (Figure 1B). Prior evidence indicates that Tax may exert these effects via downregulation of FOXP3 expression (20, 32). Transgenic mice expressing Tax exhibit reduced CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> Tregs (33) and develop arthritis (34), and transgenic rats expressing HTLV-1 env-pX develop destructive arthropathies, Sjogren syndrome, vasculitis, and polymyositis (35). Collectively, these observations suggest that Tax expression can lead to inflammatory disease by weakening immune tolerance and disrupting homeostasis.

It has long been suspected that in addition to reducing FOXP3 expression, Tax may have the ability to induce IFN- $\gamma$  production, thereby converting once-suppressive cells into proinflammatory cells. Indeed, intracellular Tax expression has been associated with the rapid upregulation of IFN- $\gamma$  in infected cells, and researchers have theorized that this upregulation may contribute to the pathogenesis of HTLV-1-associated inflammatory disorders, including HAM/TSP (19, 36, 37). Here we showed at the mRNA level that *Tax* expression stimulated *IFNG* expression; moreover, the effect appeared delayed (Figure 2A), in a manner suggestive of 1 or more intermediate steps in the pathway, rather than direct transcriptional activation. Several candidate pathways have been proposed—such as via NF- $\kappa$ B, STAT1, or STAT5—but none have been confirmed experimentally (38, 39).

We provided convincing evidence that Tax induces IFN- $\gamma$  production in infected cells indirectly by amplifying the effects of Sp1 binding to—and increasing the activity of—the *TBX21* promoter: the resulting amplification of T-bet expression was responsible for the rise in IFN- $\gamma$  production.

T-bet is said to be a Th1-specific T box transcription factor that controls the expression of the hallmark Th1 cytokine, IFN- $\gamma$  (6). *TBX21*-deficient mice exhibit greater resistance to a variety of inflammatory and autoimmune diseases than their wild-type counterparts (40). Thus, it has been of interest that elevated *TBX21* levels have been found in the PBMCs of HAM/TSP patients (41). We showed that *TBX21* expression was elevated in the CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> cells of HAM/TSP patients, but not ATLL patients (Figure 2B and Supplemental Figure 1), which suggests that this trait is specific to HAM/TSP pathogenesis. Furthermore, we interpreted the lack of elevation in CD4<sup>+</sup>CD25<sup>-</sup>CCR4<sup>-</sup> cells to indicate that elevated *TBX21* is characteristic of infected cells. Importantly, we clearly demonstrated for the first time that Tax induced T-bet expression (Figure 2, C and E, and Supplemental Figures 2 and 3). Moreover, we showed that this pathway was active in CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> cells of HAM/TSP patients by silencing Tax expression and observed a corresponding reduction in *TBX21* expression; in the reverse scenario, inducing Tax expression in otherwise-normal CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> cells from HDs resulted in heightened *TBX21* expression (Figure 2D and Supplemental Figure 2). Finally, we confirmed that this correlation extended to

protein production and clearly showed how Tax induces T-bet and subsequently IFN- $\gamma$  production over time in culture (Figure 2E).

Tax has been reported to stably bind Sp1, a known positive transcriptional regulator of *TBX21* (25, 42). More specifically, interaction with Tax is thought to increase the DNA binding activity of Sp1 (42). Here we used co-IP with samples from the HTLV-1-infected MT-2 cell line to show that endogenous Tax interacted with Sp1 (Figure 3A). Subsequently, ChIP assays revealed that both Sp1 and Tax associated with the *TBX21* promoter region (Figure 3B), a novel finding that supports our theory that Tax and Sp1 together activate *TBX21* transcription. Finally, we showed that in the absence of Sp1, Tax had no significant effect on *TBX21* expression; however, in the presence of Sp1, Tax induced *TBX21* expression in a concentration-dependent manner (Figure 3C). This finding further substantiates our claim that Tax does not directly bind the promoter, but rather acts via Sp1. It should be noted that Tax may induce *TBX21* expression via multiple pathways: it has been reported that Tax enhances STAT1 gene expression in HTLV-1-transformed T cell lines and CdCl<sub>2</sub>-stimulated JPX-9 cells (38), which suggests that Tax may also induce *TBX21* expression indirectly via STAT1.

The presence of T cell infiltrates in the CNS, indicative of spinal cord inflammation, is a well-known feature of HAM/TSP. Researchers have worked to characterize these cells over the years; together, their findings suggest that the infiltrates are dominated by CD4<sup>+</sup> T cells with relatively high proviral loads and elevated Tax and IFN- $\gamma$  expression (43–45). We hypothesized that a substantial portion of the infiltrate may be made up of infected CD4<sup>+</sup>CCR4<sup>+</sup> T cells exhibiting Th1-like properties, including IFN- $\gamma$  production. We used immunohistochemistry to investigate this theory and were able to establish the presence of CD4<sup>+</sup>CCR4<sup>+</sup>CXCR3<sup>+</sup>T-bet<sup>+</sup>IFN- $\gamma$ <sup>+</sup> cells in spinal cord tissue and HTLV-1-infected CCR4<sup>+</sup> cells in the CSF of HAM/TSP patients (Figure 4, A and B). We used FACS analysis to confirm that CD4<sup>+</sup>CCR4<sup>+</sup>CXCR3<sup>+</sup> cells made up the majority of the CD4<sup>+</sup> T cells in the HAM/TSP patient CSF (Figure 4C). For the sake of continuity between this and our previous study (19), we also confirmed that the majority of these CD4<sup>+</sup>CCR4<sup>+</sup>CXCR3<sup>+</sup> cells were also CD25<sup>+</sup> (Supplemental Figure 5), further suggestive of a Treg identity.

We interpret the observation that these CD4<sup>+</sup>CCR4<sup>+</sup>CXCR3<sup>+</sup> cells were virtually nonexistent among PBMCs in HAM/TSP patients (Figure 4D) to mean that the cells had migrated to the CNS, leaving few behind in the periphery. The surprising observation that the Ki67 marker for cell proliferation was negative in the overwhelming majority of CD4<sup>+</sup>CCR4<sup>+</sup>CXCR3<sup>+</sup> cells in the CSF (Figure 4E) signifies that the cells are indeed proliferating elsewhere and subsequently migrating to the CNS. It has in fact been said that HTLV-1-infected cells may be extraordinarily capable of crossing the blood-brain barrier (46). Due to the high proportion of CCR4 positivity among HTLV-1-infected cells (19), the high proviral load in the CSF of HAM/TSP patients (47), and the elevated levels of CCL22 in HAM/TSP patient peripheral blood (30), one might hypothesize that the infected cells migrate across the blood-brain barrier in response to chemokine ligands of CCR4, namely CCL22. However, we found that the CSF of HAM/TSP patients contained only negligible amounts of CCL22, instead favoring the CXCR3 ligand CXCL10 (48). We now postulate that

CD4<sup>+</sup>CCR4<sup>+</sup>CXCR3<sup>+</sup> T cells and other CXCR3<sup>+</sup> cells may migrate to the CNS via chemotaxis induced by CXCL10 secreted by astrocytes in the CNS. Previously, we showed that these astrocytes produce CXCL10 in response to IFN- $\gamma$ , and these levels are further amplified by the invading CXCR3<sup>+</sup> cells (27). Together, these findings indicate that a positive feedback loop involving the recruitment of proinflammatory cells to the CNS is the source of chronic inflammation in HAM/TSP, and that the original trigger is the migration of IFN- $\gamma$ -producing HTLV-1-infected cells to the CNS. Where these proinflammatory cells are primarily proliferating, and why they proliferate at different rates in different settings, are questions to be addressed in future studies.

Our findings in this and previous studies imply that targeting CCR4<sup>+</sup> cells could constitute an effective treatment for HAM/TSP. Indeed, this strategy is already in play for ATLL patients, the majority of whom suffer from CCR4<sup>+</sup> T cell-derived cancers (7). The humanized defucosylated anti-CCR4 monoclonal antibody KW-0761, which has been shown to induce CCR4-specific ADCC, has been approved as a treatment for ATLL (49, 50). The observation that the majority of infected CD4<sup>+</sup> PBMCs in HAM/TSP patients were CCR4<sup>+</sup> (Figure 5A) suggests that an anti-CCR4 antibody with ADCC properties might be used to effectively treat HAM/TSP patients as well. Steroids are currently the standard of care for HAM/TSP patients, but this approach is not considered optimal: as with many nonspecific treatments, the effectiveness is limited, and the side effects are numerous (51). Here we compared the effects of the defucosylated chimeric anti-CCR4 monoclonal antibody KM2760 (21) with those of the steroid PSL on ex vivo cultures of cells from HAM/TSP patients. Although PSL had more potent effects per microgram, both treatments successfully reduced cell proliferation and IFN- $\gamma$  production (Figure 5, D, E, and G). In addition, even a small dose of the antibody effectively reduced proviral load, whereas PSL treatment had no significant effect (Figure 5, C and F). These findings support the main premise of this paper, namely, that CCR4<sup>+</sup> cells are major viral reservoirs and producers of IFN- $\gamma$ . Our study is the first to test the effects of such an antibody-based treatment on cells from HAM/TSP patients; the results were promising, and a clinical trial investigating the in vivo effectiveness in HAM/TSP patients is now underway. Importantly, our research indicates that even if the antibody does not cross the blood-brain barrier, it could be therapeutically effective against spinal cord inflammation by eliminating the proinflammatory CCR4<sup>+</sup> cells in the peripheral blood that would have migrated to the CNS.

Until very recently, there had been no reports of T cell character changing from suppressive to inflammatory in response to internal transcriptional alterations induced intracellularly by viral products. There have been many reports of Tregs transforming in the presence of inflammation due to the influence of cytokines, including instances where FOXP3 expression is lost and even cases where IFN- $\gamma$  production is gained (12, 13). The only report of a similar phenomenon occurring via an intracellular virus-induced pathway states that the HTLV-1 basic leucine zipper (HBZ) gene product can reduce the expression of FOXP3 in HBZ-transgenic mouse Tregs (52). Here we showed for the first time that the HTLV-1 virus can similarly affect gene expression in human cells, inducing IFN- $\gamma$  production, as well as reduce suppressive function. Collectively, the research to date suggests that HTLV-1 may preferentially infect

CCR4<sup>+</sup> cells, including Tregs, and induce transcriptional changes via Tax that not only reduce FOXP3 expression, but also induce T-bet expression and consequently IFN- $\gamma$  production, yielding a proinflammatory immune imbalance. While there is considerable evidence to support this theory, further experiments are necessary to prove that this pathway is indeed the origin of HAM/TSP chronic inflammation. However, here we have directly shown that the HTLV-1 protein product Tax can induce the expression of the Th1 master transcription factor T-bet, which certainly implies that HTLV-1 is capable of activating inherent plasticity in T cells and shifting their gene expression profiles toward a Th1-like state.

## Methods

**Patient selection and sample preparation.** The study included HTLV-1-noninfected HDs ( $n = 8$ , 4 male and 4 female; mean age, 36 yr), asymptomatic carriers ( $n = 6$ , 4 male and 2 female; mean age, 56 yr), ATLL patients ( $n = 6$ , 2 male and 4 female; mean age, 68 yr), and HAM/TSP patients ( $n = 31$ , 9 male and 22 female; mean age, 61 yr). Diagnosis of ATLL was based on the criteria established by Shimoyama (53). HTLV-1 seropositivity was determined by a particle agglutination assay (Serodia-HTLV-1) and confirmed by Western blot (SRL Inc.). HAM/TSP was diagnosed according to WHO guidelines (54).

Samples of PBMCs were prepared using density gradient centrifugation (Pancoll; PAN-Biotech) and viably cryopreserved in liquid nitrogen (Cell Banker 1; Mitsubishi Chemical Medience Corp.). CSF samples were taken from 17 HAM/TSP patients. CSF cells were isolated by centrifugation and cryopreserved in the aforementioned freezing medium until use. Thoracic spinal cord tissue samples from 1 HAM/TSP patient were obtained postmortem, fixed in 10% formalin, and embedded in paraffin.

**Antibodies.** For FACS studies, labeled anti-CD3 (UCHT1), anti-CD4 (OKT4), anti-GATA3 (TWAJ), and anti-FOXP3 (PCH101) were purchased from eBioscience, and labeled anti-CCR4 (1G1), anti-CD25 (BC96), anti-CXCR3 (1C6), anti-T-bet (4B10), and anti-Ki67 (B56) were purchased from BD Biosciences. For IP studies, anti-Sp1 (PEP2) and normal IgG were purchased from Santa Cruz Biotechnology Inc., and anti-Tax (Lt-4) was prepared as described previously (55). For immunofluorescence studies, anti-CCR4, anti-IFN- $\gamma$ , and anti-CXCR3 were purchased from Abcam; anti-T-bet was purchased from Santa Cruz Biotechnology Inc.; and Alexa Fluor 488- and Alexa Fluor 594-conjugated secondary antibodies were purchased from Invitrogen. Kyowa Hakko Kirin Co. Ltd. provided KM2760, a chimeric anti-CCR4 IgG1 monoclonal antibody (21).

**Plasmids.** The *TBX21*-Luc reporter gene plasmid was constructed as described previously (25). The 100-bp promoter fragment (-101 to -1) in the 5'-flanking region of the human *TBX21* gene was obtained by PCR using human PBMC genomic DNA as the template. Primers used for PCR were 5'-CGCCTCGAGGGCGGGGTGGGGCGAGGCGG-3' and 5'-CCCAAGCTTCTGTACTAGAGTCGCAGCGCTTT-3'. The amplified PCR product was digested with XhoI/HindIII and cloned into pPicaGene-Basic vector II (Toyo-ink), which yielded *TBX21*-Luc. Creation of the human Sp1 construct with HA-tag added to the N terminus was accomplished via real-time RT-PCR amplification of human PBMC cDNA with the following primers: Sp1 forward, 5'-CGC-GAATTCATGAGCGACCAAGATCACTCCATGGA-3'; Sp1 reverse, 5'-CGCCTCGAGTCAGAAGCCATTGCCACTGATATTAATG-GAC-3'. The amplified fragment was digested with EcoRI/XhoI and



subcloned into HA-tagged pcDNA3 (Invitrogen). Tax construct with FLAG-tag added to the N terminus was prepared via PCR amplification of template DNA (56) with the following primers: Tax forward, 5'-CGCGAATTCATGGCCCACTTCCCAGGGTTT-3'; Tax reverse, 5'-CGCCTCGAGTCAGACTTCTGTTTCACGGAAATGTTTTTC-3'. The amplified fragment was digested with EcoRI/XhoI and subcloned into FLAG-tagged pcDNA3. The plasmid HTLV-1 provirus (pUC/HTLV-1) was provided by T. Watanabe (University of Tokyo, Tokyo, Japan) (57). A lentiviral vector, CSIICMV, was used as a null expression vector for lentiviral infection (provided by H. Miyoshi, RIKEN BioResource Center, Tsukuba, Japan) (58). CSIICMV/GFP and CSIICMV/GFP-Tax, which express GFP and GFP fused Tax, were constructed by inserting digested GFP and GFP-Tax from pEGFP (Clontech) and pEGFP-Tax, respectively, into CSIICMV.

**Flow cytometric analysis.** PBMCs and CSF cells were immunostained with various combinations of the following fluorescence-conjugated antibodies that tag cell surface markers: CD3 (UCHT1), CD4 (OKT4), CD25 (BC96), CCR4 (1G1), CXCR3 (1C6). In some experiments, cells were fixed with a staining buffer set (eBioscience), then intracellularly stained with antibodies against T-bet (4B10), FOXP3 (PCH101), and GATA3 (TWAJ). Cells were stained with a saturating concentration of antibody in the dark (4°C, 30 minutes) and washed twice before analysis using FACSCalibur or LSR II (BD Biosciences). Data were processed using FlowJo software (TreeStar). For cell sorting, JSAN (Bay Bioscience) was used, and the purity exceeded 95%.

**Cell isolation.** CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> cells, CD4<sup>+</sup>CD25<sup>-</sup>CCR4<sup>-</sup> cells, CD4<sup>+</sup>GATA3<sup>+</sup> cells, and CD4<sup>+</sup>FOXP3<sup>+</sup> cells were separated by FACS sorting. CD4<sup>+</sup> T cells were isolated from PBMCs using negative selection with magnetic beads (MACS CD4<sup>+</sup> T cell isolation kit; Miltenyi Biotec). CD4<sup>+</sup>CCR4<sup>-</sup> or CD4<sup>+</sup>CCR4<sup>+</sup> cells were then isolated from these CD4<sup>+</sup> T cells using positive selection with anti-CCR4 Ab (1G1) and rat anti-mouse IgG1 microbeads (Miltenyi Biotec).

**Cell culture conditions.** HEK293 cells were cultured in MEM (Wako Pure Chemical Industries) supplemented with 10% heat-inactivated FBS (Gibco, Invitrogen) and 1% penicillin/streptomycin (P/S) (Wako Pure Chemical Industries). HEK293T cells were cultured in DMEM-high glucose (Sigma-Aldrich) supplemented with 10% FBS and 1% P/S. Jurkat, MT-2, and JPX-9 cells were cultured in RPMI 1640 medium (Wako Pure Chemical Industries) supplemented with 10% FBS and 1% P/S. JPX-9 is a subline of Jurkat carrying Tax under the control of the metallothionein promoter (provided by M. Nakamura, Tokyo Medical and Dental University, Tokyo, Japan) (59), by which Tax expression is inducible by the addition of 20 μM CdCl<sub>2</sub> (Nacalai Tesque Inc.). PBMCs, CD4<sup>+</sup>CCR4<sup>+</sup> cells, CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> cells, and CD4<sup>+</sup>CD25<sup>-</sup>CCR4<sup>-</sup> cells isolated from HDs or HAM/TSP patients were cultured in RPMI 1640 medium supplemented with 5% human AB serum (Gibco, Invitrogen) and 1% P/S.

**Gene expression profiling and analyses.** For transcriptional profiling, CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> T cells from a HAM/TSP patient, an ATLL patient, and an HD were separated using FACS sorting. Total RNA was prepared using ISOGEN (Nippon gene) following the manufacturer's recommendations. RNA was amplified and labeled with cyanine 3 (Cy3) using an Agilent Quick Amp Labeling Kit, 1-color (Agilent Technologies), following the manufacturer's instructions. For each hybridization, Cy3-labeled cRNA were fragmented and hybridized to an Agilent Human GE 4x44K Microarray (design ID 014850). After washing,

microarrays were scanned using an Agilent DNA microarray scanner. Intensity values of each scanned feature were quantified using Agilent feature extraction software (version 9.5.3.1), which performs background subtractions. All data were analyzed using GeneSpring GX software (Agilent Technologies). There were a total of 41,000 probes on Agilent Human GE 4x44K Microarray (design ID 014850), not including control probes. Microarray data were deposited in GEO (accession no. GSE57259).

**Real-time PCR and real-time RT-PCR.** The HTLV-1 proviral DNA load was measured using ABI Prism 7500 SDS (Applied Biosystems) as described previously (19). For real-time RT-PCR analysis, total RNA isolation and cDNA synthesis were performed as described previously (19). Real-time PCR reactions were carried out using TaqMan Universal Master Mix (Applied Biosystems) and Universal Probe Library assays designed using ProbeFinder software (Roche Applied Science). ABI Prism 7500 SDS was programmed to have an initial step of 2 minutes at 50°C and 10 minutes at 95°C, followed by 45 cycles of 15 seconds at 95°C and 1 minute at 60°C. The primers used were as follows: *TBX21*, 5'-TGTGGTCCAAGTTTAATCAGCA-3' (forward) and 5'-TGACAGGAATGGGAACATCC-3' (reverse) (probe no. 9; Roche Applied Science); *Tax*, 5'-ATACAACCCCAACATTTCCA-3' (forward) and 5'-TTTCGGAAGGGGAGTATTT-3' (reverse) (probe no. 69; Roche Applied Science). The primers and probes for detecting *Tax*, *IFNG*, and *GAPDH* mRNA were described previously (19). Relative quantification of mRNA was performed using the comparative Ct method using *GAPDH* as an endogenous control. For each sample, target gene expression was normalized to the expression of *GAPDH*, calculated as  $2^{-(Ct[\text{target}] - Ct[\text{GAPDH}])}$ .

**Virus preparation and cell infection.** 293T cells ( $1 \times 10^6$ ) plated in 100-mm dishes were cotransfected with the appropriate lentiviral-GFP or lentiviral-GFP-Tax expression vector (17 μg), vesicular stomatitis virus G expression vector VSV-G (pMD.G) (5 μg), rev expression vector pRSVRev (5 μg), and gag-pol expression vector pMDLg/pRRE (12 μg) (60) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. After 4 hours, cells were washed 3 times with PBS, 5 ml of new medium was added, and cells were incubated for 48 hours. Culture supernatants were harvested and filtered through 0.45-μm pore size filters. Lentivirus was concentrated approximately 40-fold by low centrifugation at 6,000 g for 16 hours and resuspended in 2 ml RPMI 1640 medium. Freshly isolated CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> T cells were activated using Treg Suppression Inspector (Anti-Biotin MACSiBead Particles preloaded with biotinylated CD2, CD3, and CD28 antibodies) according to the manufacturer's protocol (Miltenyi Biotec). After being cultured for 36 hours, cells were transduced with equal amounts of the GFP or GFP-Tax lentivirus (MOI 15), followed by centrifugation for 1 hour at 780 g, 32°C. After being cultured for 4 hours at 32°C, cells were washed with culture medium and cultured in round-bottomed 96-well plates at 37°C.

**Treg suppression assay.** A study was conducted to compare the capacities of GFP versus GFP-Tax lentivirus-infected CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> T cells to suppress cell proliferation. T cell samples were taken from HDs, and  $5 \times 10^4$  CD4<sup>+</sup>CD25<sup>+</sup> T cells were stimulated with the Treg Suppression Inspector (see above) according to the manufacturer's instructions. These cells were then cocultured with  $5 \times 10^4$  GFP lentivirus-infected CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> T cells or GFP-Tax lentivirus-infected CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> T cells. After culturing for 4 days, cell proliferation was measured using a <sup>3</sup>H-thymidine incorporation assay as described previously (19).

**RNA interference assay.** siRNA was synthesized chemically at Hokkaido System Science. The sequences of siRNA oligonucleotides were as follows: Tax, 5'-GGCCUUAUUUGGACAUUUATT-3' and 5'-UAAAGUCCAAUAAGGCCTT-3' (31); Luc, 5'-CGUACGCG-GAAUACUUCGATT-3' and 5'-UCGAAGUAUUCGCGUACGTT3'. Next, 100 pmol annealed RNA duplex was transfected using Human T cell Amaxa Nucleofector Kit according to the manufacturer's recommendations (Lonza). 100 pmol Luc siRNA was used as a negative control. Cells were incubated for 48 hours and then harvested and subjected to real-time RT-PCR analysis.

**Measurement of IFN- $\gamma$ .** IFN- $\gamma$  concentration in the culture supernatant was measured with a cytometric bead array kit (BD Biosciences) using a FACSCalibur flow cytometer (BD Biosciences) according to the manufacturer's instructions.

**IP.** Approximately 1 mg of MT-2 nuclear extracts were incubated with 5  $\mu$ g anti-Tax, anti-Sp1, or normal IgG coupled with protein G-agarose (Roche Applied Science) in IP buffer (10 mM HEPES [pH 7.9], 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1% NP-40, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 5 mM NaF, 2  $\mu$ g/ml aprotinin, 2  $\mu$ g/ml leupeptin, and 2  $\mu$ g/ml pepstatin) for 2 hours. The precipitated proteins were washed with the IP buffer, separated by 10% SDS-PAGE, and immunoblotted with anti-Tax or anti-Sp1 antibodies.

**ChIP assay.** ChIP assay was performed using a ChIP assay kit (Upstate Biotechnology) with some modifications. Briefly, 5  $\times$  10<sup>6</sup> MT-2 cells were fixed with 1% formaldehyde at 37°C for 25 minutes and washed twice with PBS. Cells were subsequently harvested and sonicated in lysis buffer. Precleared chromatin samples were immunoprecipitated with 5  $\mu$ g anti-Tax antibody, anti-Sp1 antibody, or normal IgG for 16 hours at 4°C. Immune complexes were collected with salmon sperm DNA/protein G-sepharose for 90 minutes with rotation, washed, and then incubated at 65°C for 6 hours for reverse cross-linking. Chromatin DNA was extracted and analyzed using PCR with primers for the *TBX21* promoter region (-179 to -59; forward, 5'-GCCAAGAGCGTAGAATTGTC-3'; reverse, 5'-CGCTTT-GCTGTGGCTTTATG-3') (25, 61). Amplification was performed using ExTaq (Takara Bio) with 1 cycle at 95°C for 5 minutes followed by 30 cycles of 95°C for 30 seconds, 54°C for 30 seconds, and 72°C for 30 seconds. Amplified products were analyzed using 8% polyacrylamide gel electrophoresis.

**Luciferase assay.** For transient transfection, HEK293 cells were seeded at 5  $\times$  10<sup>4</sup> cells/well into 24-well plates. After 12 hours, medium was changed to MEM supplemented with 10% FBS and 1% P/S, and each plasmid was transfected with CellPfect Transfection Kit according to the manufacturer's recommendations (GE Healthcare). 50 ng pRSV- $\beta$ gal plasmid was included in each transfection experiment to control for the efficiency of transfection. The total amount of transfected DNA was kept constant with pcDNA3 in all samples. After 48 hours, cells were lysed with Passive Lysis Buffer (Promega), and luciferase activity was measured using the Promega luciferase assay system and MicroLumat Plus LB96V (Berthold Technologies). Values were normalized to  $\beta$ -galactosidase activity as an internal control.

**Tissue staining.** Formalin-fixed thoracic spinal cord tissue sections were deparaffinized in xylene and rehydrated in a series of graded alcohols and distilled water. The antigenicity of the tissue sections was recovered using a standard microwave heating technique. For immunofluorescence, the slides were incubated in PBS with 10% goat

serum for 1 hour at room temperature, then in anti-CCR4 antibody, anti-T-bet antibody, anti-IFN- $\gamma$  antibody, and anti-CXCR3 antibody overnight at 4°C, labeled with Alexa Fluor 488- or Alexa Fluor 594-conjugated secondary antibody, and examined under a fluorescence microscope (Nikon eclipse E600 with fluorescence filter Nikon F-FL; Nikon Instech) with rabbit or goat IgG as the negative control. Tissue sections were also stained with H&E.

**Immunofluorescence staining and immunofluorescence-FISH.** Jurkat cells, MT-2 cells, and cells from the CSF of 3 HAM/TSP patients were attached to slides using a cytospin centrifuge (Thermo Fisher Scientific) and fixed in 4% paraformaldehyde (Wako Pure Chemical Industries) for 30 minutes. The slides were washed with PBS and then pretreated as follows: slides were immersed in room temperature 0.2M HCl for 20 minutes, followed by 0.2% Triton-X/PBS for 10 minutes, and finally 0.005% pepsin/0.1M HCl heated to 37°C for 5 minutes. After pretreatment, the slides were stained using the immunofluorescence Can Get Signal kit (TOYOBO) according to the manufacturer's instructions with anti-CCR4 as the primary antibody and Alexa Fluor 488-conjugated anti-goat IgG as the secondary antibody. After again being fixed with 4% paraformaldehyde, cells were incubated with a nick-translated (Spectrum Red) pUC/HTLV-1 DNA probe, first for 5 minutes at 70°C and then overnight at 37°C. Images were obtained under an automated research microscope (Leica DMRA2) and analyzed with CW4000 FISH software (Leica Microsystems).

**Proliferation assay.** PBMCs from HAM/TSP patients were plated into 96-well round-bottomed plates (1  $\times$  10<sup>5</sup> cells/well) and cultured without any mitogenic stimuli. Cell proliferation was measured using a <sup>3</sup>H-thymidine incorporation assay as described previously (19).

**Statistics.** Paired 2-tailed Student's *t* test and Wilcoxon test were used for within-group comparisons. Unpaired 2-tailed Student's *t* test or Mann-Whitney *U* test was used for between-group comparisons. 1-way ANOVA was used for multiple comparisons, followed by Dunnett or Tukey test. Friedman test was used for paired multiple comparisons, followed by Dunn test. Statistical analyses were performed using Graphpad Prism 5 (GraphPad Software Inc.). A *P* value less than 0.05 was considered significant.

**Study approval.** Written informed consent was obtained from all patients before the study, which was reviewed and approved by the Institutional Ethics Committee at St. Marianna University and conducted in compliance with the tenets of the Declaration of Helsinki.

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