

Table II. Synthetic oligopeptides used in this study

Peptide	Sequence
Tax103-127	P S F L Q A M R K Y S P F R N G Y M E P T L G Q H
Tax120-144	M E P T L G Q H L P T L S F P D P G L R P Q N L Y
Tax137-161	G L R P Q N L Y T L W G G S V V C M Y L Y Q L S P
Tax154-178	M Y L Y Q L S P P I T W P L L P H V I F C H P G Q
Tax171-195	V I F C H P G Q L G A F L T N V P Y K R I E E L L
Tax188-212	Y K R I E E L L Y K I S L T T G A L I I L P E D C
Tax205-229	L I I L P E D C L P T T L F Q P A R A P V T L T A
Tax222-246	R A P V T L T A W Q N G L L P F H S T L T T P G I
Tax146-160	L W G G S V V C M Y L Y Q L S
Tax151-165	V V C M Y L Y Q L S P P I T W
Tax154-168	M Y L Y Q L S P P I T W P L L
Tax155-169	Y L Y Q L S P P I T W P L L P
Tax156-170	L Y Q L S P P I T W P L L P H
Tax161-175	P P I T W P L L P H V I F C H
Tax166-180	P L L P H V I F C H P G Q L G
Tax155-168	Y L Y Q L S P P I T W P L L
Tax155-167	Y L Y Q L S P P I T W P L
Tax155-166	Y L Y Q L S P P I T W P

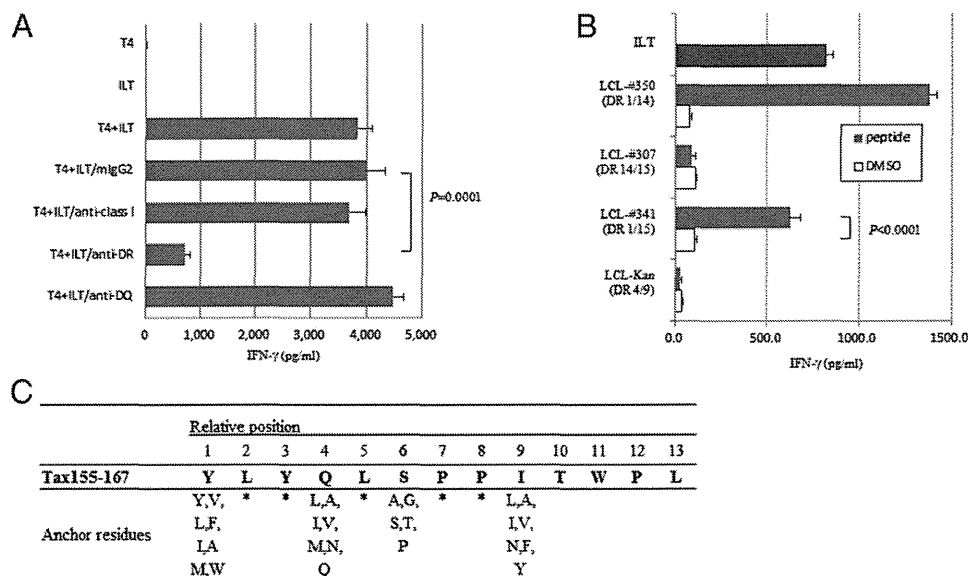
Tax155-167-specific CD4<sup>+</sup> T cells were detected ex vivo in the patient #350 (0.11%) and proliferated to 11.6% among CD4<sup>+</sup> T cells at 13 d poststimulation with Tax155-167 peptide. In the patient #364, tetramer-binding CD4<sup>+</sup> T cells were undetectable in fresh PBMCs but expanded to 0.37% by in vitro stimulation with Tax155-167 peptide (Fig. 6A). In an HLA-DRB1\*0101<sup>+</sup>-seronegative donor #365, Tax155-167-specific CD4<sup>+</sup> T cells were not found in fresh PBMCs and did not become detectable at 13 d after stimulation with Tax155-167 peptide (Fig. 6A). This result indicates that Tax155-167-specific CD4<sup>+</sup> T cells are maintained and possesses the abilities to proliferate in response to HTLV-1 Tax in these patients.

We further examined whether Tax155-167-specific CD4<sup>+</sup> T cells existed in two HTLV-1-infected individuals carrying HLA-DRB1\*0101, an AC #310 and a HAM/TSP patient #294, and detected 0.18 and 0.31% of tetramer-binding cells in peripheral

CD4<sup>+</sup> T cells, respectively (Fig. 6B). These results suggest that Tax155-167-specific CD4<sup>+</sup> T cells are maintained in HTLV-1-infected individuals expressing an HLA-DRB1\*0101 allele, regardless of HSCT.

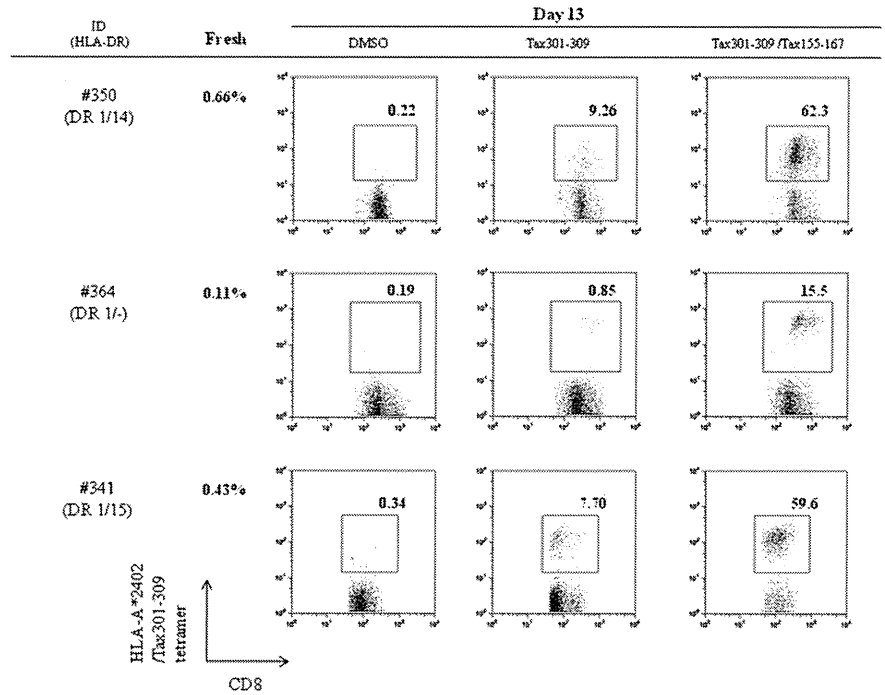
### Discussion

In this study, we demonstrated Tax-specific CD4<sup>+</sup> T cell responses in some ATL patients post-allo-HSCT and identified a novel HLA-DRB1\*0101-restricted CD4 T cell epitope, Tax155-167, which was recognized by HTLV-1-specific CD4<sup>+</sup> T cells and consequently led to robust Tax-specific CD8<sup>+</sup> T cell expansion. We also found that Tax155-167-specific CD4<sup>+</sup> T cells existed in all HTLV-1-infected HLA-DRB1\*0101<sup>+</sup> individuals tested, regardless of HSCT, by newly generated HLA-DRB1\*0101/Tax155-167 tetramers. These results suggest that Tax155-167 might be a dominant epitope recognized by HTLV-1-specific CD4<sup>+</sup> T cells



**FIGURE 4.** HLA-DRB1\*0101 restriction of Tax155-167 recognition by established T4 cells. **(A)** T4 cells were cocultured for 6 h with ILT-#350 in the presence or absence of the following blocking Abs (10 μg/ml): anti-human HLA-DR; anti-human HLA-DQ; anti-HLA-class I; or isotype control. IFN-γ production from T4 cells was measured by ELISA. **(B)** The T4 cells were cocultured for 6 h with autologous (#350) or allogeneic (#307, #341, and Kan) LCLs pulsed with (closed bar) or without (open bar) Tax155-167 for 1 h or with recipient-derived ILT-#350. The HLA-DR alleles of each LCL line are indicated in parentheses. IFN-γ production of T4 cells was assessed by ELISA. **(A and B)** Representative data of three independent experiments are shown. The error bars represent SD of triplicate wells. Statistical significance was analyzed by the unpaired *t* test. **(C)** The amino acid sequence between residues 155 and 167 of Tax contained a putative HLA-DRB1\*0101 anchor motif (33).

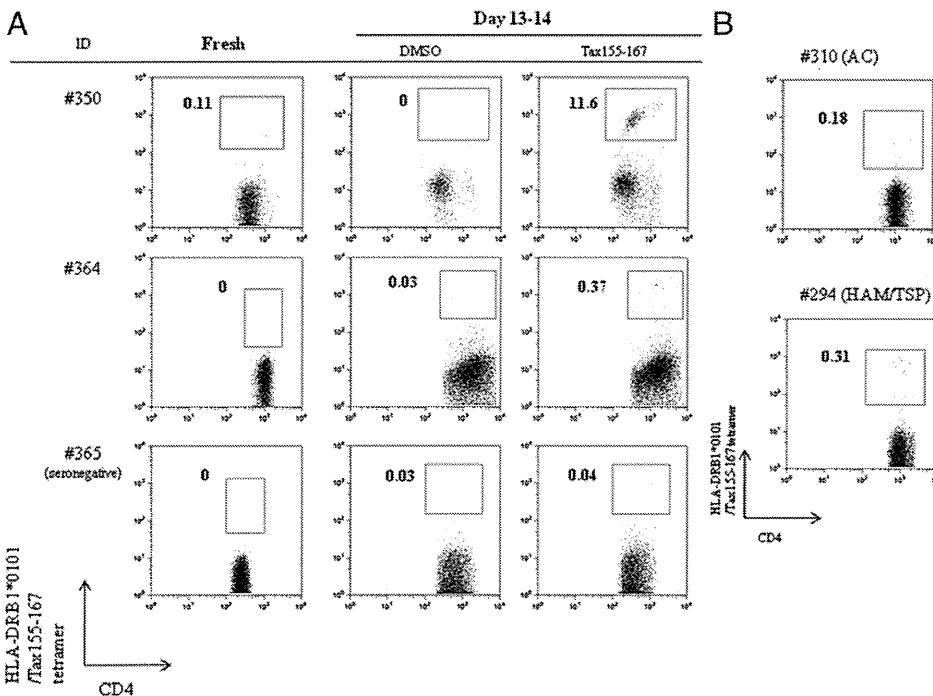
**FIGURE 5.** Augmentation of Tax-specific CD8<sup>+</sup> T cell expansion by costimulation with CTL epitope and Tax155–167 peptides. PBMCs from HLA-DRB1\*0101- and HLA-A24-expressing ATL patients (#350, #364, and #341) who underwent allo-HSCT with RIC were cultured for 13 d in the presence of DMSO, 100 nM CTL epitope (Tax301–309), or a mixture of Tax301–309 (100 nM) and Tax155–167 (100 nM) peptides. Data indicate percentages of HLA-A\*2402/Tax301–309 tetramer<sup>+</sup> cells among CD3<sup>+</sup>CD8<sup>+</sup> T cells. Fresh indicates frequency of HLA-A\*2402/Tax301–309 tetramer<sup>+</sup>CD8<sup>+</sup> T cells detected in fresh peripheral blood.



in HTLV-1-infected individuals expressing HLA-DRB1\*0101 and that Tax-specific CD4<sup>+</sup> T cells might efficiently induce HTLV-1-specific CTL expansion to strengthen the graft-versus-ATL effects in ATL patients after allo-HSCT.

In HTLV-1 infection, analysis of virus-specific CD4<sup>+</sup> T cell responses appears to be limited because CD4<sup>+</sup> T cells are preferentially infected with HTLV-1 (24, 34, 35), and HTLV-1 Ags are produced from infected cells at a few hours postculture (34, 36). In this study, we used blood samples from 18 ATL patients after allo-HSCT with RIC and from HLA identical-related or unrelated donors and found that these recipients had undetectable or very low proviral loads (Table I), as previously shown (7–9). We previously reported that Tax-specific CTLs were induced in some patients with complete remission after allo-HSCT for ATL and

might contribute to the graft-versus-leukemia effect (10). In the current study, Tax-specific T cell responses or tetramer-binding CD8<sup>+</sup> T cells were detected in 68.8% (11 of 16) or 82.4% (14 of 17) of patients tested, respectively (Fig. 1A, Table I). In addition, helper function of Tax-specific CD4<sup>+</sup> T cells to enhance Tax-specific CD8<sup>+</sup> T cell expansion was observed in PBMCs from all three HLA-DRB1\*0101<sup>+</sup> patients tested (Fig. 5). These data suggest that both CD8<sup>+</sup> and CD4<sup>+</sup> Tax-specific T cell responses might contribute to elimination of remaining leukemic and/or infected cells in some patients having T cell responses against Tax. However, given the fact that not all ATL patients who achieved complete remission after allo-HSCT had Tax-specific CD8<sup>+</sup> T cells, graft-versus-host reaction may mainly contribute to achieve complete remission after allo-HSCT. It is of note that Tax-specific



**FIGURE 6.** Detection of Tax155–167-specific CD4<sup>+</sup> T cells in HTLV-1-infected HLA-DRB1\*0101<sup>+</sup> individuals. (A) In two ATL patients after allo-HSCT (#350 and #364) and an HLA-DRB1\*0101<sup>+</sup>-seronegative donor (#365), frequency of HLA-DRB1\*0101/Tax155–167 tetramer-binding CD4<sup>+</sup> T cells was analyzed in fresh PBMCs and PBMCs cultured for 13–14 d in the presence of Tax155–167 (100 nM) peptide. Data indicate percentages of tetramer<sup>+</sup> cells in CD3<sup>+</sup>CD4<sup>+</sup> T cells. (B) Frequency of HLA-DRB1\*0101/Tax155–167 tetramer-binding CD4<sup>+</sup> T cells in fresh PBMCs from an AC #310 and an HAM/TSP patient #294 was analyzed. Data indicate percentages of tetramer<sup>+</sup> cells in CD3<sup>+</sup>CD4<sup>+</sup> T cells.

T cell responses were detected in 57.1% (four of seven) or 87.5% (seven of eight) of the patients after allo-HSCT with RIC from HTLV-1-seronegative sibling or unrelated donors, respectively. A Tax-specific T cell response was not detected in three patients who underwent allo-HSCT from seropositive donors (Fig. 1, Table I).

It has been proposed that CTLs are the main effector cells against many pathogenic viruses, including HTLV-1. To date, many CTL epitopes recognized by HTLV-1-specific CTLs have been identified, some of which are thought to be the candidates of peptide-based T cell immunotherapy (10, 20, 32, 37–40). CD4<sup>+</sup> T cells have also been known to be critical for induction and maintenance of Ag-specific CD8<sup>+</sup> T cells (15–19). With respect to HTLV-1 infection, there are several reports identifying HLA-DRB1\*0101-restricted epitopes recognized by CD4<sup>+</sup> T cells against Env or Tax (Env380–394 (21), Env436–450, Env451–465, Env456–470 (23), and Tax191–205 (22)), which were established by stimulating PBMCs from uninfected or infected individuals with synthetic peptides. In this study, for determination of an epitope recognized by HTLV-1-specific CD4<sup>+</sup> T cells, we established an HTLV-1-specific CD4<sup>+</sup> T cell line from the patient #350 at 180 d after allo-HSCT by several stimulations with an HTLV-1 Ags-expressing T cell line (ILT-#350) from the same patient. In addition, we found that Tax155–167-specific CD4<sup>+</sup> T cells were present in peripheral blood from patient #350 at 180 and 540 d after allo-HSCT, indicating that the epitope, Tax155–167, identified in this study is naturally presented on HTLV-1-infected cells and predominantly recognized by HTLV-1-specific CD4<sup>+</sup> Th cells in the patient #350 at least within 540 d after allo-HSCT. Another HLA-DRB1\*0101-restricted Tax epitope, Tax191–205, has been reported previously (22). In this study, the amino acid sequence within this region was revealed to be conserved in the infected T cell line, ILT-#350 established from the patient #350 (data not shown), indicating that Tax191–205 can be presented on APCs and Tax191–205-specific CD4<sup>+</sup> T cells may be induced in patient #350. However, Tax155–167-specific but not Tax191–205-specific CD4<sup>+</sup> T cells were revealed to predominantly appear in the HTLV-1-specific T4 cell line, established from PBMCs in the patient #350 at 180 d after allo-HSCT. This suggests that in the case of patient #350 at 180 d after allo-HSCT, Tax191–205-specific CD4<sup>+</sup> T cells may not be the most frequent population among HTLV-1-specific CD4<sup>+</sup> T cells.

It has been known that Ag-specific effector and memory CD4<sup>+</sup> T cells are typically present at much lower frequencies than their CD8<sup>+</sup> counterparts and that MHC class II tetramer might have a weak TCR–MHC affinity (41). Although this limited affinity of MHC class II tetramer might preclude detection of Ag-specific low-affinity CD4<sup>+</sup> T cells, the low-affinity CD4<sup>+</sup> T cells, below detection with MHC class II tetramers, were also proved to be critical effectors in Ag-specific responses (42). In the current study, MHC class II tetramer analysis revealed that Tax155–167-specific CD4<sup>+</sup> T cells were present in HLA-DRB1\*0101<sup>+</sup> HTLV-1-infected individuals: two ATL patients after allo-HSCT (day 180 for #350 and day 360 for #364), an AC #310, and a HAM/TSP patient #294 (Fig. 6). Because of a shortage of blood sample from patient #341, we could not perform the direct detection for Tax155–167-specific CD4<sup>+</sup> T cells by the MHC class II tetramers. However, enhanced expansion of Tax301–309-specific CD8<sup>+</sup> T cells was observed in patient #341 at 360 d after allo-HSCT when PBMCs were stimulated with Tax301–309 in the presence of Tax155–167 (Fig. 5). So far, Tax155–167-specific CD4<sup>+</sup> T cells were detected in fresh and/or Tax155–167-stimulated PBMCs of all HTLV-1-infected HLA-DRB1\*0101<sup>+</sup> individuals tested, although their frequencies were various. These results suggest that Tax155–167 may be the dominant epitope recognized by Tax-

specific CD4<sup>+</sup> T cells in HTLV-1-infected HLA-DRB1\*0101<sup>+</sup> individuals. In ATL patients after HSCT, the donor-derived T cells reconstituted in recipients will first encounter HTLV-1 Ags, because HTLV-1 still persists in the patients even though proviral loads become undetectable in the peripheral bloods. Indeed, we found that donor-derived Tax155–167-specific CD4<sup>+</sup> T cells were present in three ATL patients after allo-HSCT from seronegative donors. This finding also suggests that Tax155–167-specific naive CD4<sup>+</sup> T cells may pre-exist in HLA-DRB1\*0101<sup>+</sup> individuals and can be primed with HTLV-1 Ags during the primary infection. In this study, Tax155–167-specific CD4<sup>+</sup> T cells were also detected in an AC and a HAM/TSP patient (Fig. 6B), suggesting that Tax155–167-specific CD4<sup>+</sup> T cells may be maintained in some HLA-DR1<sup>+</sup> individuals during the chronic phase of HTLV-1 infection. However, it has been reported that epitope hierarchies may change because of T cell escape mutants (43, 44) and unresponsiveness or deletion of epitope-specific T cells because of prolonged Ag stimulation during chronic infection (45, 46). Further longitudinal studies with a number of samples will be required to confirm that Tax155–167 is a dominant epitope of HTLV-1-specific CD4<sup>+</sup> T cells in HLA-DRB1\*0101<sup>+</sup>-infected individuals in the course of HTLV-1 infection.

Among three patients (#241, #350, and #364) showing high T cell responses against recombinant Tax protein, two patients (#350 and #364) were found to carry HLA-DRB1\*0101 and have efficient CD4<sup>+</sup> Th cell responses against Tax155–167. Intriguingly, it has been reported that HLA-DRB1\*0101 is associated with susceptibility to HAM/TSP (47, 48). In addition, CD4<sup>+</sup> T cells have been shown to be the dominant cells infiltrating in early active inflammatory spinal cord lesions (28, 29) with spontaneous production of proinflammatory cytokines (30). These observations suggest that HLA-DRB1\*0101 might be associated with susceptibility to HAM/TSP via an effect on high CD4<sup>+</sup> T cell activation. Further studies are needed to clarify whether HLA-DRB1\*0101 is associated with high Tax-specific CD4<sup>+</sup> T cell responses in HTLV-1-infected individuals.

Early studies using lymphocytic choriomeningitis virus showed that CD4<sup>+</sup> T cell help is critical for maintenance of CD8<sup>+</sup> T cell function during chronic infections (18). It has also been suggested that CD4<sup>+</sup> T cells are required for optimal CTL responses during HTLV-1 infection (49). Aubert et al. (50) showed that both Ag-specific naive and effector CD4<sup>+</sup> T cell help rescued exhausted CD8<sup>+</sup> T cells in vivo, resulting in a decrease in viral burden. In the current study, we determined a novel HLA-DRB1\*0101-restricted Th epitope, Tax155–167, which was capable of augmenting Tax-specific CD8<sup>+</sup> T cell expansion by stimulating Tax155–167-specific CD4<sup>+</sup> T cells. This epitope would be a useful tool for investigating the roles of HTLV-1-specific CD4<sup>+</sup> T cells in antitumor immunity and in pathogenesis of HTLV-1-related inflammatory diseases such as HAM/TSP and developing novel vaccines to prevent progression or recurrence of ATL.

## Disclosures

The authors have no financial conflicts of interest.

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RESEARCH

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# HTLV-1 modulates the frequency and phenotype of FoxP3<sup>+</sup>CD4<sup>+</sup> T cells in virus-infected individuals

Yorifumi Satou<sup>1,5\*</sup>, Atee Utsunomiya<sup>2</sup>, Junko Tanabe<sup>1</sup>, Masanori Nakagawa<sup>3</sup>, Kisato Nosaka<sup>4</sup> and Masao Matsuoka<sup>1\*</sup>

## Abstract

**Background:** HTLV-1 utilizes CD4 T cells as the main host cell and maintains the proviral load via clonal proliferation of infected CD4<sup>+</sup> T cells. Infection of CD4<sup>+</sup> T cells by HTLV-1 is therefore thought to play a pivotal role in HTLV-1-related pathogenicity, including leukemia/lymphoma of CD4<sup>+</sup> T cells and chronic inflammatory diseases. Recently, it has been reported that a proportion of HTLV-1 infected CD4<sup>+</sup> T cells express FoxP3, a master molecule of regulatory T cells. However, crucial questions remain unanswered on the relationship between HTLV-1 infection and FoxP3 expression.

**Results:** To investigate the effect of HTLV-1 infection on CD4<sup>+</sup> T-cell subsets, we used flow cytometry to analyze the T-cell phenotype and HTLV-1 infection in peripheral mononuclear cells (PBMCs) of four groups of subjects, including 23 HTLV-1-infected asymptomatic carriers (AC), 10 patients with HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP), 10 patients with adult T-cell leukemia (ATL), and 10 healthy donors. The frequency of FoxP3<sup>+</sup> cells in CD4<sup>+</sup> T cells in AC with high proviral load and patients with HAM/TSP or ATL was higher than that in uninfected individuals. The proviral load was positively correlated with the percentage of CD4<sup>+</sup> T cells that were FoxP3<sup>+</sup>. The CD4<sup>+</sup>FoxP3<sup>+</sup> T cells, themselves, were frequently infected with HTLV-1. We conclude that FoxP3<sup>+</sup> T-cells are disproportionately infected with HTLV-1 during chronic infection. We next focused on PBMCs of HAM/TSP patients. The expression levels of the T<sub>reg</sub> associated molecules CTLA-4 and GITR were decreased in CD4<sup>+</sup>FoxP3<sup>+</sup> T cells. Further we characterized FoxP3<sup>+</sup>CD4<sup>+</sup> T-cell subsets by staining CD45RA and FoxP3, which revealed an increase in CD45RA<sup>-</sup>FoxP3<sup>low</sup> non-suppressive T-cells. These findings can reconcile the inflammatory phenotype of HAM/TSP with the observed increase in frequency of FoxP3<sup>+</sup> cells. Finally, we analyzed ATL cells and observed not only a high frequency of FoxP3 expression but also wide variation in FoxP3 expression level among individual cases.

**Conclusions:** HTLV-1 infection induces an abnormal frequency and phenotype of FoxP3<sup>+</sup>CD4<sup>+</sup> T cells.

**Keywords:** HTLV-1, ATL, HAM/TSP, FoxP3, Tax, HBZ

## Background

Human T-cell leukemia virus type 1 (HTLV-1) is a delta type retrovirus, which causes leukemia of HTLV-1-infected CD4<sup>+</sup> T cells, known as adult T-cell leukemia (ATL) [1-4], in 2 to 5 % of infected individuals. HTLV-1 is also associated with chronic inflammatory diseases [5,6], including HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP), uveitis, alveolitis [7], and dermatitis [8]. It has been

estimated that 20 million people are infected with HTLV-1 in the world. HTLV-1 has a characteristic proliferative strategy; HTLV-1 increases its copy number not via vigorous production of cell-free viral particle but mainly via proliferation of infected host cells, which contain the integrated HTLV-1 provirus in the host genome [9,10]. Given the fact that HTLV-1 utilizes CD4<sup>+</sup> T cells as the major host cell population, the pathogenesis by this virus may be due to abnormalities of CD4<sup>+</sup> T cells in HTLV-1-infected individuals. However the precise characteristics of the putative CD4<sup>+</sup> T-cell abnormality still remain to be elucidated.

In addition to viral structural proteins, such as Gag, Pol, and Env, HTLV-1 encodes several regulatory and

\* Correspondence: y.satou@imperial.ac.uk; mmatsuok@virus.kyoto-u.ac.jp  
<sup>1</sup>Laboratory of Virus Control, Institute for Virus Research, Kyoto University, Kyoto, 606-8507, Japan

<sup>5</sup>Current address: Immunology Section, Division of Infectious Diseases, Department of Medicine, Imperial College, London, W2 1PG, UK  
Full list of author information is available at the end of the article

accessory proteins, including Tax, Rex, p30, p12, and HTLV-1 bZIP factor (HBZ), which regulate viral gene expression or proliferation of infected host cells [4]. After the HTLV-1 provirus is integrated into the host genome, the virus expresses these regulatory and accessory proteins to induce host cell proliferation or viral latency, resulting in persistent infection *in vivo*. Tax is known to influence various host cell-signaling pathways, for example activation of NF- $\kappa$ B, and to contribute to proliferation and survival of infected cells [11,12]. Another viral gene, the HBZ, which is encoded in the minus strand of HTLV-1 [13] and expressed constitutively in the infected host cells [14,15], also contributes to proliferation of the infected cells [14,16], dysregulation of differentiation and function of CD4<sup>+</sup> T cells [17], and the pathogenesis of diseases such as T-cell lymphoma and chronic inflammatory diseases [17,18]. On the other hand, viral protein expression induces the host immune response to eliminate the virus, which includes both antibody and cytotoxic T lymphocytes (CTL) against the viral antigens [19-21]. It has been reported that the CTL response against this virus determines HTLV-1 proviral load; yet, the host immune system cannot eliminate the HTLV-1 completely, which allows HTLV-1 to establish persistent infection in almost all infected individuals.

Recent studies have clarified the presence of various CD4<sup>+</sup> T-cell subsets. CD4<sup>+</sup> T cells can be divided into two major categories, effector T cells and regulatory T cells. Effector T cells induce the activation of immune responses by secreting pro-inflammatory cytokines whereas regulatory T cells, which express the transcription factor FoxP3 [22-24], suppress the immune response by both cell-contact dependent and independent mechanisms [25]. As an example of cell contact dependent suppression, expression of the immune suppressive molecule CTLA-4 on the cell surface inhibits the activation of surrounding neighboring T cells [26]. In addition, a recent report demonstrated that human FoxP3<sup>+</sup>CD4<sup>+</sup> T cells were composed of three phenotypically and functionally different subsets according to the degree of FoxP3 expression and CD45RA expression, namely CD45RA<sup>+</sup>FoxP3<sup>low</sup> resting T<sub>reg</sub> cells (rT<sub>reg</sub> cells), CD45RA<sup>+</sup>FoxP3<sup>high</sup> activated T<sub>reg</sub> cells (aT<sub>reg</sub> cells), or CD45RA<sup>-</sup>FoxP3<sup>low</sup> non-suppressive T cells (FoxP3<sup>low</sup> non-Treg cells) [27]. Both rT<sub>reg</sub> cells and aT<sub>reg</sub> cells have suppressive function, but FoxP3<sup>low</sup> non-Treg cells are not suppressive.

Previous studies have reported that the HTLV-1 provirus is enriched in effector/memory T cells [28,29], and the phenotype of ATL cells shares certain characteristics with regulatory T cells based on the finding of FoxP3 expression [30,31]. However there are few studies that systematically and specifically investigate which recently

described CD4<sup>+</sup> T-cell subset is infected by HTLV-1 in asymptomatic carriers (AC), HAM/TSP patients, and ATL patients. To elucidate this point, we analyzed peripheral mononuclear cells (PBMCs) from naturally HTLV-1-infected individuals, including AC, HAM/TSP, and ATL patients, by using multicolor flow cytometric analysis combined with the detection of the viral antigen Tax to identify the presence of HTLV-1 [32]. We found the specific CD4<sup>+</sup>FoxP3<sup>+</sup> T-cell subset is frequently infected with HTLV-1, which may allow the virus to achieve persistent infection *in vivo*, and should also contribute to the pathogenesis of the virus-associated diseases.

## Results

### The frequency of FoxP3<sup>+</sup> cells is positively correlated with HTLV-1 proviral load

Previous studies reported that the HTLV-1 provirus was frequently detected in effector/memory CD4<sup>+</sup> T cells [28], but at that time the analysis did not distinguish between effector/memory CD4<sup>+</sup> T cell and regulatory T cells (T<sub>reg</sub> cells). Also further subsets of CD4<sup>+</sup> T cells have been identified recently, such as the division of FoxP3<sup>+</sup>CD4<sup>+</sup> T cells into three distinct subsets [27]. In order to uncover the impact of HTLV-1 infection on the CD4<sup>+</sup> T-cell subset, it is necessary to re-evaluate the CD4<sup>+</sup> subsets in HTLV-1-infected individuals. We analyzed 23 ACs, 10 HAM/TSP patients, 10 ATL patients, and 10 healthy donors in this study as shown in Table 1. Almost all ATL cells express CD4, and indeed the percentage of CD4<sup>+</sup> T-cells in ATL patients was significantly higher than that of uninfected healthy donors ( $p = 0.0051$ , Figure 1A). There were no significant differences in the percentage of CD4<sup>+</sup> T cells between HD, AC, and HAM/TSP individuals ( $p = 0.2153$  and  $0.4597$ , respectively, Figure 1A). To characterize the CD4<sup>+</sup> T-cell subset in more detail, we stained PBMCs with anti-CD4, anti-CD45RA, and anti-FoxP3 antibodies. In this analysis we divided CD4<sup>+</sup> T cells into three distinct subsets, which include two FoxP3<sup>-</sup> populations (CD45RA<sup>+</sup> naive T cells and CD45RA<sup>-</sup> effector/memory T cells) and a FoxP3<sup>+</sup> population. As shown in Figure 1B, the percentage of naive CD4<sup>+</sup> T cells was decreased in ATL patients ( $p = 0.0097$ ), but did not differ significantly between HD, AC and HAM/TSP ( $p = 0.8381$  and  $0.2567$ , respectively). The percentages of effector/memory CD4<sup>+</sup> T cells were not significantly different among the four studied subject groups (Figure 1C). However, frequencies of FoxP3<sup>+</sup> cells in HTLV-1 infected individuals (AC<sup>high</sup>, ATL, HAM/TSP) were remarkably higher than those of HD ( $p = 0.0054$ ,  $0.0002$  and  $0.0002$ , respectively, Figure 1D). The frequencies of FoxP3<sup>+</sup> cells in AC were significantly correlated with HTLV-1 proviral load (PVL) ( $r = 0.60$ ,  $p = 0.0051$ , Figure 1E). Additionally, the absolute number

**Table 1 Characteristics of participants**

Characteristics	HD	AC	ATL	HAM/TSP
Participant number	10	23	10	10
Age, median years (IQR)	54 (49–62)	59 (50–70)	65 (61–76)	60 (54–62)
Male sex, no (%)	3 (30)	6 (26)	5 (50)	3 (30)
WBC (IQR)/ $\mu$ L	4,930 (1,437)	5,157 (1,100)	17,030 (12,975)	5,900 (1,500)
Lymphocyte (IQR)/ $\mu$ L	1,717 (503)	1,697 (601)	8,443 (10,764)	1,739 (560)
PVL median (IQR)	-	1.8 (0.5–5.0)	59.6 (18.3–67.1)	9.6 (5.6–12.0)

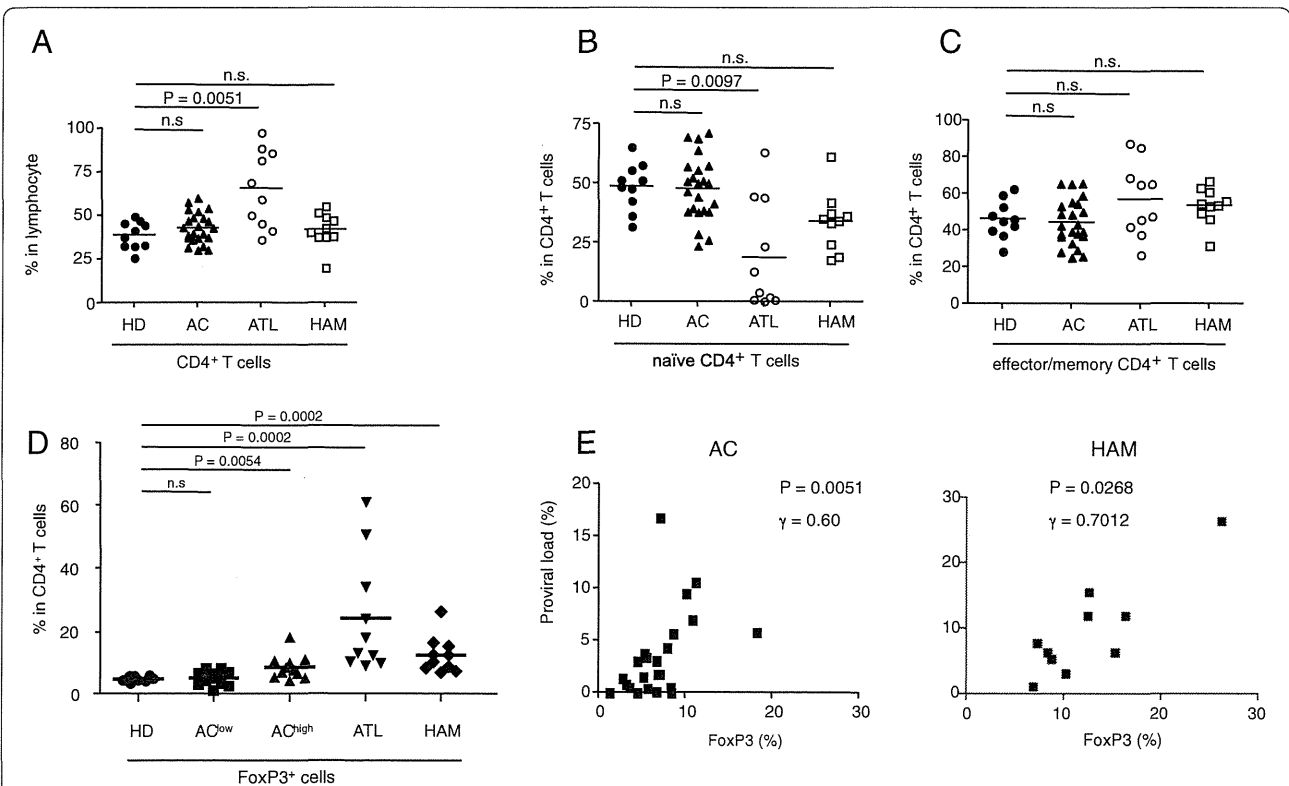
NOTE. HD healthy donor; AC asymptomatic HTLV-1 carrier; ATL adult T-cell leukemia; HAM/TSP HTLV-1 associated myelopathy/tropic spastic paraparesis; IQR interquartile range; PVL proviral load. ATL patients consist of 2 acute, 4 smoldering and 4 chronic types of ATL cases.

of each T-cell subset showed the same tendency as well as the frequency (Additional file 1: Figure S1). These results collectively suggested that HTLV-1 infection increased the frequency of FoxP3<sup>+</sup>CD4<sup>+</sup> T cells.

**Tax expression after *ex vivo* culture is well correlated with proviral load**

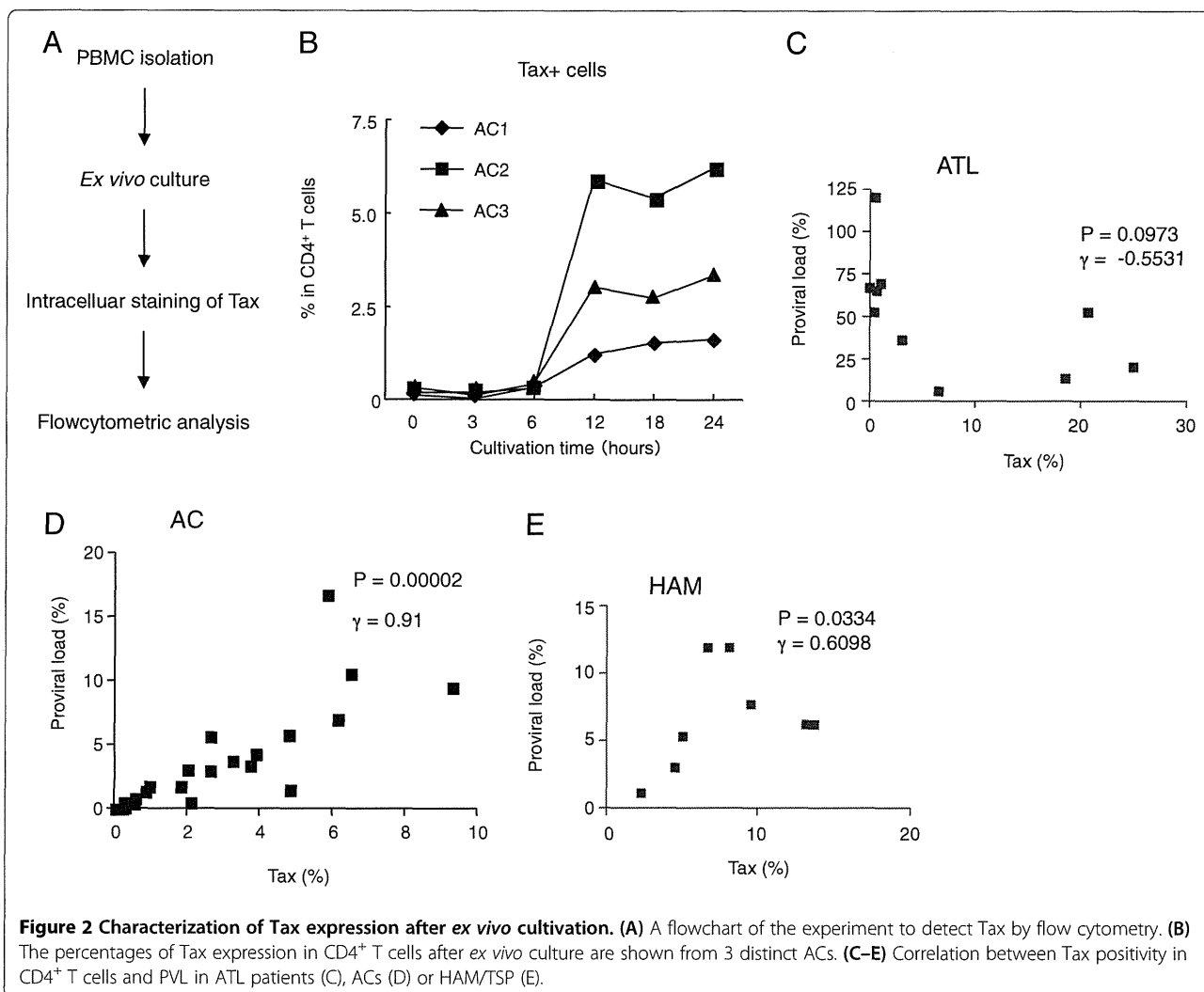
It has been reported that Tax expression increases spontaneously during *ex vivo* cultivation [32], which is useful to detect HTLV-1 infected cells at single cell level. We,

therefore, used the same method to detect HTLV-1 infected cells by flow cytometry (Figure 2A), in which we can detect both Tax and various markers of CD4<sup>+</sup> T-cell subsets at the same time. We first evaluated the detection system by using a series of samples collected at different time points after *ex vivo* cultivation. We found that a small number of Tax-expressing cells could be detected after *ex vivo* cultivation for 6 hours; significant expression could be observed after 12 hours cultivation; and Tax expression continued for 24 hours of cultivation



**Figure 1 CD4<sup>+</sup>T-cell subset in HTLV-1 infected individuals.** (A) Percentages of CD4<sup>+</sup> T cells in 4 distinct subjects. Data shown are gated on lymphocyte fraction based on the dot plot pattern of SSC and FSC. (B and C) Proportion of FoxP3<sup>+</sup>CD45RA<sup>+</sup> naive CD4<sup>+</sup> T cells (B) or FoxP3<sup>+</sup>CD45RA<sup>-</sup> effector/memory CD4<sup>+</sup> T cells (C). (D) Percentages of FoxP3<sup>+</sup> cells in CD4<sup>+</sup> T cells. (E) Frequency of FoxP3<sup>+</sup>CD4<sup>+</sup> cells in ACs or HAM/TSP patients showed significant correlation with HTLV-1 proviral load by Spearman's rank correlation ( $P = 0.0051$  or  $P = 0.0268$ ,  $r = 0.60$  or  $r = 0.7012$ , respectively).





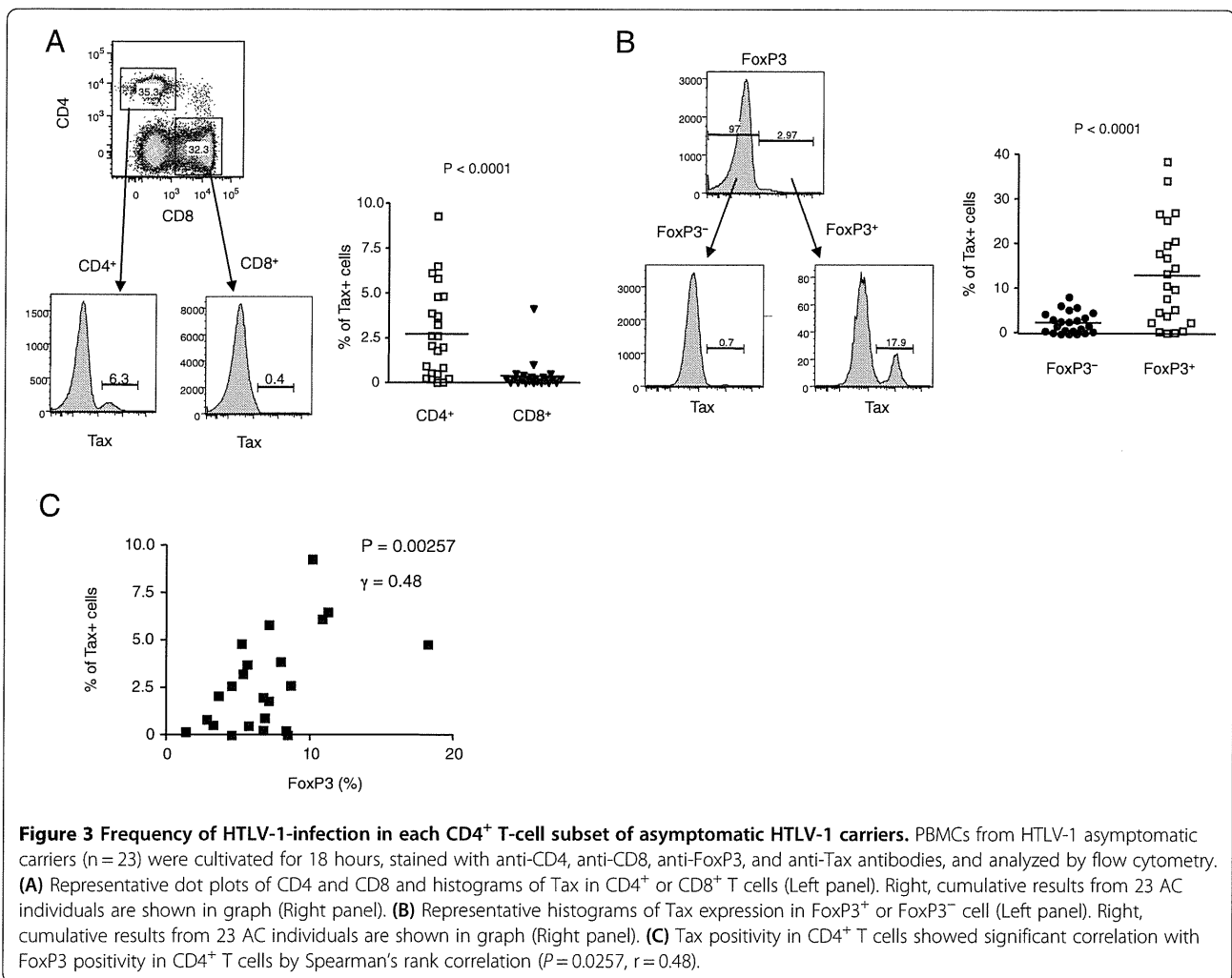
(Figure 2B). In order to confirm the efficiency of this system, we analyzed the correlation between HTLV-1 proviral load and the percentage of Tax expression in this system.

Consistent with previous reports that Tax expression is frequently silenced in ATL cells, Tax expression after *ex vivo* cultivation of ATL cells was not correlated with the proviral load (Figure 2C). The percentage of Tax positive cells tended to be lower than the proviral load even after *ex vivo* culture in AC and HAM/TSP patients, but we found that Tax positivity showed a significant correlation with the proviral load both in AC and HAM/TSP ( $r = 0.91$  or  $0.61$ ,  $p = 0.00002$  or  $0.0334$ , respectively, Figure 2D and E). In order to investigate whether T-cell subset markers, including FoxP3 and CD45RA, are influenced by *ex vivo* cultivation, we analyzed their expression both before and after cultivation. The results showed that the frequency of FoxP3 or CD45RA was not significantly changed during *ex vivo* culture (Additional file 2: Figure S2). These findings collectively

indicate the usefulness of this Tax detection system for this study.

#### The frequency of HTLV-1 infection in each CD4<sup>+</sup> T-cell subset

We next investigated which T-cell subset is frequently infected with HTLV-1. We cultivated PBMCs isolated from HTLV-1 infected individuals *ex vivo* for 12–18 hours and stained with antibodies to Tax and various T-cell subset markers such as CD4, CD8, and FoxP3. Consistent with the previous reports, the frequency of Tax positivity in CD4<sup>+</sup> T cells was much higher than that in CD8<sup>+</sup> T cells ( $p < 0.0001$ , Figure 3A). Among CD4<sup>+</sup> T cells, the FoxP3 positive cell population contained a significantly higher ratio of Tax positive cells than that in FoxP3 negative cells ( $p < 0.0001$ , Figure 3B). In line with the finding in Figure 1E, the frequencies of FoxP3<sup>+</sup> cells were significantly correlated with Tax positivity in CD4<sup>+</sup> T cells. ( $r = 0.48$ ,  $p = 0.0257$ , Figure 3C). These results indicated



that the increased FoxP3<sup>+</sup> cells in HTLV-1-infected individuals were frequently infected with HTLV-1.

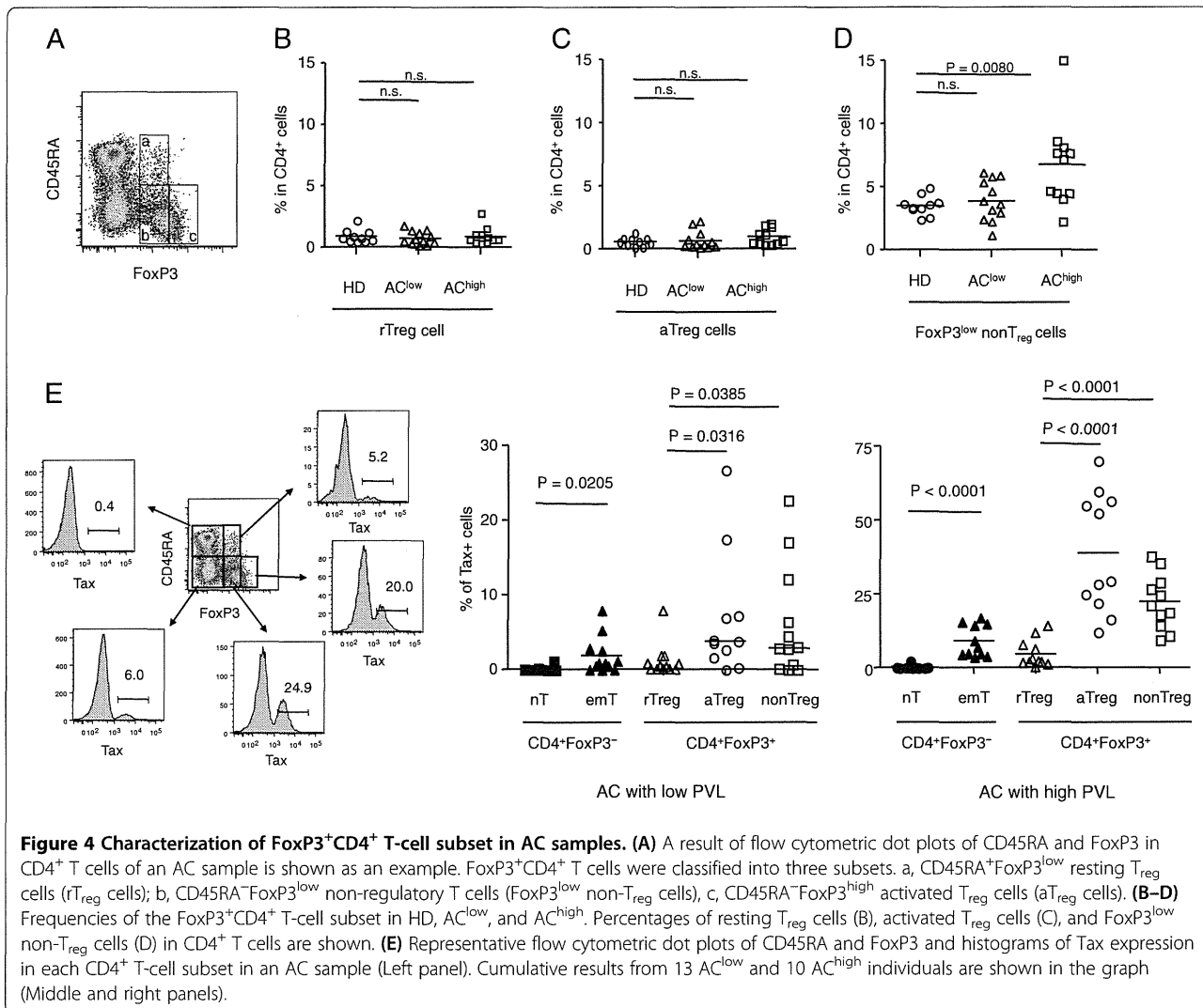
#### Characterization of FoxP3<sup>+</sup>CD4<sup>+</sup> T-cell subset in AC

We further focused on the FoxP3<sup>+</sup>CD4<sup>+</sup> T-cell subset as defined previously (Figure 4A) [27]. First, we investigated the frequency of FoxP3<sup>+</sup>CD4<sup>+</sup> T-cell subset in HD or AC. The results showed that the frequencies of rT<sub>reg</sub> or aT<sub>reg</sub> in AC<sup>low</sup> or AC<sup>high</sup> were not significantly different from that in HD (Figure 4B and 4 C), but FoxP3<sup>low</sup> non-T<sub>reg</sub> cells were significantly more frequent in the AC<sup>high</sup> population ( $p = 0.0080$ , Figure 4D). We next analyzed the presence of HTLV-1 in each CD4<sup>+</sup> T-cell subset by using AC sample. We observed that Tax positivity in FoxP3<sup>-</sup> effector/memory CD4<sup>+</sup> T cells was higher than that of FoxP3<sup>-</sup> naïve CD4<sup>+</sup> T cells ( $p < 0.0001$ , Figure 4E). Since effector/memory CD4 T cells are the most dominant in total CD4 T cells in terms of absolute cell number, the Tax-expressing cells are most abundant in effector/memory CD4 T cells (Additional file 3: Figure

S3). More interestingly, Tax positivity in aT<sub>reg</sub> cells or FoxP3<sup>low</sup> non-T<sub>reg</sub> cells was much higher than that of rT<sub>reg</sub> cells in ACboth AC<sup>low</sup> and AC<sup>high</sup> subjects ( $p < 0.0001$  or  $0.0001$ , respectively, Figure 4E). These results indicated that HTLV-1 is frequently present in aT<sub>reg</sub> cells or FoxP3<sup>low</sup> non-T<sub>reg</sub> cells.

#### Characteristics of T-cell subsets in HAM/TSP patients

To investigate the inflammatory aspects of HTLV-1 infection, we next focused on PBMCs of HAM/TSP patients. There were no significant differences in the percentage of CD4<sup>+</sup> or CD8<sup>+</sup> T cells between HD and HAM/TSP groups ( $p = 0.3073$  and  $0.1509$ , respectively, Figure 5A). The result of Tax staining showed that HTLV-1 infection was predominantly detected in CD4<sup>+</sup> T cells, and at a higher frequency in CD4<sup>+</sup>FoxP3<sup>+</sup> T cells than CD4<sup>+</sup>FoxP3<sup>-</sup> T cells ( $p = 0.0069$ , Figure 5B). To characterize the phenotype of FoxP3<sup>+</sup> cells in HAM/TSP patients, we investigated the expression levels of T<sub>reg</sub> associated molecules, and found that the expression of GITR or CTLA-4 in HAM/TSP patients



**Figure 4** Characterization of FoxP3<sup>+</sup>CD4<sup>+</sup> T-cell subset in AC samples. (A) A result of flow cytometric dot plots of CD45RA and FoxP3 in CD4<sup>+</sup> T cells of an AC sample is shown as an example. FoxP3<sup>+</sup>CD4<sup>+</sup> T cells were classified into three subsets. a, CD45RA<sup>+</sup>FoxP3<sup>low</sup> resting T<sub>reg</sub> cells (rT<sub>reg</sub> cells); b, CD45RA<sup>-</sup>FoxP3<sup>low</sup> non-regulatory T cells (FoxP3<sup>low</sup> non-T<sub>reg</sub> cells); c, CD45RA<sup>-</sup>FoxP3<sup>high</sup> activated T<sub>reg</sub> cells (aT<sub>reg</sub> cells). (B–D) Frequencies of the FoxP3<sup>+</sup>CD4<sup>+</sup> T-cell subset in HD, AC<sup>low</sup>, and AC<sup>high</sup>. Percentages of resting T<sub>reg</sub> cells (B), activated T<sub>reg</sub> cells (C), and FoxP3<sup>low</sup> non-T<sub>reg</sub> cells (D) in CD4<sup>+</sup> T cells are shown. (E) Representative flow cytometric dot plots of CD45RA and FoxP3 and histograms of Tax expression in each CD4<sup>+</sup> T-cell subset in an AC sample (Left panel). Cumulative results from 13 AC<sup>low</sup> and 10 AC<sup>high</sup> individuals are shown in the graph (Middle and right panels).

was significantly lower than that in HD ( $p=0.0328$  or  $0.00002$ , respectively, Figure 5C). On the contrary, CD25 expression was high in HAM/TSP patients ( $p=0.0099$ , Figure 5C). We further evaluated FoxP3<sup>+</sup>CD4<sup>+</sup> T-cell subset in HAM/TSP patients. The frequencies of rT<sub>reg</sub> were not significantly different from that in HD ( $p=0.9096$ , Figure 5D), but aT<sub>reg</sub> cells or FoxP3<sup>low</sup> non-T<sub>reg</sub> cells were remarkably increased ( $p=0.0250$  or  $0.0004$ , Figure 5E and 5F). Furthermore, aT<sub>reg</sub> cells or FoxP3<sup>low</sup> non-T<sub>reg</sub> cells showed a high frequency of Tax<sup>+</sup> cells compared with rT<sub>reg</sub> cells ( $p=0.0069$  or  $0.0069$ , respectively, Figure 5G) as observed in ACs (Figure 4E). These data indicated that HTLV-1 infection significantly influenced not only the frequency but also the phenotype of CD4<sup>+</sup>FoxP3<sup>+</sup> T cells in an inflammatory disease HAM/TSP.

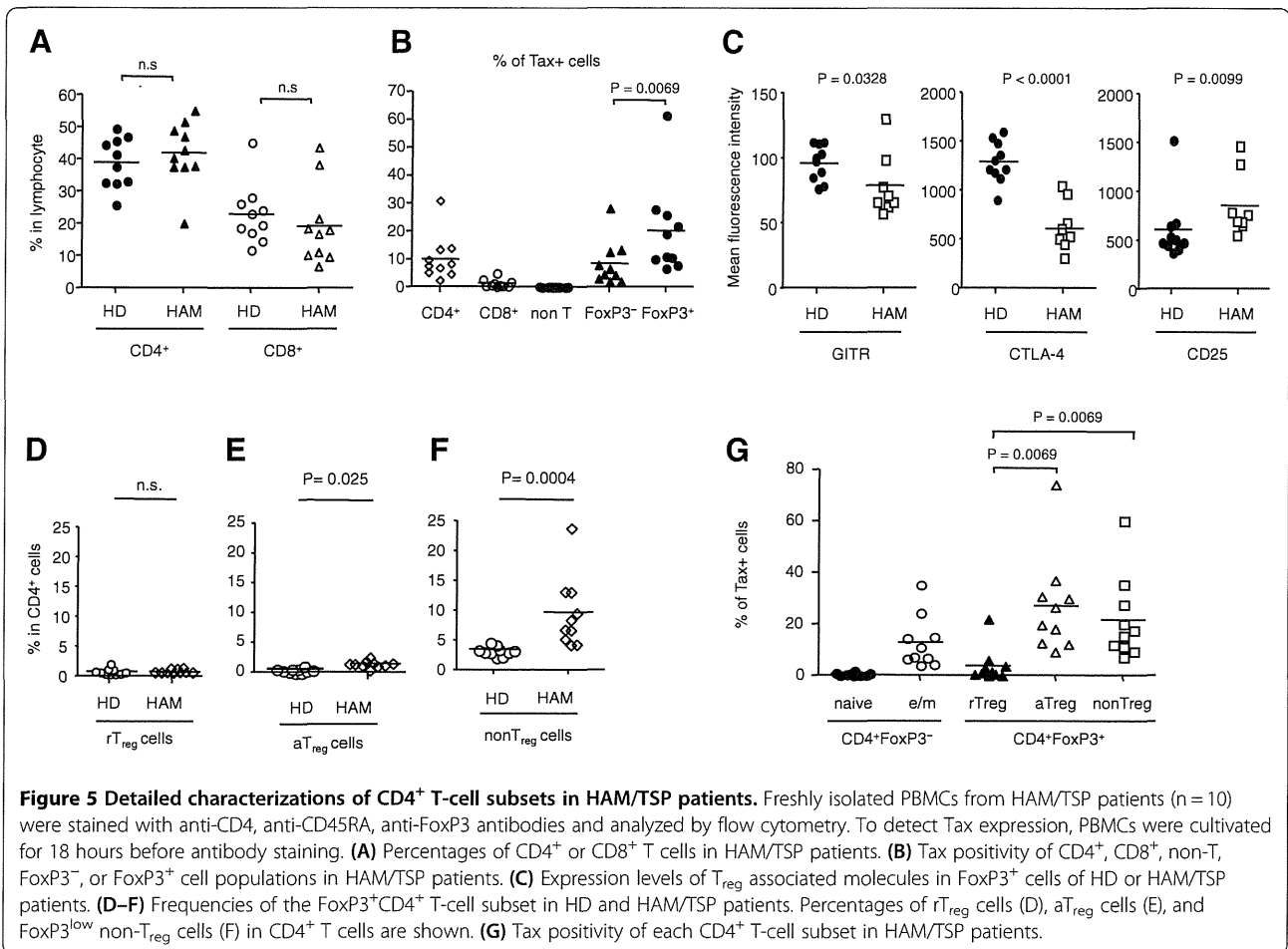
#### Phenotypical analyses of ATL cells

Previous studies reported that some ATL cells express FoxP3 or CD25 [30,31,33], but the precise information

about FoxP3<sup>+</sup>CD4<sup>+</sup> T-cell subset of ATL cells remains unknown. We, therefore, analyzed CD4<sup>+</sup> T-cell subsets for ATL cases. FoxP3 positivity was 80% in ATL cases; yet the expression level was different among the cases (Figure 6A), which is consistent with previous reports [30,31]. In line with the finding in asymptomatic HTLV-1-infected carriers that the percentage of HTLV-1 in FoxP3<sup>low</sup> non-T<sub>reg</sub> cells or aT<sub>reg</sub> cells was high (Figure 4E), ATL cells analyzed in this study did not express CD45RA, suggesting that FoxP3-expressing ATL cells might be derived from FoxP3<sup>low</sup> non-T<sub>reg</sub> or aT<sub>reg</sub> cells. CD25 expression on ATL cells was generally high, but there was also much variation among the cases (Figure 6B).

#### Discussion

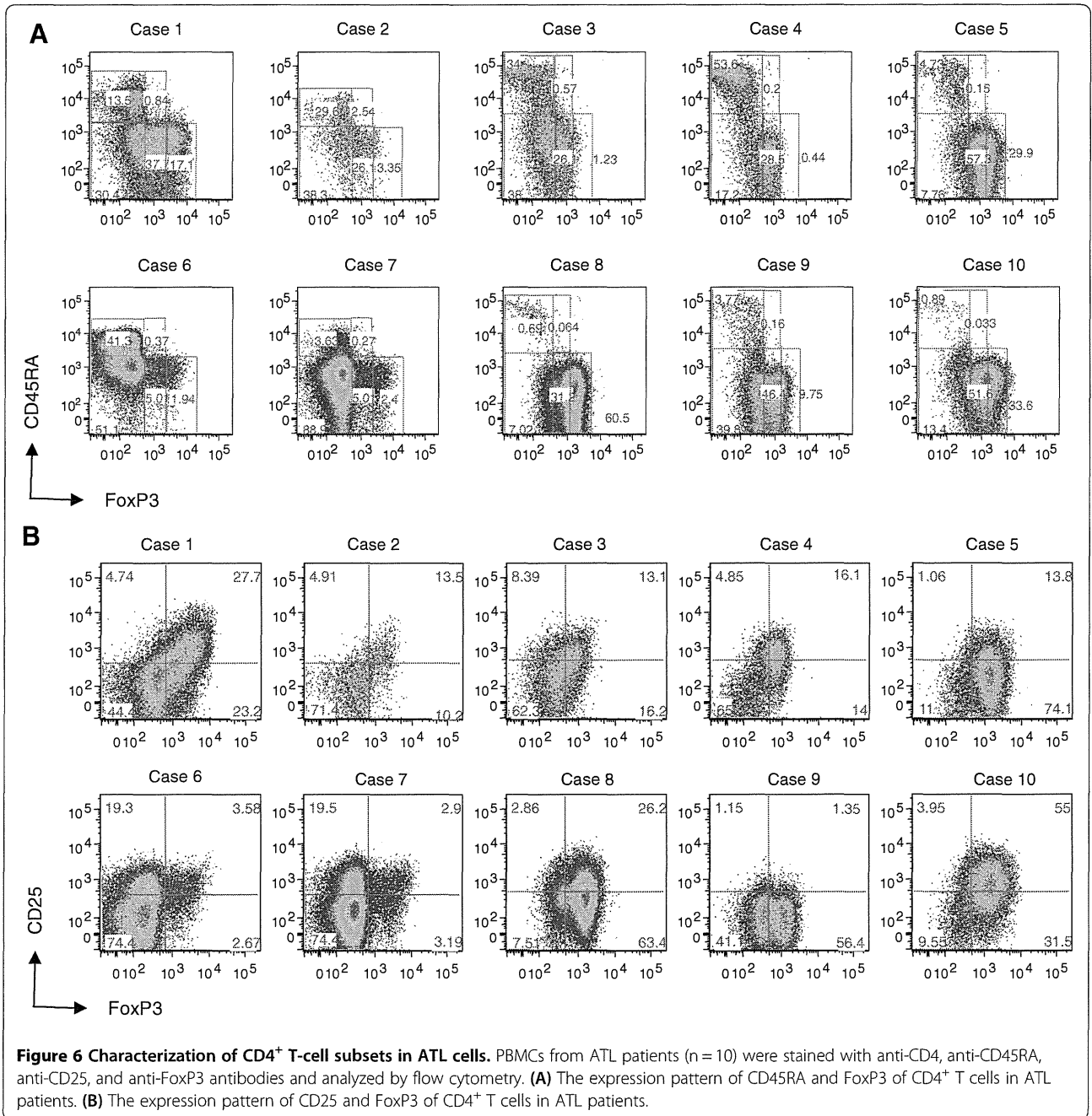
FoxP3<sup>+</sup> T<sub>reg</sub> cells play a crucial role in persistent infection and pathogenesis of chronic viral infection. Previous studies have suggested that T<sub>reg</sub> cells suppress virus-



specific CD8<sup>+</sup> T-cell effector functions in chronic human viral infections such as human immunodeficiency virus, hepatitis C virus and cytomegalovirus [34,35]. Regarding this point, FoxP3<sup>+</sup> T<sub>reg</sub> cells play a role in facilitating viral persistence. In HTLV-1 infection, the frequency of FoxP3<sup>+</sup> cells is indeed correlated with the impairment of CTL activity against the viral antigen Tax in HAM/TSP patient [36]. On the other hand, FoxP3<sup>+</sup> T<sub>reg</sub> cells could prevent tissue damage caused by excessive immune response triggered by viral infection. In addition to these general roles of FoxP3<sup>+</sup> T<sub>reg</sub> cells in chronic viral infection, FoxP3<sup>+</sup> T<sub>reg</sub> cells should have some specific role in HTLV-1 infection, because FoxP3<sup>+</sup> T<sub>reg</sub> cells are comprised in CD4<sup>+</sup> T cells, which are a main host cell population of HTLV-1. Here we performed a comprehensive analysis of CD4<sup>+</sup> T-cell subsets in individuals naturally infected with HTLV-1 and revealed that the frequency of HTLV-1 infection is positively correlated with the frequency of FoxP3<sup>+</sup> T cells (Figure 1E). The increased FoxP3<sup>+</sup> T cells themselves are frequently infected with HTLV-1 (Figure 3B), suggesting that HTLV-1 utilizes the FoxP3<sup>+</sup> T cells as a host cell. What is the advantage for HTLV-1 to exist in FoxP3<sup>+</sup> T cells? There are two

possibilities for this preference. First, FoxP3<sup>+</sup> T cells are known as hyper-proliferating cells *in vivo* with a doubling time of 8 days [37], which could contribute to clonal expansion of infected cells. Second, HTLV-1 can evade the host immune system by directly infecting this potentially immuno-suppressive cell population. Thus, HTLV-1-infection of FoxP3<sup>+</sup> T cells should enable the virus to increase or maintain proviral load and achieve persistent infection.

How then does HTLV-1 infection target FoxP3<sup>+</sup> T cells? This could be explained by the following two mechanisms. First, FoxP3<sup>+</sup> T cells are known to contact with dendritic cells (DCs) frequently [38], which could increase the chance of *de novo* viral infection between DCs and FoxP3<sup>+</sup> T cells. A recent study demonstrated that cell-free HTLV-1 efficiently infects DCs, and the infected DCs promote *de novo* infection of CD4<sup>+</sup> T cells [39]. This notion is consistent with the finding that effector/memory-type CD45RA<sup>-</sup> T<sub>reg</sub> cells, including FoxP3<sup>low</sup> non-T<sub>reg</sub> cells and FoxP3<sup>high</sup> aT<sub>reg</sub> cells, are more frequently infected with HTLV-1 than CD45RA<sup>+</sup> rT<sub>reg</sub> cells (Figure 4E). Second, once FoxP3<sup>-</sup> T cells are infected with HTLV-1, HBZ should be expressed in the host cells. Since HBZ is recently



reported to induce FoxP3 expression via enhancing TGF- $\beta$  signaling pathway [17,40], HTLV-1 infection is likely to convert FoxP3<sup>-</sup> cells into FoxP3<sup>+</sup> cells. In addition, HTLV-1 has a cell-extrinsic effect on FoxP3<sup>+</sup> cell generation. HTLV-1 infected cells secrete CCL22 via expression of Tax, which indirectly contributes to the generation and maintenance of HTLV-1 uninfected FoxP3<sup>+</sup> cells [41,42]. This would contribute to an increased number of HTLV-1-uninfected FoxP3<sup>+</sup> cells.

Since FoxP3<sup>+</sup> T<sub>reg</sub> cells play a crucial role in suppressing immune response, the increase of FoxP3<sup>+</sup> cells

observed in HTLV-1 infection may contribute to immunodeficiency, which is frequently observed in HTLV-1 infection [43]. On the other hand, the high frequency of FoxP3<sup>+</sup> T cells observed in HAM/TSP patients is paradoxical, because the pathogenesis of HAM/TSP is believed to be inflammatory. Therefore, we analyzed the phenotype of the increased FoxP3<sup>+</sup> cells and observed that CTLA-4 and GITR expression of FoxP3<sup>+</sup> T cells in HAM/TSP patient was significantly reduced compared to uninfected individuals (Figure 5C). A similar observation was reported previously that the expression level of

FoxP3, GITR, or CTLA-4 mRNA in CD4<sup>+</sup>CD25<sup>+</sup> T cells of HAM/TSP patients is lower than that of HD [44]. That report used CD4<sup>+</sup>CD25<sup>+</sup> as a marker of T<sub>reg</sub> cells, but CD4<sup>+</sup>CD25<sup>+</sup> T cells contain not only FoxP3<sup>+</sup> T<sub>reg</sub> cells but also FoxP3<sup>-</sup> activated T cells. Particularly the proportion of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>-</sup> activated T cells is up-regulated in HAM/TSP patients, which is likely to reduce the proportion of FoxP3<sup>+</sup> T<sub>reg</sub> cells in CD4<sup>+</sup>CD25<sup>+</sup> T cells of HAM/TSP patients. Thus, the expression level of GITR or CTLA-4 in FoxP3<sup>+</sup> T cells of HAM/TSP patients has not been elucidated yet. To avoid this concern, we utilized the multicolor flow cytometry, which enabled us to show that CTLA-4 and GITR were clearly down regulated in FoxP3<sup>+</sup> T cells of HAM/TSP patients.

Then what is the underlying mechanism of this phenomenon? We reported recently that HBZ-Tg mice showed a pro-inflammatory phenotype in spite of the increase of Foxp3<sup>+</sup> T cells [17], which is similar to HAM/TSP patients (Figure 1D). T<sub>reg</sub> associated molecules were also down regulated in Foxp3<sup>+</sup> T cells of HBZ-Tg mice. Thus, HBZ-mediated FoxP3 dysfunction may play a role in the abnormality regarding FoxP3<sup>+</sup> cells in HAM/TSP patients. It has been reported that Tax also contributes to the dysregulation of FoxP3<sup>+</sup> T<sub>reg</sub> cells. Tax suppresses FoxP3 expression at transcriptional level [45], which alternatively or additionally could contribute to the abnormal phenotype of FoxP3<sup>+</sup> cells. These findings collectively indicate that the increased FoxP3<sup>+</sup> T<sub>reg</sub> cells were functionally impaired in HAM/TSP patients. Furthermore, FoxP3<sup>+</sup> CD4<sup>+</sup> T cells in HAM/TSP patient contain an increased FoxP3<sup>+</sup> non-Treg population (Figure 5F), which would contribute to the inflammatory phenotype of HAM/TSP via generation of pro-inflammatory cytokine-producing CD4<sup>+</sup> T cells such as T<sub>HAM</sub> cells [46] or exFoxp3 cells [47].

In the current study, we did not observed FoxP3 repression during Tax expression by *ex vivo* cultivation. This result seems to be inconsistent with a previous report that Tax represses FoxP3 expression [45]. There are two possible explanation of this inconsistency. First, there is the difference of the ways to express Tax. In the previous study, the authors used transfection of plasmid that induces Tax expression by the CMV promoter. We used endogenous HTLV-1 provirus to express Tax. Therefore, the expression level of Tax in our current study should be much lower than that of the previous study. In addition, Tax expression was induced in a proportion of FoxP3<sup>+</sup> cell in our current study. Second, there are differences in incubation time for Tax expression. In the previous study, the authors evaluated FoxP3 expression after 48 hours of transfection, whereas we evaluated FoxP3 expression within 24 hours after Tax expression.

High expression levels of CD25 are also well documented in HTLV-1 infection [33]. Consistent with previous

findings, CD25 expression is upregulated in FoxP3<sup>+</sup> cells of HAM/TSP patient (Figure 5C). One determinant of the susceptibility to HAM/TSP is host genetic polymorphism such as MHC class 1, which influences the efficiency of CTL against HTLV-1 [48,49]. HTLV-1-infected individuals who have HLA class I susceptible for HAM/TSP may allow high expression of Tax and/or HBZ, which could cause up-regulation of CD25 molecules in the FoxP3<sup>+</sup> cell population (Figure 5C).

It is controversial whether ATL is a leukemia of FoxP3<sup>+</sup> T<sub>reg</sub> cells or not. However, there is no *a priori* reason to assume that ATL cells must be exclusively derived from FoxP3<sup>+</sup> T<sub>reg</sub> cells or non-T<sub>reg</sub> cells. Indeed, there are previous reports to support both possibilities. Some studies have reported that ATL cells have regulatory functions [50,51], whereas other studies reported no regulatory function in ATL [52,53]. We showed here that HTLV-1 is frequently detectable in CD4<sup>+</sup>FoxP3<sup>+</sup> T cells (Figure 3B) in AC. More than half of ATL cells express FoxP3 (Figure 6), even though FoxP3 expression in ATL cells is variable as shown in the present and previous studies [30,31]. These findings prompt us to propose an idea that more than a half of ATL cells are possibly derived from FoxP3<sup>+</sup> T<sub>reg</sub> cells. We reported previously that HBZ expression is constitutively active but Tax expression is frequently silenced in ATL cells, which possibly contributes to high frequency of FoxP3<sup>+</sup> ATL.

## Conclusion

This study demonstrated that HTLV-1 infection induced the abnormality of frequency and phenotype of FoxP3<sup>+</sup> T cells, suggesting that HTLV-1 has evolved a sophisticated strategy to achieve persistent infection by directly affecting the central regulator of the host immune system. HTLV-1-mediated dysregulation of FoxP3<sup>+</sup> T cells is likely to be a critical cellular mechanism for the understanding HTLV-1 pathogenicity.

## Methods

### Clinical samples and ethics statement

PBMCs were obtained from asymptomatic HTLV-1 infected carriers (n = 23), HAM/TSP patients (n = 10), ATL patients (n = 10), and age-matched healthy controls (n = 10). Characteristics of each group are presented in Table 1. ATL patients consist of 2 acute, 4 smoldering and 4 chronic types of ATL cases. Genomic DNA extracted from PBMCs was used to determine proviral load (PVL) as described previously [29]. Briefly, PVL was quantified by real time PCR and calculated by using genomic DNA of TL-Om1, an ATL cell line with one copy of complete HTLV-1 provirus, as a standard of 100%. We defined AC with less than 2% of proviral load as AC<sup>low</sup> and AC with more than 2% of proviral load as AC<sup>high</sup>. This study was conducted according to the

principles expressed in the Declaration of Helsinki and approved by the Institutional Review Board of Kyoto University (844 and E-921). All patients provided written informed consent for the collection of samples and subsequent analysis.

#### Antibodies

The following antibodies were purchased from BD Pharmingen; purified monoclonal antibody (mAb) for human CD3 (UCHT1), CD4 (RPA-T4), CD8a (RPA-T8), CD45RA (NI100) and CTLA-4 (BNI3). Purified mAbs for human CD25 (BC96), GITR (eBio AITR) and FoxP3 (236A/E7) were purchased from eBioscience.

#### Flow cytometric analysis

PBMCs were isolated with Ficoll-Isopaque (GE Healthcare) gradient centrifugation. Flow cytometric analyses were carried out using a FACS CantoII with Diva Software (BD Pharmingen), and the data were analyzed by FlowJo software (Treestar). To discriminate dead cells, we used LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (Invitrogen). For cell surface staining,  $10^6$  cells were incubated with mAbs for 30 minutes at 4°C, and then analyzed. For intracellular staining, we used a human FoxP3 staining kit according to the manufacturer's protocol (eBioscience). To distinguish FoxP3<sup>+</sup> and FoxP3<sup>-</sup> cell population clearly, we used isotype control according to the manufacturer's recommendation. To detect the viral antigen Tax, we cultured PBMCs from ACs or HAM/TSP patients for 12–18 hours and stained with monoclonal antibodies against FoxP3 or Tax (MI-73) [54], and then analyzed by flow cytometry.

#### Statistical analysis

To compare 2 groups when data were determined to have a Gaussian distribution, the Student *t* test was used. If data did not have a Gaussian distribution, the Mann–Whitney *U* test was used for unpaired data, and the Wilcoxon signed-ranks test was used for paired data. The AC group and HD did not differ significantly in sex or age, using chi-squared test and Mann–Whitney *U* test. Differences with  $P < 0.05$  were considered to be statistically significant. Correlations were evaluated using Spearman's rank correlation.

#### Additional files

**Additional file 1: Figure S1.** Absolute cell numbers of each CD4<sup>+</sup>T-cell subset in HTLV-1 infected individuals. (A) Absolute cell numbers of CD4<sup>+</sup> T cells in 4 distinct subjects. Data shown are gated on lymphocyte fraction based on the dot plot pattern of SSC and FSC. (B and C) Absolute cell numbers of FoxP3<sup>-</sup>CD45RA<sup>+</sup> naïve CD4<sup>+</sup> T cells (B) or FoxP3<sup>-</sup>CD45RA<sup>+</sup> effector/memory CD4<sup>+</sup> T cells (C). (D) Absolute cell numbers of FoxP3<sup>+</sup> cells in CD4<sup>+</sup> T cells.

**Additional file 2: Figure S2.** Effect of *ex vivo* cultivation on FoxP3 and

CD45RA expression. The percentages of FoxP3 and CD45RA expression in CD4<sup>+</sup> T cells both before and after *ex vivo* culture are shown from 5 distinct ACs.

**Additional file 3: Figure S3.** Frequency of each CD4 T-cell subset in Tax-expressing cell population in AC. Cumulative results from 23AC individuals are shown in the graph.

#### Competing interests

The authors declare that they have no competing interests.

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#### Author details

<sup>1</sup>Laboratory of Virus Control, Institute for Virus Research, Kyoto University, Kyoto, 606-8507, Japan. <sup>2</sup>Department of Hematology, Imamura Bun-in Hospital, Kagoshima, 890-0064, Japan. <sup>3</sup>Department of Neurology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto, 602-8566, Japan. <sup>4</sup>Department of Hematology, Kumamoto University School of Medicine, Kumamoto, 860-8556, Japan. <sup>5</sup>Current address: Immunology Section, Division of Infectious Diseases, Department of Medicine, Imperial College, London, W2 1PG, UK.

#### Authors' contributions

This study was designed by YS and MM. Laboratory analysis was performed by YS and JT. Data analysis was performed by YS, AU, JT and MM. Clinical samples and data were provided by AU, MN and KN. YS and MM wrote the paper. All authors read and approved the final manuscript.

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## HTLV-I virological and histopathological analysis in two cases of anti-centromere-antibody-seropositive Sjögren's syndrome

Hideki Nakamura · Yoshiro Horai · Ayuko Tokuyama · Shunsuke Yoshimura ·  
Hideki Nakajima · Kunihiro Ichinose · Satoshi Yamasaki · Tatsufumi Nakamura ·  
Tomayoshi Hayashi · Atsushi Kawakami

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### Abstract

**Introduction** The aim of this study was to show the clinical and pathological characteristics of anti-centromere-antibody (ACA)-seropositive Sjögren's syndrome (SS) in two anti-human T-cell leukemia virus type I (HTLV-I)-seropositive patients.

**Methods** One patient was an HTLV-I carrier whereas the other was diagnosed with HTLV-I-associated myelopathy (HAM). Background data including serum HTLV-I titers, viral loads, and cytokine profiles were recorded. Azocarmine with aniline blue (Azan)–Mallory staining and immunohistochemistry of the labial salivary glands (LSGs) and a muscle biopsy specimen from the HAM patient were performed.

**Results** Serum transforming growth factor beta (TGF- $\beta$ ), tumor necrosis factor alpha (TNF- $\alpha$ ), and HTLV-I viral load were high in the HAM-SS patient compared with the HTLV-I carrier. Fibrous change in LSG was prominent in the HAM-SS patient. Although TGF- $\beta$  expression was similar in the two patients, expression of HTLV-I-related proteins including p12, p28, group-specific antigen (GAG), and nuclear factor kappa-B (NF- $\kappa$ B) in the LSG were dominantly detected in the HAM-SS patient. Frequency of TGF- $\beta$  staining in HTLV-I-seropositive SS patients without ACA, HTLV-I-seronegative SS patients with ACA, and HTLV-I-seronegative SS patients without ACA was lower than that of the previous two patients.

**Conclusion** A high HTLV-I viral load in situ is supposed to promote the production of cytokines, especially TGF- $\beta$ , resulting in the fibrous change of LSG in ACA-seropositive SS patients.

**Keywords** HTLV-I infection · Anti-centromere antibody · Sjögren's syndrome · Cytokine

### Abbreviations

ACA	Anti-centromere antibody
ANA	Anti-nuclear antibody
CSF	Cerebrospinal fluid
HAM	HTLV-I-associated myelopathy
HTLV-I	Human T-cell leukemia virus type I
IFN- $\gamma$	Interferon gamma
MNC	Mononuclear cell
LSG	Labial salivary gland
SS	Sjögren's syndrome
TGF- $\beta$	Transforming growth factor beta
TNF- $\alpha$	Tumor necrosis factor alpha

H. Nakamura (✉) · Y. Horai · A. Tokuyama · K. Ichinose ·  
S. Yamasaki · A. Kawakami  
Unit of Translational Medicine,  
Department of Immunology and Rheumatology,  
Nagasaki University Graduate School of Biomedical Sciences,  
1-7-1 Sakamoto, Nagasaki, Nagasaki 852-8501, Japan  
e-mail: nakamura\_hideki911@yahoo.co.jp;  
nhideki@nagasaki-u.ac.jp

S. Yoshimura · H. Nakajima  
Unit of Translational Medicine, Department of Neurology,  
Nagasaki University Graduate School of Biomedical Sciences,  
Nagasaki, Japan

T. Nakamura  
Department of Molecular Microbiology and Immunology,  
Nagasaki University Graduate School of Biomedical Sciences,  
Nagasaki, Japan

T. Hayashi  
Department of Pathology, Nagasaki University Hospital,  
Nagasaki, Japan

## Introduction

Human T-cell leukemia virus type I (HTLV-I) is known to be one of the causative agents of Sjögren's syndrome (SS) [1, 2]. Our previous epidemiologic studies show a close association between HTLV-I and SS [3, 4]. In addition, we found a significantly high prevalence of SS in patients with HTLV-I-associated myelopathy (HAM) [3, 5]. On the other hand, anti-centromere antibody (ACA) is known as a second class of autoantibodies in SS patients [6, 7]. Our previous report revealed that ACA is detected in only 4 % of HTLV-I-seropositive SS cases, demonstrating that HTLV-I might not be involved in the pathogenesis in ACA-seropositive SS patients [8]. However, if HTLV-I infection coincidentally occurs in ACA-seropositive SS patients, the influence of ACA on HTLV-I-associated SS might become obvious. In this study, we report two cases of ACA-seropositive SS patients who were also seropositive for anti-HTLV-I antibody. One patient was complicated with HAM, whereas the other was an HTLV-I carrier. The variation in HTLV-I viral load in these patients appears to explain the differences in labial salivary gland (LSG) histopathology and cytokine profile.

## Patients and methods

### Patients

#### Case 1

This was a 61-year-old female patient who complained of sicca symptoms. Both ACA and anti-HTLV-I antibody measured by chemiluminescent enzyme immunoassay (CLEIA) were highly positive, as shown in Table 1. As no other symptoms or signs, including in the neuromuscular systems, were found in this patient, she was classified as an HTLV-I carrier.

#### Case 2

A 57-year-old female patient who complained of sicca symptoms and myalgia was diagnosed with HAM based on the diagnostic guidance for HAM determined by the Ministry of Health, Labour and Welfare. She had slowly progressive and symmetrical pyramidal tract damage with positive anti-HTLV-I antibody in both serum and cerebrospinal fluid (CSF). Antibodies against gp46, p53, p24, and p19 of HTLV-I in CSF were all positive. Serum ACA was also positive at a high titer (Table 1). She also suffered from inflammatory myopathy as evidenced by the elevation of muscle enzymes and by magnetic resonance imaging and muscle biopsy findings.

Both patients were diagnosed with SS according to the revised criteria [9], as proposed by the American–European Consensus Group. In both cases, HTLV-I viral loads in sera and serum cytokines including tumor necrosis factor alpha (TNF- $\alpha$ ), interferon gamma (IFN- $\gamma$ ), and transforming growth factor beta (TGF- $\beta$ ) were measured. For comparison, we studied the three groups of patients: (1) HTLV-I-seropositive SS patients without ACA, (2) HTLV-I-seronegative SS patients with ACA, and (3) HTLV-I-seronegative SS patients without ACA with respect to TGF- $\beta$  immunostaining of LSG (four patients each in three groups).

### LSG biopsy

LSG biopsy from the lower lip was performed under local anesthesia in SS patients. Informed consent to use biopsy samples was obtained from all participating patients at the commencement of the study. The study was conducted with the approval of the human ethical committee of our institution. The classifications of Chisholm and Mason [10] were used to determine the severity of mononuclear cell (MNC) infiltration.

### Azan–Mallory staining and immunohistochemistry of labial salivary glands

Formalin-fixed, paraffin-embedded sections (3- $\mu$ m thick) from the LSGs of these ACA-seropositive SS patients were used for azocarmine with aniline blue (Azan)–Mallory staining and immunohistochemistry. The sections were then stained using the Histofine Simple Stain Kit (Nichirei Co., Tokyo, Japan) with mouse anti-human CD4, CD8, CD20, and CD68 antibodies (DakoCytomation, Glostrup, Denmark), mouse anti-HTLV-I [p19, p28, and group-specific antigen (GAG)] antibody (Chemicon International Inc., Temecula, CA, USA), mouse anti-nuclear factor kappa B (NF- $\kappa$ B) p65 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and mouse anti-TGF- $\beta$  antibody (LifeSpan BioSciences, Inc., Seattle, WA, USA). Briefly, endogenous peroxidase was inactivated in a 3 % hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) solution after microwave epitope retrieval. These sections were then blocked with 5 % normal horse serum, followed by incubation with monoclonal and polyclonal antibodies in a humid chamber for 60 min at room temperature. After incubation, all sections, including the negative control sections, were treated with peroxidase-conjugated secondary antibodies for 30 min. The color was developed by soaking the sections in 3,3'-diaminobenzidine (DAB) and H<sub>2</sub>O<sub>2</sub> for 10 min, followed by counterstaining by soaking the sections in hematoxylin solution. Negative

**Table 1** Background information and serum data of the human T-cell leukemia virus type I (HTLV-I)-associated anti-centromere antibody (ACA)-seropositive patients

	Case 1 HTLV-I carrier with ACA-seropositive SS	Case 2 HAM with ACA-seropositive SS
Age and gender	61 years old, female	57 years old, female
Xerostomia	Positive	Positive
Xerophthalmia	Positive	Negative
Schirmer test (right/left mm; <5 mm: positive)	5/4	11/11
Saxon test (g/2 min; <2 g: positive)	1.47	2.7
ANA: pattern	160×, centromere	640×, centromere
Anti-SS-A antibody: normal 10–30 U/ml	0.7	0.9
Anti-SS-B antibody: normal 15–25 U/ml	0.9	0.5
ACA: normal <16 index	172.8	165.0
IgG: normal 870–1,700 mg/dl	1,712	1,623
Rheumatoid factor: normal <15 IU/ml	11.4	17.0
Sialography <sup>a</sup> (Rubin and Holt)	Stage 1	Stage 2
Lip biopsy grade <sup>b</sup> (Chisholm and Mason)	3	3
LST (cpm)	105,936/617	184,859/19,319
PHA(+)/no stimulation		
LST (cpm)	160,934/617	102,299/19,319
ConA(+)/no stimulation		
Serum anti-HTLV-I antibody: normal <1.0 COI	>45	>45
Serum viral load (copies/10 <sup>4</sup> cells)	<53	373
Serum TNF- $\alpha$ : normal 0.6–2.8 pg/ml	1.0	2.9
Serum IFN- $\gamma$ : normal <0.1 IU/ml	<0.1	<0.1
Serum TGF- $\beta$ : normal 1.56–3.24 ng/ml	2.76	12.6

Anti-SS-A Ab and anti-SS-B Ab (Mesacup SS-A/Ro test and SS-B/La test; Medical and Biological Laboratories, Nagoya, Japan) and ACA (Mesacup-2 test CENP-B; Medical and Biological Laboratories, Nagoya, Japan) were measured using an enzyme-linked immunosorbent assay (ELISA) kit. Serum anti-HTLV-I antibody was measured by chemiluminescent enzyme immunoassay, and HTLV-I viral load was measured by the FastStart DNA Master Hybridization probe method. Serum TNF- $\alpha$  and TGF- $\beta$  were measured by ELISA. Serum IFN- $\gamma$  was measured by enzyme immunoassay. Data shown represent the period before treatments with agents such as glucocorticoids or immunosuppressive agents

SS Sjögren's syndrome, ANA anti-nuclear antibody, COI cutoff index, ConA concanavalin A, cpm count per minute, HAM HTLV-I-associated myelopathy, Ig-G immunoglobulin G, LST lymphocyte stimulation test, PHA phytohemagglutinin, TNF tumor necrosis factor, IFN interferon TGF transforming growth factor

<sup>a</sup> Sialography grading was determined by Rubin and Holt. Stages 1 and 2 represent punctate and globular patterns, respectively

<sup>b</sup> Grading defined by Chisholm and Mason: the presence of at least one focus of mononuclear cells per 4 mm<sup>2</sup> section = grade 3

control sections were treated with mouse immunoglobulin (Ig)G1.

## Results

### Clinical and serological data with cytokine profile

As shown in Table 1, a high ACA titer was detected in both patients. Serum IgG was almost normal, which is characteristic in ACA-seropositive SS patients [6]. As patient 2 was diagnosed with HAM, spontaneous proliferation of MNCs was significantly higher than in patient 1. Serum HTLV-I viral load was 373 copies/10<sup>4</sup> cells in patient 2, which is obviously higher than in patient 1 (<53 copies/10<sup>4</sup> cells). Serum TNF- $\alpha$  and TGF- $\beta$  levels in patient 2

were increased compared with those in patient 1, although serum IFN- $\gamma$  in both patients was within normal limits.

### Azan–Mallory staining and immunohistochemical analysis

MNC infiltration was similar in both patients; however, Azan–Mallory staining showed a stronger fibrosis in patient 2 than in patient 1 (Fig. 1). In patient 2, TGF- $\beta$  was highly stained in infiltrating MNCs and vessels, except in ductal and acinar cells. TGF- $\beta$  staining, although weaker than MSG, was also performed in the muscle in patient 2. Accordingly, infiltration of CD4+ lymphocytes, which were dominant compared with CD20 and CD68, was shown in the LSGs of both patients (Fig. 2). Although CD8+ lymphocytes were also scattered in LSGs, CD4+