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Potential Contribution of a Novel Tax Epitope–Specific CD4+ T Cells to Graft-versus-Tax Effect in Adult T Cell Leukemia Patients after Allogeneic Hematopoietic Stem Cell Transplantation

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Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is an effective treatment for adult T cell leukemia/lymphoma (ATL) caused by human T cell leukemia virus type 1 (HTLV-1). We previously reported that Tax-specific CD8+ cytotoxic T lymphocyte (CTL) contributed to graft-versus-ATL effects in ATL patients after allo-HSCT. However, the role of HTLV-1–specific CD4+ T cells in the effects remains unclear. In this study, we showed that Tax-specific CD4+ as well as CD8+ T cell responses were induced in some ATL patients following allo-HSCT. To further analyze HTLV-1–specific CD4+ T cell responses, we identified a novel HLA-DRB1*0101–restricted epitope, Tax155–167, recognized by HTLV-1–specific CD4+ Th1-like cells, a major population of HTLV-1–specific CD4+ T cell line, which was established from an ATL patient at 180 d after allo-HSCT from an unrelated seronegative donor by in vitro stimulation with HTLV-1–infected cells from the same patient. Costimulation of PBMCs with both the identified epitope (Tax155–167) and known CTL epitope peptides markedly enhanced the expansion of Tax-specific CD8+ T cells in PBMCs compared with stimulation with CTL epitope peptide alone in all three HLA-DRB1*0101+ patients post–allo-HSCT tested. In addition, direct detection using newly generated HLA-DRB1*0101/Tax155–167 tetramers revealed that Tax155–167-specific CD4+ T cells were present in all HTLV-1–infected individuals tested, regardless of HSCT. These results suggest that Tax155–167 may be the dominant epitope recognized by HTLV-1–specific CD4+ T cells in HLA-DRB1*0101+–infected individuals and that Tax-specific CD4+ T cells may augment the graft-versus-Tax effects via efficient induction of Tax-specific CD8+ T cell responses. *The Journal of Immunology*, 2013, 190: 000–000.

uman T cell leukemia virus type 1 (HTLV-1) is the causative agent of a highly aggressive CD4+ T cell malignancy, adult T cell leukemia/lymphoma (ATL) (1, 2). This virus has infected 10–20 million people worldwide, especially in southern Japan, the Caribbean basin, South America, Melanesia, and equatorial Africa (3). Approximately 5% of HTLV-1–seropositive individuals develop ATL, and another 2–3% develop a slow progressive neurologic disorder known as HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP) or various chronic inflammatory diseases (4). The majority of HTLV-1–infected individuals remain asymptomatic throughout their lives.

ATL is characterized by extremely poor prognosis, mainly because of intrinsic drug resistance to cytotoxic agents. It has been reported that allogeneic hematopoietic stem cell transplantation (allo-HSCT), but not autologous HSCT, improved the outcome of ATL (5, 6). In previous clinical studies carried out by the ATL allo-HSCT Study Group, the overall survival rate within 3 y after allo-HSCT with reduced intensity conditioning (RIC) was 36% (7). HTLV-1 proviral load became and remained undetectable in some ATL patients with complete remission after allo-HSCT, suggesting that it is an effective treatment for ATL (7–9). In these studies, we reported that donor-derived HTLV-1 Tax-specific CD8+ CTLs were induced in some ATL patients who achieved complete remission after allo-HSCT (10). These CTLs were able to lyse recipient–derived HTLV-1–infected T cells in vitro, suggesting potential contributions to graft-versus-leukemia effects. CD8+ T cells, especially CTLs, generally play an important role in controlling viral replication in various infections, such as those

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involving HIV, hepatitis B virus, and hepatitis C virus. In HTLV-1 infection, HTLV-1–specific CD8$^+$ T cells predominantly recognize the Tax Ag and are believed to contribute to controlling infected cells (11, 12). A high frequency of functional Tax-specific CD8$^+$ T cells can be detected in HAM/TSP patients and some asymptomatic carriers (ACs), whereas most ATL patients and a small population of ACs show severely reduced Tax-specific CD8$^+$ T cell responses (13, 14). The mechanism underlying the suppression of HTLV-1–specific CD8$^+$ T cell responses in these patients has not yet been fully elucidated.

For induction and maintenance of virus-specific CTLs, virus-specific CD4$^+$ Th cell responses are required in many virus infections (15–19). However, there are only a few reports of HTLV-1–specific Th cell responses (20–23), presumably because of their susceptibility to HTLV-1 infection in vivo and in vitro (24). Preferential HTLV-1 infection in HTLV-1–specific CD4$^+$ T cells could be one of the reasons for immune suppression in ATL patients. In addition, it has been reported that a higher frequency of CD4$^+$FOXP3$^+$ regulatory T (Treg) cells is observed in infected individuals compared with uninfected healthy donors. The frequency of Tax$^-$ Treg cells, which are a major population of Treg cells in infected individuals, is negatively correlated with HTLV-1–specific CTL responses (25). HTLV-1 basic leucine zipper factor might also be involved in immune suppression, because HTLV-1 basic leucine zipper was constitutively expressed in infected cells (26) and inhibited the activity of IFN-γ promoters by suppressing NFAT and AP-1 signaling pathways, resulting in the impaired secretion of Th1 cytokines from CD4$^+$ Th cells in a transgenic mouse model (27) These reports suggest that both the dysfunction of HTLV-1–specific CD4$^+$ Th cells and the increased number of uninfected Treg cells might be implicated in the immunosuppression observed in ATL patients. Conversely, in HAM/TSP patients, CD4$^+$ T cells are predominantly found in early active inflammatory spinal cord lesions (28, 29) with spontaneous production of proinflammatory, neurotoxic cytokines, such as IFN-γ and TNF-α (30), suggesting their contributions to the pathogenesis of HAM/TSP. However, the precise roles of HTLV-1–specific CD4$^+$ T cells in HTLV-1 infection remain unclear.

In some ATL patients who achieved complete remission after allo-HSCT, it has been suggested that donor-derived HTLV-1 Tax-specific CTLs may contribute to elimination of ATL cells (graft-versus-Tax effects) (10). We believe that CD4$^+$ T cells also play a critical role in the graft-versus-ATL effects because CD4$^+$ T cells are required for induction and maintenance of optimal CTL responses (15–19). It therefore is important to clarify the role of HTLV-1–specific CD4$^+$ T cells in the effects for understanding HTLV-1–specific T cell immunity in ATL patients after allo-HSCT and for developing new vaccine strategies to prevent recurrence of ATL.

Several studies have reported some HTLV-1–specific CD4$^+$ T cell epitopes restricted by different HLA haplotypes (20–23). The helper functions of these epitopes in HTLV-1–specific CTL responses in HTLV-1–infected individuals have not been well understood. However, Jacobson et al. (20) showed that CD4$^+$ T cells specific for Env gp46 196–209, an epitope restricted by HLA-DQ5 or -DRw16, exhibited a cytotoxic function by directly recognizing HTLV-1–infected cells. This observation raises the possibility that some HTLV-1–specific CD4$^+$ T cells may contribute to the graft-versus-ATL effects through their cytotoxic function in ATL patients after allo-HSCT.

In the current study, we demonstrated that both CD4$^+$ and CD8$^+$ Tax-specific T cell responses were induced in patients after allo-HSCT with RIC for ATL. To further analyze HTLV-1–specific CD4$^+$ T cell responses in ATL patients after allo-HSCT, we de-

termined a novel HLA-DRB1*0101–resricted epitope, Tax155–167, recognized by HTLV-1–specific CD4$^+$ Th1-like cells, a major population of HTLV-1–specific CD4$^+$ T (T4) cell line, which was established from a patient in complete remission following allo-HSCT with RIC. Costimulation with oligopeptides corresponding to the Th1 epitope, Tax155–167, together with a known CTL epitope led to robust expansion of Tax-specific CD8$^+$ T cells in PBMCs from three HLA-DRB1*0101$^+$ patients after allo-HSCT tested. Furthermore, Tax155–167-specific CD4$^+$ T cells were found to be maintained in all HTLV-1–infected HLA-DRB1*0101$^+$ individuals tested, regardless of HSCT, by direct detection with newly generated HLA-DRB1*0101/Tax155–167 tetramers. Our results suggest that Tax155–167 may be a dominant epitope recognized by HTLV-1–specific CD4$^+$ T cells in HTLV-1–infected individuals carrying HLA-DRB1*0101 and that Tax-specific CD4$^+$ T cells may strengthen the graft-versus-ATL effects through efficient induction of Tax-specific CTL responses.

Materials and Methods

Subjects

A total of 18 ATL patients who underwent allo-HSCT with RIC regimen, and one HTLV-1–seronegative (#365) and two seropositive donors (one AC #310 and one HAM/TSP patient #294) carrying HLA-DRB1*0101 donated peripheral blood samples after providing written informed consent. Approximately one-half of these patients received allogeneic peripheral blood stem cell transplantation from HLA-A-, B-, and -DR–identical sibling donors. The other half received allogeneic bone marrow cells from HLA-A–, B–, and DR–identical seronegative unrelated donors (Table I). These patients were the participants of clinical studies organized by the ATL allo-HSCT Study Group, supported by the Ministry of Health, Welfare, and Labor of Japan. This study was also reviewed and approved by the Institutional Ethical Committee Review Board of the Tokyo Medical and Dental University.

Generation of cell lines derived from patients and donors

PBMCs were isolated using Ficoll-Paque PLUS (GE Healthcare, Buckinghamshire, U.K.) density gradient centrifugation and stored in liquid nitrogen in Bambanker stock solution (NIPPON Genetics, Tokyo, Japan) until required. These were used in part to obtain HTLV-1–infected IL-2–dependent T cell lines (ILT) and EBV-transformed lymphoblastoid B cell lines (LCL). ILT-#350 was spontaneously immortalized during long-term culture of PBMCs from patient #350 before allo-HSCT and maintained in RPMI 1640 medium (Life Technologies, Grand Island, NY) containing 20% FCS (Sigma Aldrich, St. Louis, MO) and 30 U/ml recombinant human IL-2 (rhIL-2; Shionogi, Osaka, Japan). LCL-#307, -#341, and -#350 were established by maintaining PBMCs from ATL patients #307, #341, and #350, respectively, after allo-HSCT. These PBMCs were maintained in RPMI 1640 medium containing 20% FCS, following infection with the EBV-containing culture supernatant of the B95-8 cell line, LCL-Kan, derived from a healthy individual was also used.

Synthetic peptides

A total of 18 overlapping peptides, 12- to 25-mer in length, spanning the central region of Tax (residues 103–246) were purchased and used for epitope mapping (Scrum Tokyo, Japan) (Table II). HLA-A*2402–restricted CTL epitopes (Tax301–309, SFHSLHLLF) (10) were used for in vitro stimulation of Tax-specific CTLs (Hokudo, Sapporo, Japan).

GST–Tax fusion protein–based immunoassay

HTLV-1 Tax-specific T cell responses were evaluated using GST–fusion proteins of the N-terminal (residues 1–127), central (residues 113–237), and C-terminal (residues 224–353) regions of HTLV-1 Tax (GST–Tax-A, -B, and -C, respectively) as described previously (13, 31). PBMCs (1 × 10^6 cells/ml) were incubated with or without a mixture of GST–Tax-A, -B, and -C proteins (GST–TaxABC) in 200 μl RPMI 1640 medium supplemented with 10% FCS. After 4 d, the supernatant was collected, and the concentration of IFN-γ in the supernatant was determined using an OptiEIA Human IFN-γ ELISA Kit (BD Biosciences, San Jose, CA). The minimum detectable dose for this assay was determined to be 23.5 pg/ml IFN-γ. CD8$^+$ cells were depleted from PBMCs by negative selection using Dynabeads M-450 CD8 (Invitrogen, Carlsbad, CA), according to the

manufacturer's instructions. For cytokine profiling of a HTLV-1–specific CD4⁺ T cell line, cells were stimulated with formaldehyde-fixed ILT-#350 for 48 h. Culture supernatant was collected, and various cytokines were measured using a Human Th1/Th2/Th17 Cytokine Kit for a Cytokine Beads Array (BD Biosciences).

Induction of HTLV-1–specific CD4⁺ T cell line (T4 cells)

PBMCs (1 × 10⁶ cells/ml) from patient #350, in complete remission at 180 d after allo-HSCT, were cultured for 2 wk with 100 nM Tax301–309 peptide in 96-well round-bottom tissue culture plate (BD Biosciences) in a final volume of 200 μl RPMI 1640 medium with 20% FCS and 10 U/ml rhIL-2. CD4⁺ cells were then isolated by negative selection using a Human CD4 T lymphocyte Enrichment Set-DM (BD Biosciences) and maintained in RPMI 1640 medium with 20% FCS and 100 U/ml rhIL-2. Cells (1 × 10⁶ cells/ml) were stimulated with formaldehyde-fixed ILT-#350 (2.5 × 10⁵ cells/ml) every 2–3 wk. After multiple rounds of stimulation, the resulting CD4⁺ T cell line was assessed for HTLV-1 specificity by comparing IFN-γ production against ILT-#350 to that against an HTLV-1–negative cell line, LCL-#350.

RT-PCR

Total RNA from cells was isolated using Isogen (Nippon Gene, Tokyo, Japan) and Turbo DNA-free (Life Technologies). First-strand cDNA was prepared from 0.5 μg RNA using ReverTra Ace and Oligo(dT)$_{20}$ primers provided in a ReverTra Ace-α-kit (Toyobo, Osaka, Japan). PCRs were performed in 50 μl reaction mixture containing ReverTra Dash (Toyobo), 0.5 μM of each HTLV-1 pX-specific primer (pX1, 5'-CCA CTT CCC AGG GTT TAG ACA GAT CTT C-3' and pX4, 5'-TTC CTT ATC CCT CGA CTC CCC TCC TTC CCC-3'), and 2 μl cDNA. GAPDH-specific primers (GAPDH5', 5'-ACC ACA GTC CAT GCC ATC AC-3'; GAPDH3', 5'-TCC ACC ACC CTG TTG CTG TA-3') were used as an internal control. The thermal cycling conditions comprised an initial activation step at 94°C for 1 min, followed by 30 cycles of denaturation (98°C, 10 s), annealing (60°C, 2 s), and extension (74°C, 30 s). The PCR amplicons were visualized by ethidium bromide staining following 2% (w/v) agarose gel electrophoresis.

Flow cytometry

For cell surface staining, the following fluorochrome-conjugated mouse anti-human mAbs were used: CD3-FITC (UCHT1; BioLegend, San Diego, CA), CD4-FITC (RPA-T4; BioLegend), CD8-FITC (RPA-T8; BioLegend), and CD8-PE-Cy5 (HIT8a; BD Biosciences, San Jose, CA). For tetramer staining, PE-conjugated HLA-A*0201/Tax11–19, HLA-A*1101/Tax88–96, HLA-A*1101/Tax272–280, and HLA-A*2402/Tax301–309 tetramers were purchased from Medical & Biological Laboratories (Nagoya, Japan). PE-conjugated HLA-DRB1*0101/Tax155–167 tetramer were newly generated through the custom service of Medical & Biological Laboratories. Whole-blood or cultured cells were stained with PE-conjugated Tax/HLA tetramer in conjunction with CD3-FITC and CD8-PE-Cy5 or CD4-PE-

Cy5. For whole-blood samples, RBCs were lysed and fixed in BD FACS lysing solution (BD Biosciences) before washing. Samples were analyzed on a FACSCalibur (BD Biosciences), and data analyses were performed using FlowJo software (Tree Star, Ashland, OR).

Epitope mapping

T4 cells (3 × 10⁵ cells/ml) were stimulated with LCL-#350, pulsed with various concentrations of synthetic peptides for 1 h at 37°C, at a responder/stimulator (R/S) ratio of 3. The culture supernatant was collected at 6 h poststimulation, and peptide-specific IFN-γ production from T4 cells was determined by ELISA.

HLA class II restriction assay

T4 cells (5 × 10⁵ cells/ml) were cocultured for 6 h with ILT-#350 (1 × 10⁵ cells/ml) in the presence or absence of anti-human HLA-DR (10 μg/ml; L243; BioLegend), anti-human HLA-DQ (10 μg/ml; SPVL3; Beckman Coulter, Fullerton, CA), or anti–HLA-ABC (10 μg/ml; W6/32; BioLegend). The IFN-γ in the supernatant was measured by ELISA.

To identify a HLA class II molecule responsible for Ag presentation to T4 cells, Tax155–167 peptide-specific IFN-γ responses were evaluated using various HLA-typed LCLs (LCL-#350, LCL-#341, LCL-#307, and LCL-Kan). These LCLs (1 × 10⁵ cells/ml) were pulsed with 100 ng/ml Tax155–167 peptide for 1 h, fixed with 2% formaldehyde, and then cultured with T4 cells (3 × 10⁵ cells/ml) for 6 h. The culture supernatant was collected, and IFN-γ in the supernatant was measured by ELISA.

Tetramer-based proliferation assay

PBMCs (1.0 × 10⁶ cells/ml) were cultured for 13 or 14 d with or without 100 nM antigenic peptides in the presence of 10 U/ml rhIL-2. Cells were stained with HLA/Tax tetramer-PE, CD3-FITC, and CD8-PE-Cy5 or CD4-PE-Cy5 and then analyzed by flow cytometry.

Statistic analysis

Statistical significance was evaluated with the unpaired t test using Graphpad Prism 5 (Graphpad Software, La Jolla, CA). In all cases, two-tailed p values <0.05 were considered significant.

Results

Tax-specific T cell responses in ATL patients who received allo-HSCT with RIC

We previously reported that Tax-specific CD8⁺ T cells were induced in some ATL patients after allo-HSCT with RIC from HLA-identical sibling donors (10). In this study, we examined the Tax-specific T cell response in a larger number of ATL patients who received allo-HSCT with RIC. Table I provides a summary of the

Table I. Clinical information and summary for Tax-specific CD8⁺ T cells in 18 ATL patients at 180 d post–allo-HSCT with RIC

ATL Subtype	Type of Donor	Donor-HLA	Donor HTLV-1 Sero Status	Chimerism (%)[a]	Tetramer (%)[b]	Proviral Load[c]
Lymphoma	r-PB	A 26/33, DR 4/13	(−)	<5	NT	0.1
Acute	r-PB	A 2/26, DR 10/18	(−)	<5	0.00	0.1
Lymphoma	r-PB	A 24/−, DR 9/15	(−)	<5	0.07	0.1
Lymphoma	r-PB	A 24/33, DR 13/15	(−)	<5	0.00	0.0
Lymphoma	r-PB	A 24/26, DR 4/15	(+)	<5	1.34	4.8
Acute	ur-BM	A 24/33, DR 13/15	(−)	<5	0.72	0.0
Acute	r-PB	A 2/11, DR 14/15	(+)	<5	0.10	5.4
Acute	ur-BM	A 2/24, DR 14/15	(−)	<5	0.92	0.0
Acute	ur-BM	A 11/24, DR 8/9	(−)	<5	0.75	NT
Acute	r-PB	A 2/24, DR 4/8	(−)	<5	1.40	0.7
Acute	ur-BM	A 24/33, DR 1/15	(−)	<5	0.45	0.1
Lymphoma	ur-BM	A 2/24, DR 4/−	(−)	<5	0.44	0.0
Acute	r-PB	A 24/−, DR 8/15	(+)	<5	0.00	0.0
Acute	ur-BM	A 24/26, DR 1/14	(−)	<5	0.59	0.6
Acute	ur-BM	A 24/26, DR 9/12	(−)	<5	0.45	0.0
Lymphoma	r-PB	A 2/11, DR 4/14	(−)	<5	0.42	0.0
Acute	ur-BM	A 11/26, DR 8/15	(−)	<5	0.14	0.0
Acute	r-PB	A 24/26, DR 1/−	(−)	<5	0.11	0.0

[a]Indicates percentage of recipient-derived T cell chimerism.
[b]Indicates percentage of tetramer⁺ cells among CD8⁺ T cells in PBMCs.
[c]Indicates copy number per 1000 PBMCs.
F, Female; M, male; NT, not tested; r-PB, related donor-derived peripheral blood stem cell; ur-BM, unrelated donor-derived bone marrow cell.

results of Tax-specific CD8⁺ T cell detection by flow cytometry, using the Tax/HLA tetramers, in the peripheral blood of 18 ATL patients at 180 d after allo-HSCT, together with clinical information. During this period, all patients achieved a complete chimera state consisting of >95% of donor-derived hematopoietic cells. By using four available tetramers (HLA-A*0201/Tax11–19, HLA-A*2402/Tax301–309, HLA-A*1101/Tax88–96, and HLA-A*1101/Tax272–280), Tax-specific CD8⁺ T cells were found in 14 patients. Because the donors were uninfected individuals in the majority of cases (Table I), induction of the Tax-specific donor-derived CD8⁺ T cells in recipients indicated the presence of newly occurring immune responses against HTLV-1 in the recipients. This evidence strengthens our previous observation (10, 32).

We also used a GST–Tax fusion protein-based assay to evaluate Tax-specific T cell responses. The tetramer-based assay was limited to four kinds of epitopes and restricted by three HLA alleles but did not detect T cells directed to other epitopes or HLAs. The GST–Tax fusion protein-based assay can detect both CD4⁺ and CD8⁺ T cell responses, irrespective of HLA types. However, this sensitivity is not as good as single-cell analysis by flow cytometry (31). As shown in Fig. 1A, there was a wide variation in the IFN-γ responses to the Tax protein in the PBMCs among the 16 patients tested. In five patients (#247, #270, #328, #340, and #349), IFN-γ production of PBMCs against GST–TaxABC proteins was very low or not specific for the Tax protein. PBMCs from the other 11 patients (#239, #241, #301, #317, #341, #344, #350, #351, #352,

#358, and #364) produced higher amounts of IFN-γ in response to GST–TaxABC proteins compared with GST. However, the levels of IFN-γ production varied among the patients.

We also evaluated the extent to which Tax-specific CD4⁺ T cells were responsible for IFN-γ in the GST–Tax-based immunoassay system. We used PBMCs from patients #350 and #341, who showed high Tax-specific T cell responses. CD8⁺ cell–depleted PBMCs from patient #350 and #341 showed a reduced but still significant level of Tax-specific IFN-γ–producing response compared with whole PBMCs (Fig. 1B). These results indicate that not only CD8⁺ but also CD4⁺ T cells against Tax are present in the peripheral blood from patient #350 and #341 after allo-HSCT with RIC.

Induction of an HTLV-1–specific CD4⁺ T cell line from patient #350

We next attempted to induce HTLV-1–specific CD4⁺ T cells from the PBMCs of patient #350 at 180 d after allo-HSCT, using an HTLV-1–infected T cell line (ILT-#350) as APCs. Freshly isolated PBMCs were stimulated for 2 wk with Tax301–309, a dominant CTL epitope presented by HLA-A*2402, to eliminate HTLV-1–infected cells, which potentially existed in PBMCs. The CD4⁺ cells were then isolated from the cultured cells and stimulated with formaldehyde-fixed ILT-#350 every 2–3 wk. The established cell line was found to be a CD4⁺ T cell line (designated as T4 cells thereafter) because cells expressed CD3 and CD4 but not CD8
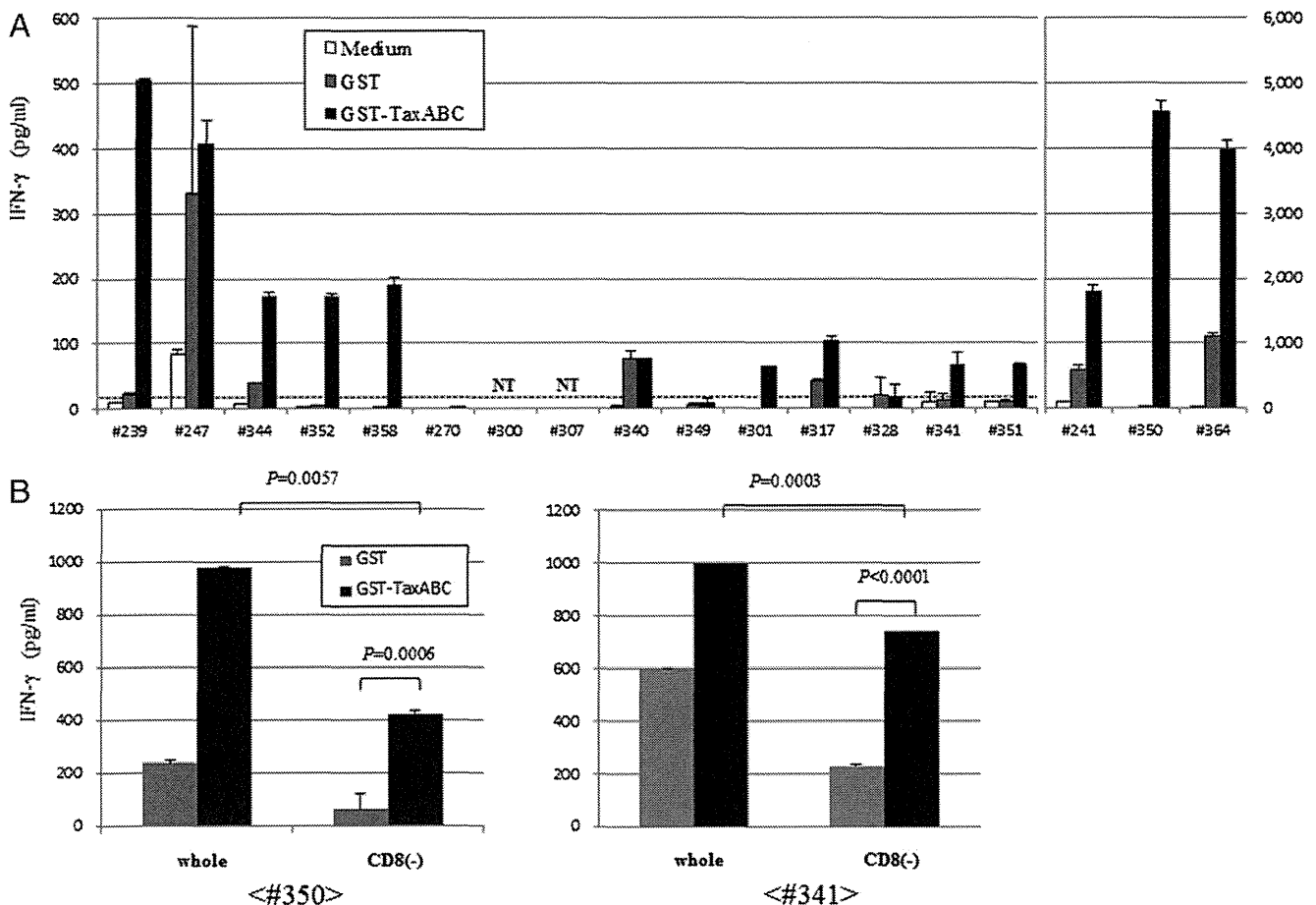
FIGURE 1. Diversity of Tax-specific T cell responses in ATL patients who received allo-HSCT with RIC. (**A** and **B**) PBMCs from 18 ATL patients at 180 d after allo-HSCT (A) or whole and CD8⁺ cell–depleted PBMCs from two patients at 540 d after allo-HSCT (#350 and #341) (B) were cultured for 4 d in the absence (open square) or presence of GST (gray square), or GST–Tax (black square) proteins. The concentration of IFN-γ in the supernatant was determined by ELISA. The y-axis on the right side indicates the results from three patients (#241, #350, and #364). The dotted horizontal line indicates the detection limit (23.5 pg/ml). The error bars represent SD of duplicated wells. The representative result of two independent experiments is shown in (B).

(Fig. 2A). Because HTLV-1 has been shown to preferentially infect CD4$^+$ T cells in vivo and in vitro (24), we examined HTLV-1 expression in T4 cells by RT-PCR (Fig. 2B). As expected, the T4 cells did not express HTLV-1 Tax, indicating that the cells were not infected with HTLV-1. We assessed expression of various cytokines in T4 cells (Fig. 2C). The T4 cells were stimulated with formaldehyde-fixed ILT-#350 or LCL-#350. The cells produced large amounts of IFN-γ and TNF-α and small amounts of IL-2, IL-4, and IL-10 in response to ILT-#350 but not against LCL-#350. IL-6 and IL-17A were not detected in the culture supernatant. These data indicate that T4 cells are mainly HTLV-1–specific CD4$^+$ Th1-like cells but contain minor populations to produce Th2 cytokines.

Determination of the minimum epitope recognized by T4 cells

Freshly isolated PBMCs in the patient #350 produced IFN-γ in response to GST–Tax (Fig. 1A). We expected that the epitope recognized by the T4 cells should be present in the Tax protein. We therefore examined whether the T4 line responded to Tax using LCL-#350 pulsed with GST–Tax proteins as APCs. As shown in Fig. 3A, the T4 cells produced significantly higher amounts of IFN-γ in response to GST–TaxABC and GST–Tax-B (residues 113–237) (31) but not GST–Tax-A (residues 1–127) (31) and -C (residues 224–353 (31), when compared with the GST control protein, indicating that the T4 cells recognized the central region (residues 113–237) of the Tax Ag. We next synthesized eight overlapping 25-mer peptides spanning the central region of Tax (residues 103–246) and analyzed their abilities to stimulate T4 cells (Table II). The cell line produced high amounts of IFN-γ only when stimulated with Tax154–178 (Fig. 3B). We then prepared four overlapping 15-mer peptides, covering residues 154–178 of Tax, to examine the IFN-γ responses of the T4 cells (Table II). Both Tax151–165 and Tax156–170-stimulated cells to induce IFN-γ responses but not at a comparable level to Tax154–178 (Fig. 3C). These results suggest that the epitope recognized by T4 cells might be present in the N-terminal half of Tax154–178. We therefore stimulated the cells with Tax154–168, Tax155–169, or Tax156–170.

The cells showed higher IFN-γ responses against Tax154–168 and Tax155–169 than Tax156–170, indicating that the minimum epitope might be within residues 155–168 of Tax (Fig. 3D). To identify the minimum epitope recognized by T4 cells, we next synthesized three overlapping peptides of 12- to 14-mer lengths beginning at residue 155 of Tax (Table II). Tax155–167 induced IFN-γ responses in cells at a similar level to Tax155–169 and Tax155–168, although Tax155–166 did not (Fig. 3E). Moreover, IFN-γ production of cells in response to various concentrations of Tax155–167 was comparable to that against Tax155–169 and Tax155–168 (Fig. 3F). These data clearly show that the minimum epitope recognized by the T4 cells is Tax155–167.

*HLA-DRB1*0101 restriction of Tax-specific T4 cells*

To analyze HLA class II molecules involved in the presentation of the minimum epitope, T4 cells were stimulated with ILT-#350 in the presence or absence of anti–HLA-DR, -DQ, and anti-HLA class I blocking Abs. As shown in Fig. 4A, the addition of an anti–HLA-DR blocking Ab abrogated IFN-γ responses of the T4 cells against ILT#-350, indicating that the epitope was HLA-DR restricted.

We further investigated the HLA-DR alleles responsible for the presentation of the minimum epitope by using four HLA-typed LCLs displaying different HLA-DRs. As shown in Fig. 4B, the T4 cells responded by producing IFN-γ when Tax155–167 was presented by autologous LCL-#350 (DR1/14) and allogeneic LCL-#341 (DR1/15). These results clearly indicate that this epitope is presented by HLA-DRB1*0101 on APCs. We searched for a known HLA-DRB1*0101 motif in the identified epitope Tax155–167 and found that this epitope contained the HLA-DRB1*0101 motif (Fig. 4C) (33).

Enhancement of Tax-specific CD8$^+$ T cell expansion by Tax155–167-specific CD4$^+$ T cell help

As T4 cells were established from PBMCs of an HTLV-1–infected patient #350, it is suggested that Tax155–167-specific CD4$^+$ T cells may be maintained in the HLA-DRB1*0101$^+$ patient #350.
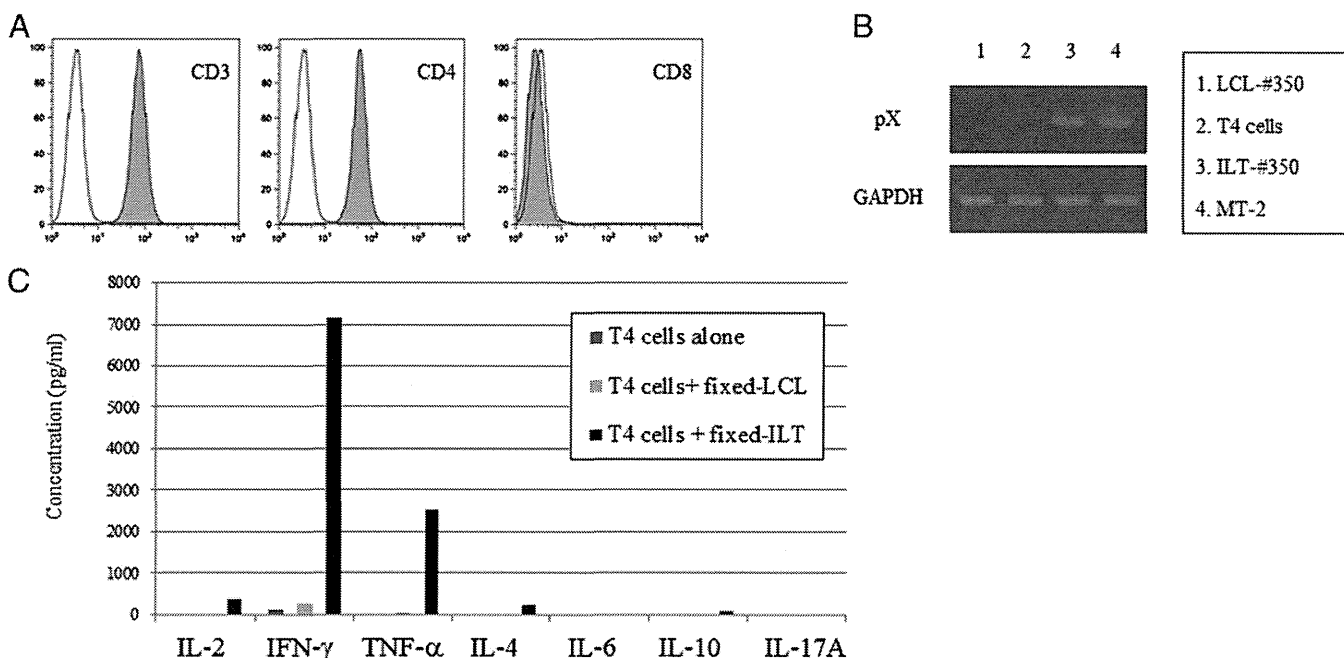
FIGURE 2. Phenotype and function of CD4$^+$ T cell line (T4) generated from patient #350. (**A**) Cell surface phenotype of T4 cells was analyzed by flow cytometry. (**B**) Total RNA was extracted from LCL-#350 (*lane 1*), T4 cells (*lane 2*), ILT-#350 (*lane 3*), and MT-2 (*lane 4*). Tax mRNA expression for each cell type was analyzed by RT-PCR. GAPDH was used as an internal control. (**C**) T4 cells were stimulated for 24 h with or without formaldehyde-fixed ILT-#350 or LCL-#350 cells. The concentration of indicated cytokines in the supernatants was measured using a cytometric bead array system.

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FIGURE 3. Identification of the dominant Tax-derived epitope recognized by established T4 cells. (**A**) Donor-derived LCL-#350 was pulsed with GST, GST–Tax-A, GST–Tax-B, GST–Tax-C, or a mixture of GST–Tax-A, -B, and -C (GST–TaxABC) for 24 h and then cocultured for 24 h with the T4 cells at a responder/stimulator (R/S) ratio of 3. IFN-γ production from T4 cells was analyzed by ELISA. (**B** and *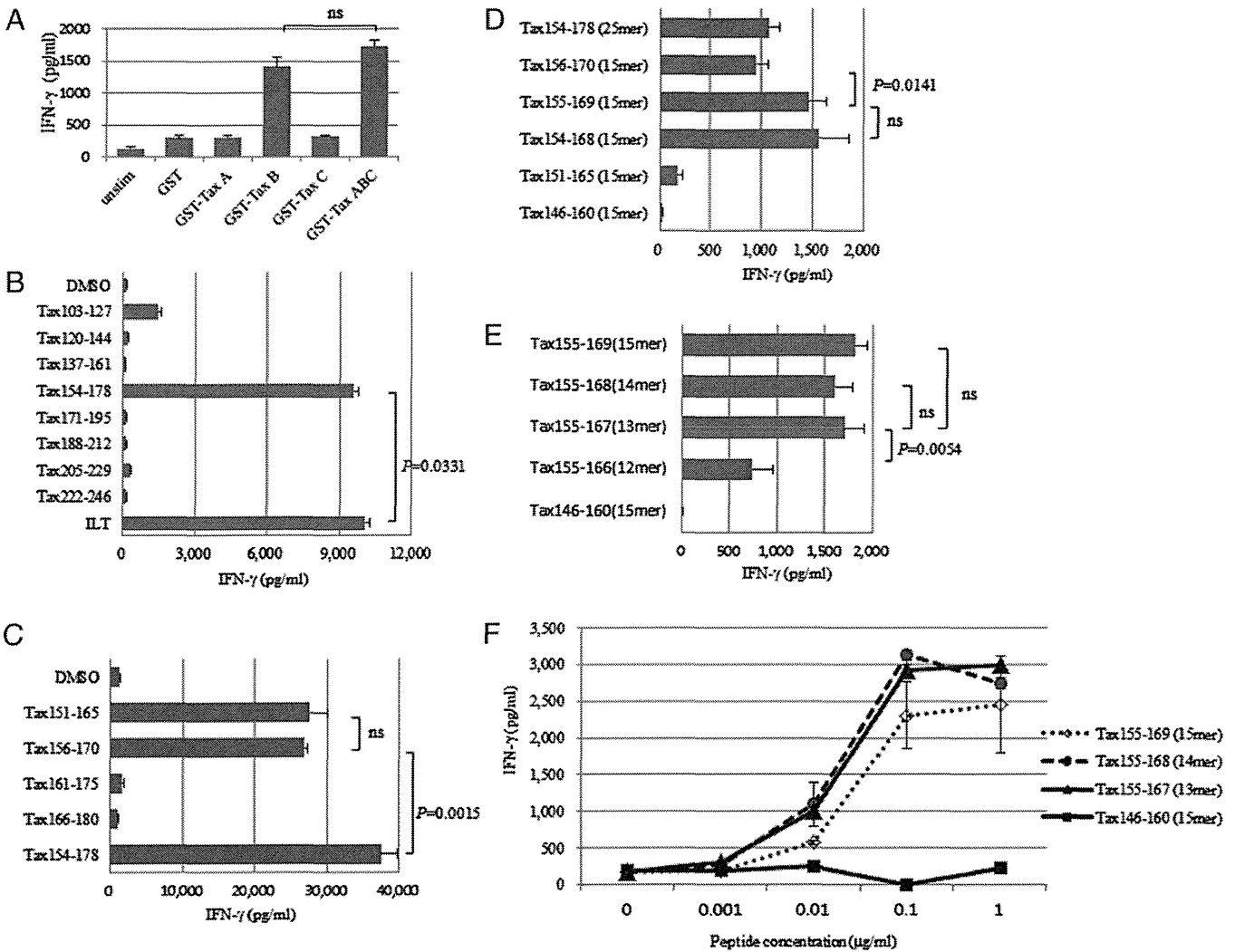*C**) LCL-#350 was pulsed with the indicated overlapping 25-mer-long (B) or 15-mer-long (C) synthetic peptides (10 μg/ml) within the Tax-B region for 1 h. Formaldehyde-fixed ILT-#350 cells were cocultured with T4 cells for 6 h. IFN-γ in the supernatant was measured by ELISA. (**D** and **E**) IFN-γ responses of T4 cells were assessed using the indicated overlapping 12- to 25-mer-long synthetic peptides (100 ng/ml). (**F**) IFN-γ responses of T4 cells against indicated concentrations of 13- to 15-mer-long peptides were assessed as in (B) and (C). (A–F) Results are representative of two or three independent experiments. The error bars represent SD of triplicate wells. Statistical significance was analyzed by the unpaired t test.

We therefore evaluated the helper function of Tax155–167-specific CD4$^+$ T cells on the expansion of dominant Tax-specific CTLs in fresh PBMCs of the patient #350. Freshly isolated PBMCs from patient #350 (A24/26, DR1/14) at 540 d after allo-HSCT were stimulated for 13 d with the HLA-A24–restricted CTL epitope peptide (Tax301–309) in the presence or absence of the HLA-DRB1*0101–restricted CD4$^+$ Th epitope peptide (Tax155–167), and Tax-specific CD8$^+$ T cell expansion was evaluated using the HLA-A*2402/Tax301–309 tetramer. As shown in Fig. 5, Tax301–309-specific CD8$^+$ T cells proliferated to 9.26% of CD8$^+$ T cells when stimulated with Tax301–309 alone. Surprisingly, a highly elevated frequency (62.3%) of tetramer-binding CD8$^+$ T cells was detected by in vitro costimulation with Tax301–309 and Tax155–167, suggesting the presence of Tax155–167-specific CD4$^+$ Th cells in patient #350.

We examined whether Tax155–167-specific CD4$^+$ T cells existed and functioned as helper cells in the other two HTLV-1–infected HLA-DRB1*0101$^+$ patients after allo-HSCT (day 360 for patient #341 and day180 for #364). These patients had detectable

levels of HLA-A*2402/Tax301–309 tetramer-binding CD8$^+$ T cells in the peripheral blood (Fig. 5). In patients #341 and #364, the tetramer-binding cells expanded to 7.7 and 0.849% of CD8$^+$ T cells at 13 d of culture when stimulated with the CTL epitope peptide, Tax301–309, alone. Costimulation of PBMCs with both peptides Tax155–167 and Tax301–309 led to a vigorous proliferation of tetramer-binding CD8$^+$ T cells (59.6% for patient #341 and 15.5% for patient #364) as observed in patient #350 (Fig. 5). These results indicate that Tax155–167-specific CD4$^+$ T cells may be present and contribute to enhancing CD8$^+$ T cell responses in HTLV-1–infected HLA-DRB1*0101$^+$ individuals after allo-HSCT.

*Tax155–167-specific CD4$^+$ T cells were maintained in HTLV-1–infected HLA-DRB1*0101$^+$ individuals*

We next generated the HLA-DRB1*0101/Tax155–167 tetramer to directly detect Tax155–167-specific CD4$^+$ T cells and examined the presence of Tax155–167-specific CD4$^+$ T cells in the PBMCs freshly isolated from two HLA-DRB1*0101$^+$ patients after allo-HSCT (day 180 for patient #350 and day 360 for patient #364).

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$^+$ T cells were detected ex vivo in the patient #350 (0.11%) and proliferated to 11.6% among CD4$^+$ T cells at 13 d poststimulation with Tax155–167 peptide. In the patient #364, tetramer-binding CD4$^+$ 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$^+$–seronegative donor #365, Tax155–167-specific CD4$^+$ 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$^+$ 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$^+$ 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$^+$ T cells, respectively (Fig. 6B). These results suggest that Tax155–167-specific CD4$^+$ 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$^+$ 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$^+$ T cells and consequently led to robust Tax-specific CD8$^+$ T cell expansion. We also found that Tax155–167-specific CD4$^+$ T cells existed in all HTLV-1–infected HLA-DRB1*0101$^+$ 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$^+$ T cells

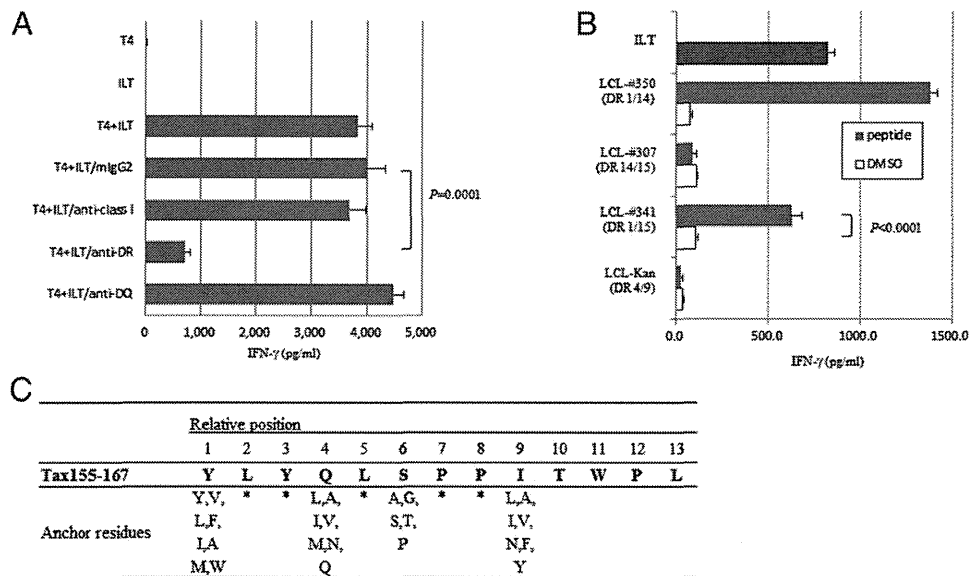|	Relative position												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Tax155-167	Y	L	Y	Q	L	S	P	P	I	T	W	P	L
Anchor residues	Y,V, L,F, I,A M,W	*	*	L,A, I,V, M,N, Q	*	A,G, S,T, P	*	*	L,A, I,V, N,F, Y				

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^{+} 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^{+} cells among CD3^{+}CD8^{+} T cells. Fresh indicates frequency of HLA-A*2402/Tax301-309 tetramer^{+}CD8^{+} T cells detected in fresh peripheral blood.
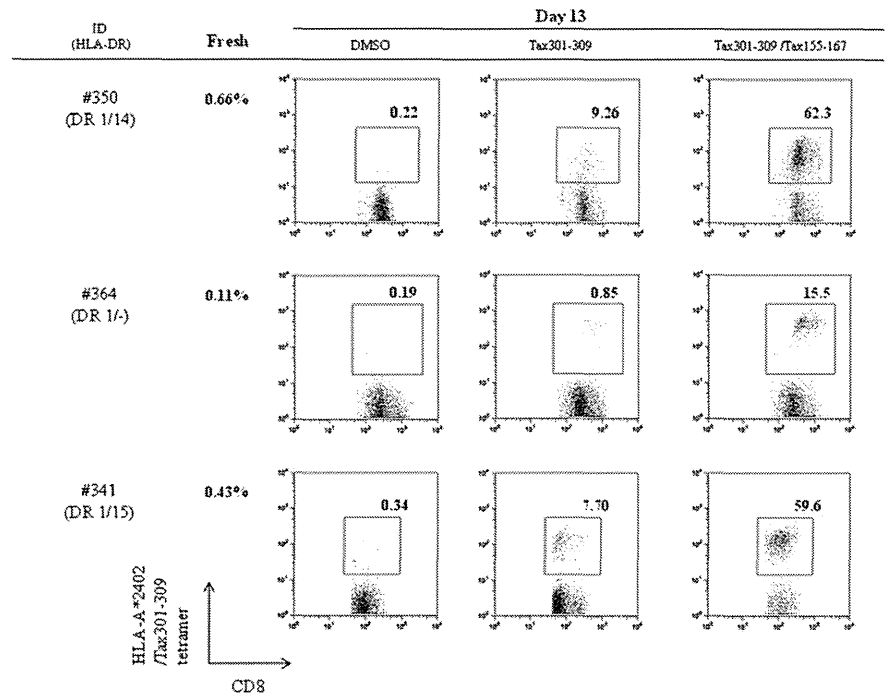
in HTLV-1–infected individuals expressing HLA-DRB1*0101 and that Tax-specific CD4^{+} T cells might efficiently induce HTLV-1–specific CTL expansion to strengthen the graft-versus-ATL effects in ATL patients after allo-HSCT.

In HTLV-1 infection, analysis of virus-specific CD4^{+} T cell responses appears to be limited because CD4^{+} 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^{+} 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^{+} T cells to enhance Tax-specific CD8^{+} T cell expansion was observed in PBMCs from all three HLA-DRB1*0101^{+} patients tested (Fig. 5). These data suggest that both CD8^{+} and CD4^{+} 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^{+} T cells, graft-versus-host reaction may mainly contribute to achieve complete remission after allo-HSCT. It is of note that Tax-specific
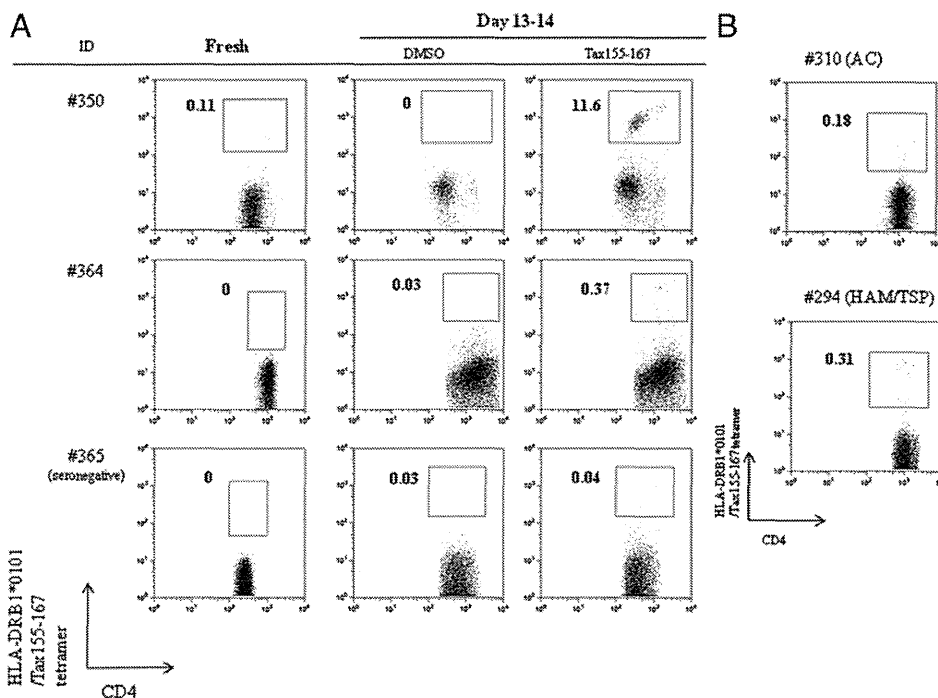
FIGURE 6. Detection of Tax155-167-specific CD4^{+} T cells in HTLV-1–infected HLA-DRB1*0101^{+} individuals. **(A)** In two ATL patients after allo-HSCT (#350 and #364) and an HLA-DRB1*0101^{+}–seronegative donor (#365), frequency of HLA-DRB1*0101/Tax155-167 tetramer-binding CD4^{+} T cells was analyzed in fresh PBMCs and PBMCs cultured for 13–14 d in the presence of Tax155-167 (100 nM) peptide. Data indicate percentages of tetramer^{+} cells in CD3^{+}CD4^{+} T cells. **(B)** Frequency of HLA-DRB1*0101/Tax155-167 tetramer-binding CD4^{+} T cells in fresh PBMCs from an AC #310 and an HAM/TSP patient #294 was analyzed. Data indicate percentages of tetramer^{+} cells in CD3^{+}CD4^{+} 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+ T cells have also been known to be critical for induction and maintenance of Ag-specific CD8+ T cells (15–19). With respect to HTLV-1 infection, there are several reports identifying HLA-DRB1*0101–restricted epitopes recognized by CD4+ 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+ T cells, we established an HTLV-1–specific CD4+ 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+ T cells were present in peripheral blood from patient #350 at 180 and 540 d after all-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+ 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+ T cells may be induced in patient #350. However, Tax155–167-specific but not Tax191–205-specific CD4+ 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+ T cells may not be the most frequent population among HTLV-1–specific CD4+ T cells.

It has been known that Ag-specific effector and memory CD4+ T cells are typically present at much lower frequencies than their CD8+ 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+ T cells, the low-affinity CD4+ 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+ T cells were present in HLA-DRB1*0101+ 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+ T cells by the MHC class II tetramers. However, enhanced expansion of Tax301–309-specific CD8+ 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+ T cells were detected in fresh and/or Tax155–167-stimulated PBMCs of all HTLV-1–infected HLA-DRB1*0101+ individuals tested, although their frequencies were various. These results suggest that Tax155–167 may be the dominant epitope recognized by Tax-specific CD4+ T cells in HTLV-1–infected HLA-DRB1*0101+ 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+ T cells were present in three ATL patients after allo-HSCT from seronegative donors. This finding also suggests that Tax155–167-specific naive CD4+ T cells may pre-exist in HLA-DRB1*0101+ individuals and can be primed with HTLV-1 Ags during the primary infection. In this study, Tax155–167-specific CD4+ T cells were also detected in an AC and a HAM/TSP patient (Fig. 6B), suggesting that Tax155–167-specific CD4+ T cells may be maintained in some HLA-DR1+ 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+ T cells in HLA-DRB1*0101+–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+ 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+ 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+ T cell activation. Further studies are needed to clarify whether HLA-DRB1*0101 is associated with high Tax-specific CD4+ T cell responses in HTLV-1–infected individuals.

Early studies using lymphocytic choriomeningitis virus showed that CD4+ T cell help is critical for maintenance of CD8+ T cell function during chronic infections (18). It has also been suggested that CD4+ 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+ T cell help rescued exhausted CD8+ 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+ T cell expansion by stimulating Tax155–167-specific CD4+ T cells. This epitope would be a useful tool for investigating the roles of HTLV-1–specific CD4+ 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

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RETROVIROLOGY

HTLV-1 modulates the frequency and phenotype of FoxP3⁺CD4⁺ T cells in virus-infected individuals

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Abstract

Background: HTLV-1 utilizes CD4 T cells as the main host cell and maintains the proviral load via clonal proliferation of infected CD4⁺ T cells. Infection of CD4⁺ T cells by HTLV-1 is therefore thought to play a pivotal role in HTLV-1-related pathogenicity, including leukemia/lymphoma of CD4⁺ T cells and chronic inflammatory diseases. Recently, it has been reported that a proportion of HTLV-1 infected CD4⁺ 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⁺ 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⁺ cells in CD4⁺ 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⁺ T cells that were FoxP3⁺. The CD4⁺FoxP3⁺ T cells, themselves, were frequently infected with HTLV-1. We conclude that FoxP3⁺ 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_{reg} associated molecules CTLA-4 and GITR were decreased in CD4⁺FoxP3⁺ T cells. Further we characterized FoxP3⁺CD4⁺ T-cell subsets by staining CD45RA and FoxP3, which revealed an increase in CD45RA⁻FoxP3^low non-suppressive T-cells. These findings can reconcile the inflammatory phenotype of HAM/TSP with the observed increase in frequency of FoxP3⁺ 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⁺CD4⁺ 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⁺ 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⁺ T cells as the major host cell population, the pathogenesis by this virus may be due to abnormalities of CD4⁺ T cells in HTLV-1-infected individuals. However the precise characteristics of the putative CD4⁺ 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

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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-κB, 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$^+$ 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$^+$ T-cell subsets. CD4$^+$ 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$^+$CD4$^+$ T cells were composed of three phenotypically and functionally different subsets according to the degree of FoxP3 expression and CD45RA expression, namely CD45RA$^+$FoxP3^{low} resting T$_{reg}$ cells (rT$_{reg}$ cells), CD45RA$^-$FoxP3^{high} activated T$_{reg}$ cells (aT$_{reg}$ cells), or CD45RA$^-$FoxP3^{low} non-suppressive T cells (FoxP3^{low} non-Treg cells) [27]. Both rT$_{reg}$ cells and aT$_{reg}$ cells have suppressive function, but FoxP3^{low} 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$^+$ 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$^+$FoxP3$^+$ 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$^+$ cells is positively correlated with HTLV-1 proviral load

Previous studies reported that the HTLV-1 provirus was frequently detected in effector/memory CD4$^+$ T cells [28], but at that time the analysis did not distinguish between effector/memory CD4$^+$ T cell and regulatory T cells (T$_{reg}$ cells). Also further subsets of CD4$^+$ T cells have been identified recently, such as the division of FoxP3$^+$CD4$^+$ T cells into three distinct subsets [27]. In order to uncover the impact of HTLV-1 infection on the CD4$^+$ T-cell subset, it is necessary to re-evaluate the CD4$^+$ 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$^+$ 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$^+$ T cells between HD, AC, and HAM/TSP individuals ($p = 0.2153$ and 0.4597, respectively, Figure 1A). To characterize the CD4$^+$ T-cell subset in more detail, we stained PBMCs with anti-CD4, anti-CD45RA, and anti-FoxP3 antibodies. In this analysis we divided CD4$^+$ T cells into three distinct subsets, which include two FoxP3$^-$ populations (CD45RA$^+$ naïve T cells and CD45RA$^-$ effector/memory T cells) and a FoxP3$^+$ population. As shown in Figure 1B, the percentage of naïve CD4$^+$ 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$^+$ T cells were not significantly different among the four studied subject groups (Figure 1C). However, frequencies of FoxP3$^+$ cells in HTLV-1 infected individuals (AC^{high}, 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$^+$ 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

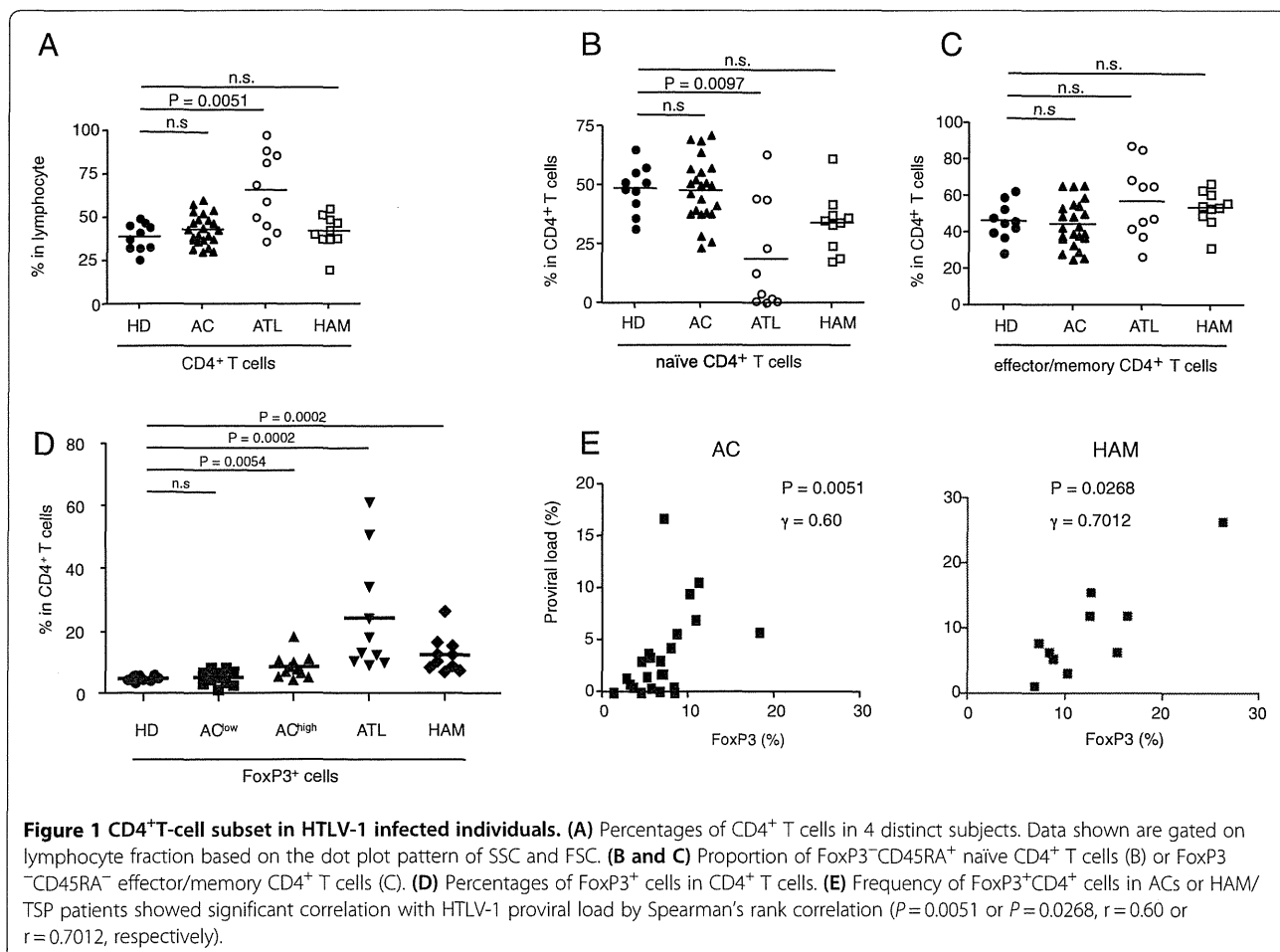| Characteristics	HD	AC	ATL	HAM/TSP
Participant number	10	23	10	10
Age, median years (IQR)	54 (49–62)	59 (50–70)	65 (61–76)	60 (54–62)
Male sex, no (%)	3 (30)	6 (26)	5 (50)	3 (30)
WBC (IQR)/µL	4,930 (1,437)	5,157 (1,100)	17,030 (12,975)	5,900 (1,500)
Lymphocyte (IQR)/µL	1,717 (503)	1,697 (601)	8,443 (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.
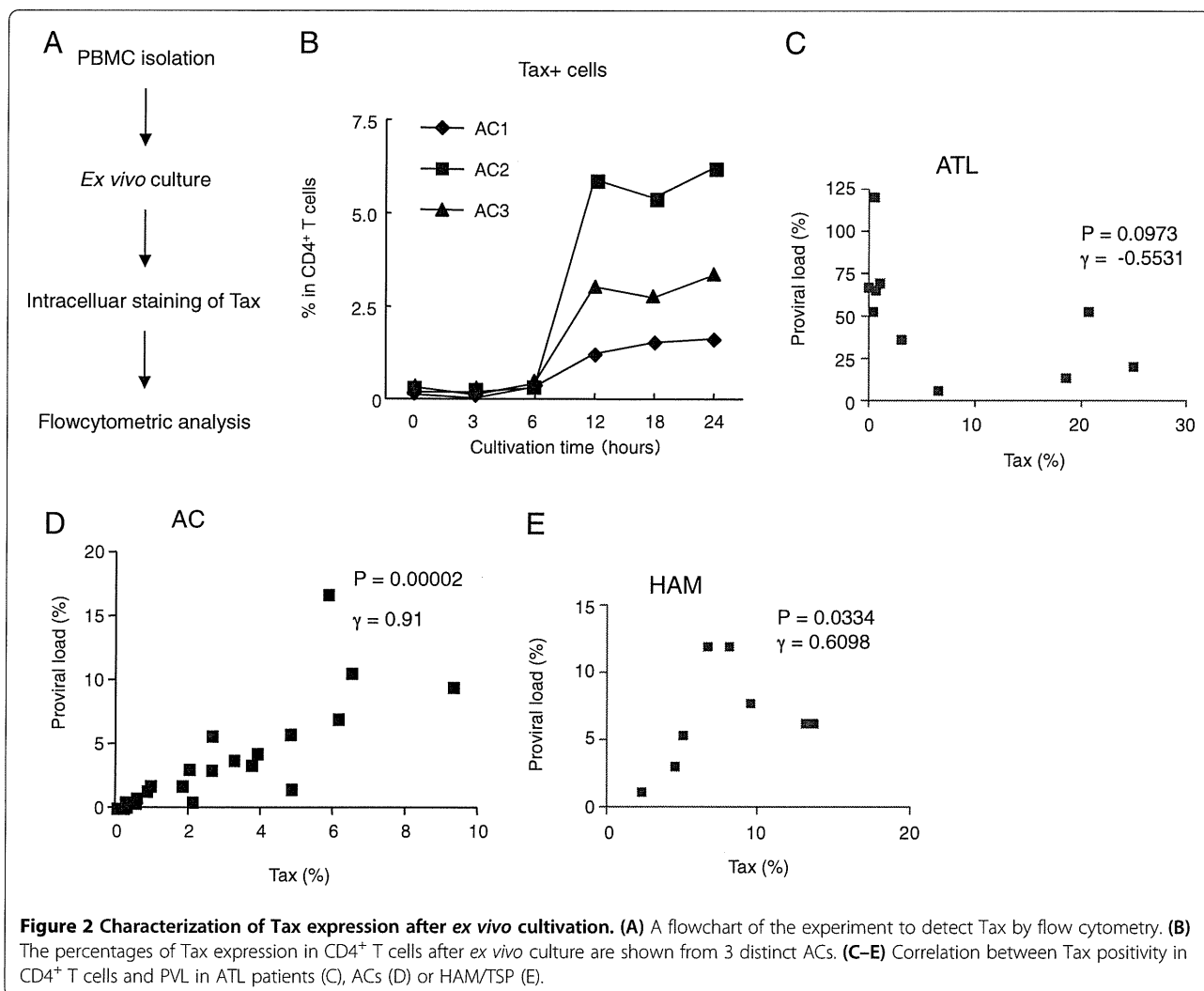
of each T-cell subset showed the same tendency as well as the frequency (Additional file 1: Figure S1). These results collectively suggested that HTLV-1 infection increased the frequency of FoxP3$^+$CD4$^+$ T cells.

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$^+$ 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$^+$T-cell subset in HTLV-1 infected individuals. (A) Percentages of CD4$^+$ 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$^-$CD45RA$^+$ naïve CD4$^+$ T cells (B) or FoxP3 $^-$CD45RA$^-$ effector/memory CD4$^+$ T cells (C). **(D)** Percentages of FoxP3$^+$ cells in CD4$^+$ T cells. **(E)** Frequency of FoxP3$^+$CD4$^+$ 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 2 Characterization of Tax expression after *ex vivo* cultivation. (A) A flowchart of the experiment to detect Tax by flow cytometry. **(B)** The percentages of Tax expression in CD4$^+$ T cells after *ex vivo* culture are shown from 3 distinct ACs. **(C–E)** Correlation between Tax positivity in CD4$^+$ T cells and PVL in ATL patients (C), ACs (D) or HAM/TSP (E).
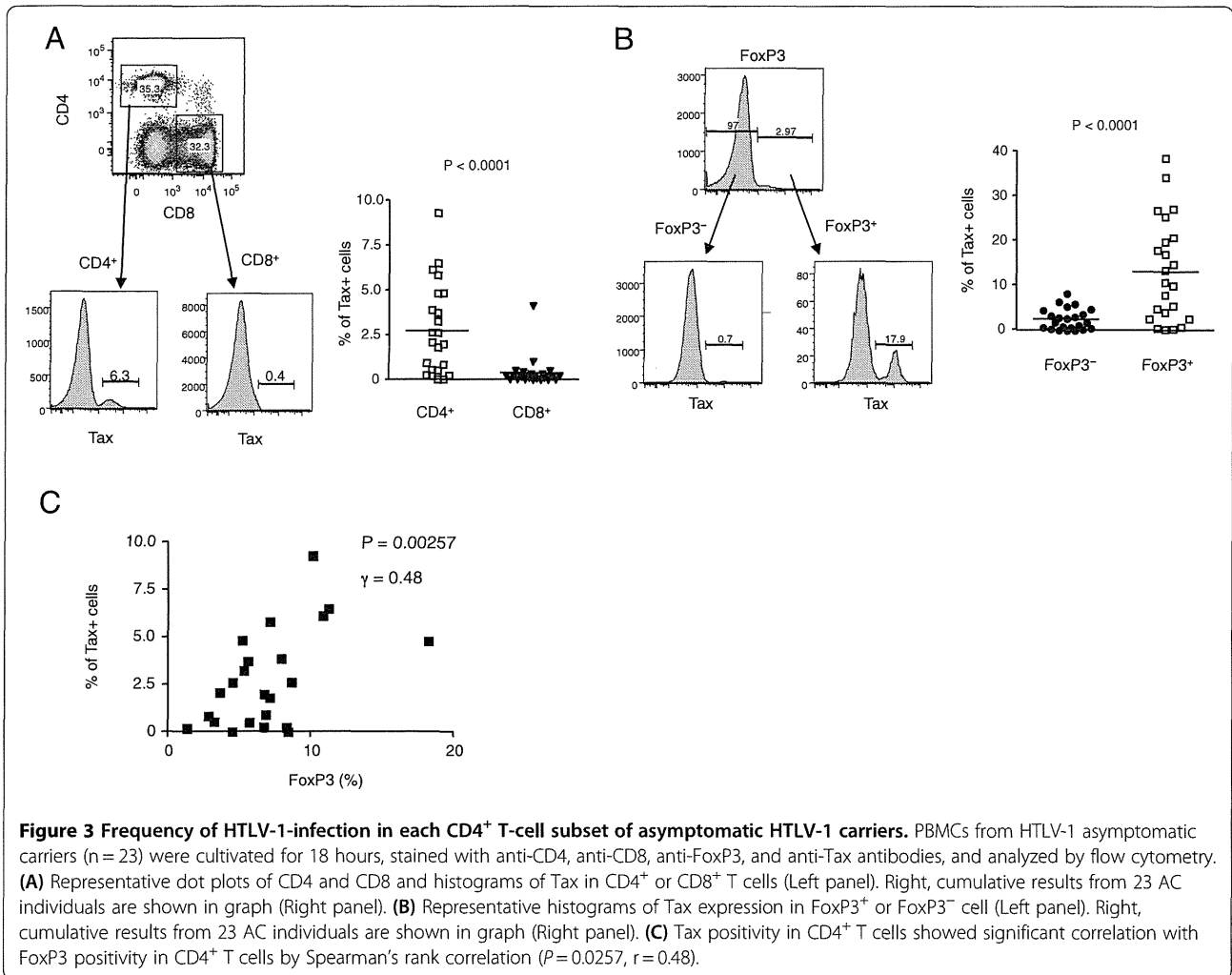
(Figure 2B). In order to confirm the efficiency of this system, we analyzed the correlation between HTLV-1 proviral load and the percentage of Tax expression in this system.

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 file2: 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$^+$ 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$^+$ T cells was much higher than that in CD8$^+$ T cells (p < 0.0001, Figure 3A). Among CD4$^+$ 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$^+$ cells were significantly correlated with Tax positivity in CD4$^+$ T cells. (r = 0.48, p = 0.0257, Figure 3C). These results indicated

Figure 3 Frequency of HTLV-1-infection in each CD4$^+$ T-cell subset of asymptomatic HTLV-1 carriers. PBMCs from HTLV-1 asymptomatic carriers (n = 23) were cultivated for 18 hours, stained with anti-CD4, anti-CD8, anti-FoxP3, and anti-Tax antibodies, and analyzed by flow cytometry. **(A)** Representative dot plots of CD4 and CD8 and histograms of Tax in CD4$^+$ or CD8$^+$ T cells (Left panel). Right, cumulative results from 23 AC individuals are shown in graph (Right panel). **(B)** Representative histograms of Tax expression in FoxP3$^+$ or FoxP3$^-$ cell (Left panel). Right, cumulative results from 23 AC individuals are shown in graph (Right panel). **(C)** Tax positivity in CD4$^+$ T cells showed significant correlation with FoxP3 positivity in CD4$^+$ T cells by Spearman's rank correlation ($P = 0.0257$, r = 0.48).

that the increased FoxP3$^+$ cells in HTLV-1-infected individuals were frequently infected with HTLV-1.

Characterization of FoxP3$^+$CD4$^+$ T-cell subset in AC

We further focused on the FoxP3$^+$CD4$^+$ T-cell subset as defined previously (Figure 4A) [27]. First, we investigated the frequency of FoxP3$^+$CD4$^+$ T-cell subset in HD or AC. The results showed that the frequencies of rT$_{reg}$ or aT$_{reg}$ in AC^{low} or AC^{high} were not significantly different from that in HD (Figure 4B and 4 C), but FoxP3^{low} non-T$_{reg}$ cells were significantly more frequent in the AC^{high} population ($p = 0.0080$, Figure 4D). We next analyzed the presence of HTLV-1 in each CD4$^+$ T-cell subset by using AC sample. We observed that Tax positivity in FoxP3$^-$ effector/memory CD4$^+$ T cells was higher than that of FoxP3$^-$ naïve CD4$^+$ 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$_{reg}$ cells or FoxP3^{low} non-T$_{reg}$ cells was much higher than that of rT$_{reg}$ cells in ACboth AC^{low} and AC^{high} subjects ($p < 0.0001$ or 0.0001, respectively, Figure 4E). These results indicated that HTLV-1 is frequently present in aT$_{reg}$ cells or FoxP3^{low} non-T$_{reg}$ 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$^+$ or CD8$^+$ 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$^+$ T cells, and at a higher frequency in CD4$^+$FoxP3$^+$ T cells than CD4$^+$FoxP3$^-$ T cells ($p = 0.0069$, Figure 5B). To characterize the phenotype of FoxP3$^+$ cells in HAM/TSP patients, we investigated the expression levels of T$_{reg}$ associated molecules, and found that the expression of GITR or CTLA-4 in HAM/TSP patients