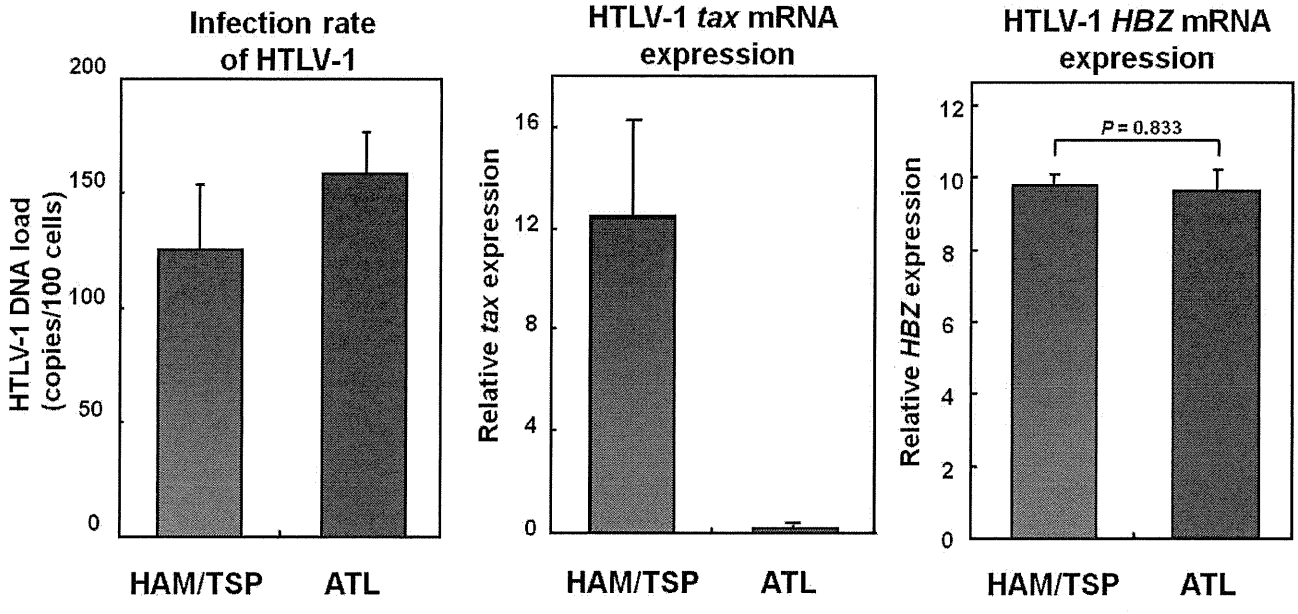
This hypothesis is currently being tested as a means of elucidating the precise molecular mechanisms by which HTLV-1 influences the fate and function of $CD4^+CD25^+CCR4^+$ T cells, especially $Foxp3^+$ Treg cells. Further research investigating this hypothesis using animal models is required, as is further work to pathologically identify the $exFoxp3^+$ cells in the spinal cord lesions of HAM/TSP patients.

Figure 6. Increased HTLV-1 *tax* mRNA expression in $CD4^+CD25^+CCR4^+$ T cells in HAM/TSP patients. The HTLV-1 proviral load in $CD4^+CD25^+CCR4^+$ T cells from HAM/TSP and ATL patients was quantified by real-time PCR (left panel, n = 3). Expression levels of HTLV-1 *tax* mRNA (center panel, HAM/TSP: n = 4, ATL: n = 3) and *HBZ* mRNA (right panel, n = 5) in $CD4^+CD25^+CCR4^+$ T cells from HAM/TSP and ATL patients were quantified by real-time RT-PCR. Data are presented as mean ± standard error.



7. Mechanisms Underlying Increased HTLV-1 Tax Expression in HAM/TSP Patients

As described above, higher levels of HTLV-1 Tax expression have been observed in HAM/TSP patients [11], and a correlation between Tax expression and disease risk [64] has been identified. Both findings, together with experimental evidence [65] and theoretical justification [66] for selective proliferation of HTLV-1 expressing T cells *in vivo*, indicate that increased HTLV-1 provirus expression may play an important role in the pathogenesis of HAM/TSP. However, the molecular mechanisms underlying the increased levels of HTLV-1 provirus expression in HAM/TSP patients are not understood. Evidence continues to accumulate that the genomic integration site of HTLV-1 provirus affects the level of provirus expression. Continued accumulation of evidence is aided by the availability of the human genome sequence, which has enabled large-scale research into HTLV-1 integration sites. This research has demonstrated that the provirus integration sites of HTLV-1 *in vivo* are not randomly distributed within the human genome but rather associated with transcriptionally active regions [67,68]; that the frequent integration into these transcription units is associated with increased levels of provirus expression; and, importantly, that the increased number of integration sites in

transcription units is associated with HAM/TSP [68]. Future research should endeavor to elucidate the mechanisms underlying the immune dysregulation observed in HAM/TSP patients.

8. Conclusion

HTLV-1 initiates persistent infection of $CD4^+$ T cells and results in the development of HAM/TSP, a chronic neuroinflammatory disorder characterized by very high strong cellular and humoral immune responses. Because a higher viral load in HTLV-1-infected individuals increases the risk of HAM/TSP and is associated with high cellular and humoral immune responses, HTLV-1 infection-induced immune dysregulation may play an important role in the development and pathogenesis of this disease. The recent discovery of Treg cells has provided new opportunities for and generated increased interest in elucidating the mechanisms underlying the induction of immune activation by HTLV-1-infected T cells. Among the $CD4^+$ T helper cell populations that play a central role in adaptive immune responses, the $CD4^+CD25^+CCR4^+$ T cell population, which primarily consists of suppressive T cell subsets, such as the Treg and Th2 subsets, in healthy individuals, is the predominant viral reservoir of HTLV-1 in both ATL and HAM/TSP patients. Interestingly, cells of this T cell subset become Th1-like cells, overproducing IFN-γ in HAM/TSP patients, while leukemogenesis develops and maintains the $Foxp3^+$ Treg phenotype in ATL patients. These results indicate that HTLV-1 may intracellularly induce T cell plasticity from Treg to IFN-γ^+ T cells, which may contribute to the development of HAM/TSP. As such, these results support the hypothesis that HTLV-1 is one of the exogenous retrovirus genes responsible for immune dysregulation through its interference in the equilibrium maintained among host immune responses. Because the majority of immune disorders are of unknown etiology, the discovery of HTLV-1 and its association with inflammatory conditions has greatly enhanced our understanding of the pathogenic mechanisms underlying organ-specific immune disorders. Further investigation of the mechanism underlying HTLV-1 action in the immune system may result in identification of new molecular pathways that will further elucidate the basic mechanisms underlying immune-mediated disorders.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

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Advantage of higher-avidity CTL specific for Tax against human T-lymphotropic virus-1 infected cells and tumors

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ABSTRACT

Strong CTL response can be observed and associated with the control of proviral load in human T-lymphotropic virus type 1 (HTLV-1) infection. However, there are few details with regard to how HTLV-1 specific CTLs work against HTLV-1 infected cells and adult T-cell leukemia cells (ATLs). In this study, using Tax-specific CTL lines with high- and low-functional avidity developed from HLA-A2-transgenic mice, we showed that higher avidity CTLs specific for Tax expressing larger numbers of TCRs and better binding strength to the antigen-HLA-A2 complex are much more efficient at eliminating HTLV-1 infected cells and, in particular, ATL tumor cells with the ability of recognizing a latent level of Tax product detected only with a real-time PCR. These findings suggest that such higher avidity CTLs specific for Tax in HTLV-1 could be responsible for preventing the development of HTLV-1 infection by detecting trace amount of antigens.

1. Introduction

The human T-lymphotropic virus type 1 (HTLV-1) causes two distinct types of disease: a $CD4^+$ T cell malignancy known as adult T cell leukemia (ATL) [1,2] and a range of inflammatory disease, of which HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is the best recognized and most widely studied [3,4]. In patients with HTLV-1 infection, the proviral load of HTLV-1 is usually stable over time [5]. However, the factors determining the set point of proviral load in each person remain to be elucidated. In particular, CTLs are active in individuals with low proviral load, in whom immunosurveillance could be more effective [6,7]. Several studies have reported that high-levels of HTLV-1-specific CTL activity can be observed in HAM/TSP patients and some asymptomatic HTLV-1 carriers, while ATL patients apparently lack HTLV-1-specific CTL activity, although it can be sporadically induced during the remission stages or after mitogenic stimulation with multiple in vitro antigenic stimulations of peripheral blood mononuclear cells [8,9]. One of the major target antigens by HTLV-1-specific CTLs in human is Tax protein [10,11], which is a molecule responsible for T-cell immortalization [12,13]. CTLs induced in ATL patients in remission are able to lyse autologous tumor cells in vitro [14]. These observations suggest that HTLV-1-specific CTLs could play a critical role in host immunosurveillance against ATLs.

While the number of HTLV-1-specific CTLs elicited is unquestionably important [7], recent studies have identified an additional parameter, functional avidity, as critical in determining the efficiency of viral clearance [15–18]. T-cell avidity is a measure of the sensitivity of T cells recognizing a cognate antigen. High-avidity CTLs are those that can recognize antigen-presenting cells (APCs) bearing very low levels of peptide-major histocompatibility complex (MHC) antigen, whereas low-avidity CTLs require much more peptide-MHC antigen to be activated or to exert effector function [15,19–21].

In this study, in order to clarify whether Tax-specific CTLs with higher avidity are critical as a deterrent to control the proliferation of ATL and the expansion of HTLV-1 infection, we developed two CTL lines specific for Tax11-19 antigen having high- and low-avidity from HLA-A2 transgenic mice in vitro. Using these CTLs, we demonstrate not only that Tax product is a critical antigen but also in particular that the specific CTLs with higher avidity for Tax11-19 have a selective advantage on recognition of human ATLs and HTLV-1 infected cells compared with those with low avidity in vitro.

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2. Materials and methods

2.1. Synthetic peptides

The Tax11-19 peptide, LLFGYPVYV, was purchased from Asahi Technoglass (Chiba, Japan) and used as an HLA-A2-restricted CTL antigen [11].

2.2. Cells

C1R.AAD cell line (HMYC1R transfected with HLA chimeric molecule containing α1 and α2 domains from human HLA-A2.1 and α3 from mouse H-2D^d) was described previously [22]. Cell lines were maintained in culture medium (CTM; 1:1 mixture of RPMI 1640 and Eagle-Hank's amino acid (EHAA)) containing 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 10 mM HEPES, 4 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

HTLV-1-infected human ATL cell lines, KK-1 and KOB, were derived from the peripheral blood and ascites of ATL patients, respectively [23,24]. Human IL-2 dependent T cell line (HCT-4) was derived from the cerebrospinal fluid of a HAM/TSP patient [25]. KK-1, KOB, and HCT-4 were used as a target. Cells were maintained in CTM with 100 units/ml of recombinant human IL-2 (Imunace®35, Shionogi, Osaka, Japan).

2.3. Mice

Transgenic HHD-2 mice (gift from Dr. François Lemonnier, Institute Pasteur, Paris, France) were bred in our colony at the Institute of the Experimental Animals at St. Marianna University. HHD-2 mice are characterized by knock-out of the murine β_2-microglobulin gene, as well as murine H-2D^b, transgenic expression of human HLA-A2.1 with a covalently-linked human β_2-microglobulin and a murine D^b-derived α3 domain to allow interaction with mouse CD8 [26]. All animal studies were approved by the Institute of Experimental animals at St. Marianna University.

2.4. Binding assay

Peptide binding to HLA-A2 molecules was measured using T2 mutant cell lines as described previously [27,28]. T2 cells (3×10^5/well) were incubated overnight in 96-well plates with culture medium (a 1:1 mixture of RPMI 1640 and Eagle-Hank's amino acid (EHAA) containing 2% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin) with 10 μg/ml human β_2-microglobulin (Sigma–Aldrich, St. Louis, MO) and different peptide concentration. On the following day, cells were washed at 190 × *g* for 5 min twice with cold PBS containing 2% FBS and incubated for 30 min at 4 °C with anti-HLA-A2.1 BB7.2 mAb (1/100 dilution of hybridoma supernatant) and 5 μg/ml FITC-labeled goat anti-mouse Ig (BD PharMingen, San Diego, CA). Cells were washed twice after each incubation; subsequently, HLA-A2.1 expression was measured by flow cytometry (FACScan; BD Biosciences, Mountain View, CA). HLA-A2.1 expression was quantified as fluorescence index (FI) according to the formula: FI = ((geometric mean fluorescence with peptide – geometric mean fluorescence without peptide)/geometric mean fluorescence without peptide). $FI_{0.5}$ is the concentration required to give an FI of 0.5, meaning a 50% increase in HLA-A2 on the cell surface. Background fluorescence without BB7.2 was subtracted for each individual value. To compare the different peptides, $FI_{0.5}$ was calculated from the titration curve for each peptide. Each sample was tested in triplicate. Values were expressed as mean in triplicate.

2.5. CTL generation in HHD-2 transgenic mice

The method for generating antigenic peptide-specific CTL lines form HHD mice was described previously [28,29]. Mice aged more than 8 weeks were immunized subcutaneously in the base of the tail with 100 μl of an emulsion containing 1:1 incomplete Freund's adjuvant (IFA), antigenic CTL peptide and cytokines (50 nmol Tax (11-19) peptide, 25 nmol HBV core 128–140 helper epitope, 3 μg of rmIL-12 and 3 μg of rmGM-CSF). Mice were boosted 2 weeks later, with the spleens removed 10–14 days after the boost. Immune spleen cells (2.5×10^6/well) were stimulated in 24-well plates with autologous spleen cells (5×10^6/well) pulsed for 30 min with 10 μM Tax11-19 peptide for the development of low-avidity CTL lines (LCTL) or with 10 nM for high-avidity CTL lines (HCTL) in CTM supplemented with 10% T-stim® (Collaborative Biochemical Products, Bedford, MA). Following a minimum of four in vitro stimulations with the peptide-pulsed syngeneic spleen cells, two CTL lines were maintained by weekly restimulation with 1×10^6 cells/well with 4×10^6 peptide-pulsed mitomycin C-treated syngeneic spleen cells as feeders.

2.6. Cytotoxic assay

CTL activity was measured with ^{51}Cr-labeled target cells. Target cells (1×10^6) were pulsed in 100 μl of 150 μCi ^{51}Cr for 1 h and were washed three times, with 5000 cells/well then added to 96-well round-bottom plates containing different peptide concentrations. Effector cells were introduced followed by additional incubation. Supernatants were then harvested and analyzed. The percentage of specific ^{51}Cr release was calculated as 100 × (experimental release – spontaneous release)/(maximum release – spontaneous release). Spontaneous release was determined from target cells that had been incubated in the absence of effector cells, while maximum release was determined in the presence of 2% TRITON® X-100 Detergent (CALBIOCHEM, La Jolla, CA). Each sample was tested in triplicate. Values were expressed as means ± SEM of triplicates.

2.7. IFN-γ ELISA assay

IFN-γ in the culture supernatant harvested at 24 h was determined using an ELISA kit (R&D, Minneapolis, MN) according to the manufacture's instructions. All samples were analyzed in triplicate. Values were expressed as means ± SEM of triplicates.

2.8. TCR Vβ screenings of CTLs

We assessed a Vβ usage pattern between HCTL and LCTL using Vβ TCR screening kit by a flow cytometry analysis (BD Bioscience Phamingen, San Diego, CA).

2.9. Flow cytometry

We used a PE-Tax11-19/HLA-A*0201 tetramer-LLFGYPVYV (Medical & Biological Laboratories, Nagoya, Japan) and PE-hamster anti-mouse CD3ε Ab (145-2C11, BD Bioscience Phamingen, San Diego, CA). Cells were centrifuged and washed twice with PBS containing 0.5% BSA, and then resuspended in 1% BSA/PBS. Cells were incubated 40 min at 4 °C with the antibody and then washed three times. The tetramer and anti CD3ε Ab were titrated for staining simultaneously.

In order to compare the affinity of T cell receptor between HCTLs and LCTLs, indexes were calculated using the following two equations: ratio of geometric mean (RGM) = (geometric mean using tetramer or anti-CD3ε Ab)/(geometric mean using control Ab). Each sample was tested in triplicate.

2.10. Western blotting

KK-1, KOB, and HCT-4 were lysed using standard lysis buffer (20 mM Tris–HCl, 250 mM NaCl, 1% NP-40, 1 mM dithiothreitol, 10 mM NaF, 2 mM Na_3VO_4, 10 mM $Na_4P_2O_7$, and protease inhibitor cocktail (Roche, Mannheim, Germany)). Lysates were stored at −80 °C until use. Protein concentration was determined using the Bradford method (Bio-Rad protein assay reagent; Bio-Rad laboratories, Hercules, CA). Equal amounts (30 μg) of protein were separated by SDS–PAGE on 10% polyacrylamide gels and transferred to PVDF membranes. Following the transfer, membranes were blocked with Difco Skim milk (BD Bioscience, San Diego, CA) overnight at 4 °C. The working concentrations of the first Abs were 1 μg/ml for anti-Tax Ab (Lt-4) [30] and anti murine β-actin Ab (SIGMA, St. Louis, MO), and 1:10,000 for HRP-conjugated anti-mouse IgG Ab (SIGMA, St. Louis, MO). The membrane was washed, and was reacted with the appropriate second antibody. Finally, signals were visualized using the extended cavity laser (ECL) system (GE Healthcare Bio-sciences KK, Tokyo, Japan).

2.11. Real-time reverse transcriptase-PCR (RT-PCR)

Total RNA was isolated from cells using TRIzol® Reagent (Invitrogen, Carisbad, CA). First-stand cDNA was synthesized with random hexamers and reverse transcriptase (ReverTraAce; Toyobo, Japan) using 1 μg of total RNA in a reaction volume of 20 μl. Real-time PCR reactions were carried out using TaqMan® Universal Master Mix (Applied Biosystems, Carisbad, CA). ABI Prism 7500 SDS was programmed to an initial step of 2 min at 50 °C and 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C. The primers and probe for detecting the HTLV-1 Tax or GAPDH mRNA were used as described previously [31]. Relative quantification of mRNA was performed using the comparative threshold cycle method with GAPDH as an endogenous control. For each sample, target gene expression was normalized against the expression of GAPDH. To determine relative expression levels, the following formula was used: target gene expression = $2^{-(Ct[target]-Ct[GAPDH])}$. Each sample was tested in triplicate. Values were expressed as means ± SEM of triplicates.

3. Results

3.1. Binding affinity of Tax11-19 for HLA-A2 molecule

Before attempting to develop Tax-specific CTL lines from HLA-A2 transgenic HHD mice, we evaluated the binding affinity of Tax11-19 peptide by T2 binding assay, which measures the cell surface stabilization of HLA-A2 molecules. Tax11-19 peptide displayed a binding capacity for the HLA-A2 molecule that was nearly equal to that of the positive control, the highly antigenic influenza virus matrix peptide (FMP58-66) [32] ($FI_{0.5}$ = 0.329 for Tax11-19, 0.284 μM for FMP58-66) (Fig. 1). These data suggest that Tax11-19 would be a very strong antigenic peptide restricted to the HLA-A2 molecule.

3.2. Recognition of Tax11-19 peptide by CTL lines of different avidity

Based on the observation that Tax11-19 showed strong antigenicity inducing specific CTLs, we next attempted to develop low-avidity CTLs (LCTL) and high-avidity CTLs (HCTL) from HLA-A2 transgenic mice. HCTL were generated by weekly stimulation using low concentrations (10 nM) of the Tax peptide pulsed onto APCs, while LCTLs were also generated using 10 μM of the Tax peptide pulsed onto APCs. Using these different CTL lines, we examined Tax-specific CTLs-mediated cytotoxicity with Tax peptide titrated over a range of concentrations. The titration curve showed a

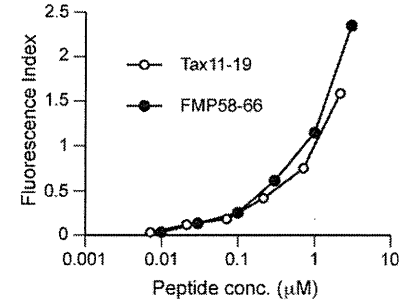

Fig. 1. Comparison of HLA-A2 binding curves between Tax11-19 and FMP58-66 peptide in T2-binding assay. The binding affinity of Tax11-19 for HLA-A2 molecule is almost as strong as that of FMP58-66 in influenza A virus.

0.5–1 $\log_{10}$ difference in functional avidity measured as the peptide concentration necessary to produce 50% lysis (Fig. 2A). Similarly, we examined their properties in antigen-specific IFN-γ production from these CTL lines (Fig. 2B). With a 24 h assay, HCTLs showed more IFN-γ production than LCTLs even at lower concentration of Tax antigen. These data suggest that the two different CTL lines specific for Tax have different functional avidity.

3.3. Different Vβ usage and binding ability to Tax-tetramer between high- and low-avidity CTLs

In order to confirm whether these CTLs with different avidity possessed different TCR structures, we assessed the difference in Vβ usage pattern between HCTLs and LCTLs using flow cytometric analysis (FCM). On FCM, antibodies available for screening were those for Vβ 2, 3, 4, 5, 6, 7, 8.1, 8.2, 8.3, 9, 10, 11, 12, 13, 14, and 17. On FCM, no Vβ were detected in LCTLs, while only Vβ5 was detected in HCTLs (Fig. 3A). The data suggested that the major TCR repertoire of HCTL is Vβ5, indicating that these two Tax-specific CTL lines have different TCR structures.

We next compared the binding affinity of TCR between HCTL and LCTL using Tax11-19/HLA-A2 tetramer-LLFGYPVYV and anti-CD3 Ab (Fig. 3B). On FCM with both Tax11-19-tetramer and anti-CD3 Ab titration, HCTLs showed a stronger fluorescence than LCTLs (Fig. 3B). On Tax11-19-tetramer assay, the ratio of fluorescence index (HCTL/LCTL) was ~5-fold at any titrated concentration, and it took 1.5 logs more tetramer to achieve the same level of staining. In the titration of anti-CD3 Ab, the ratio was ~3-fold and also it required about 3-fold more antibody to reach the same level of staining. These findings suggested that HCTLs not only have higher TCR affinity but also express greater numbers of TCR molecules on their surface when compared with LCTLs.

3.4. Recognition of human ATL targets by Tax-specific CTLs from HHD mice

We further examined whether these murine CTL lines with different functional avidity could induce cytotoxic activity against human ATL targets. We used the HTLV-1-infected human ATL cell lines, KK-1 (HLA-A2) and KOB (HLA-A30) as target cells derived from peripheral blood and ascitis of ATL patients, respectively [23,24]. These murine CTL lines did not show strong cytotoxicity against human ATL lines as against murine targets with a 4 h assay, as it was previously reported that species specificity between murine CD8 and the α3 domain of human HLA-A2 may reduce the recognition ability by CTLs [33]. However, on a 12 h assay, cytotoxicity against human ATL was observed in an HLA-A2 restricted manner (Fig. 4A). HCTLs were especially more efficient at killing at low E/T ratios. Furthermore, on kinetics assay, HCTLs showed more efficient cytotoxicity against the human ATL target (KK-1) than LCTLs (Fig. 4B).

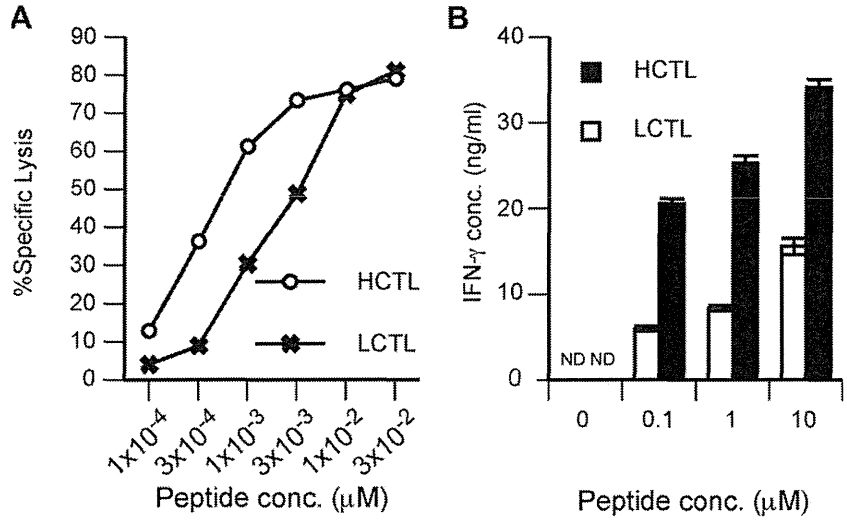

Fig. 2. Difference in functional avidity between HCTLs and LCTLs. (A) Recognition by the Tax11-19 peptide specific CTLs, HCTL and LCTL, of Tax11-19 antigenic peptide from 10^{-4} to 10 μM when presented on C1R.AAD target cells. The effector to target-cell (E/T) ratio was 20:1. Error bars were omitted because all SEMs were <3.5%. (B) Comparison of Tax11-19-specific IFN-γ production between HCTLs and LCTLs. A total of 200,000 CTL cells were cultured with 100,000 mytomicin-c treated C1R.AAD cell with 0.1–10 μM Tax11-19 peptide. Culture supernatants at 24 h were assayed using IFN-γ ELISA kit according to the manufacturer's instructions. ND, not detected.

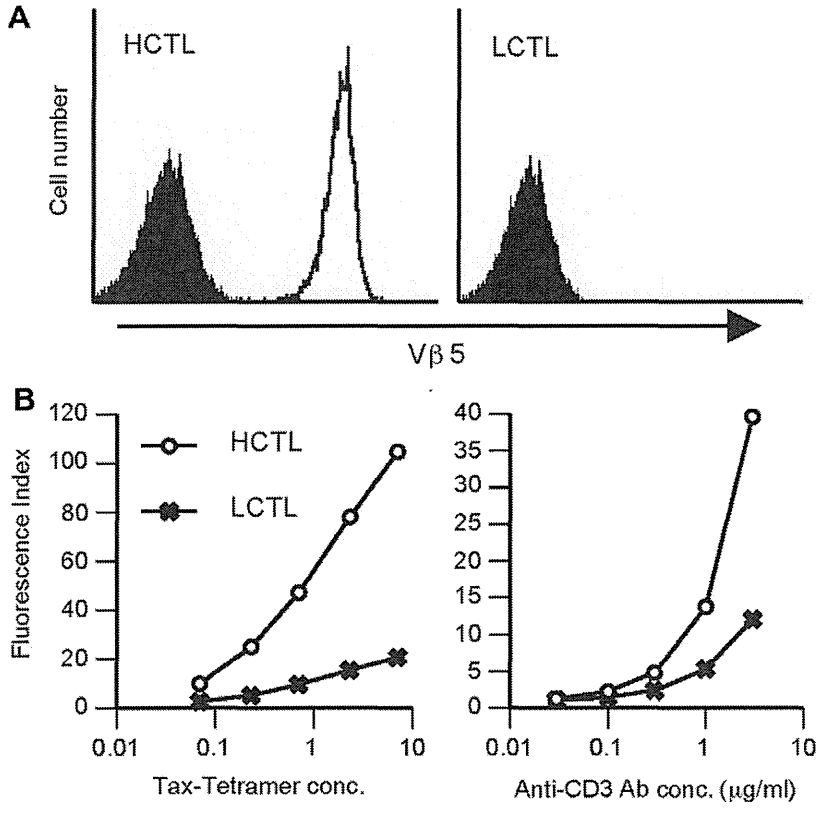

Fig. 3. TCR Vβ usage and expression level of TCR complex on Tax-specific CTLs with different functional avidity. (A) Comparison of Vβ usage pattern between HCTLs and LCTLs cytometry analysis (FCM). No Vβs among available anti-Vβ antibodies were detected in LCTL but only Vβ5 was detected in HCTL. (B) Comparison of binding curves for human Tax11-19-tetramer and anti-CD3ε Ab between HCTLs and LCTLs. HCTLs consistently showed a stronger fluorescence index than LCTLs; for Tax11-19-tetramer, the ratio of fluorescence index (LCTL/HCTL) was ~5-fold, and for anti-CD3ε Ab, it was ~3-fold.

3.5. Recognition of HTLV-1 infected human T cells by Tax-specific CTL from HHD mice

Next, in order to examine a comparison of the cytotoxicity against HTLV-1 infected non-tumor cells, we used HTLV-1 infected human T cells (HCT-4) derived from a patient with HAM/TSP [25]. On a 12 h lytic assay, HCTLs showed more efficient cytotoxicity against the HTLV-1 infected human T cells while LCTLs were not able to kill the targets under the these experimental conditions (Fig. 5A). At no time point was there detectable killing by LCTLs (Fig. 5B). These findings suggested that the superior recognition ability by the CTLs with higher functional avidity may have a more

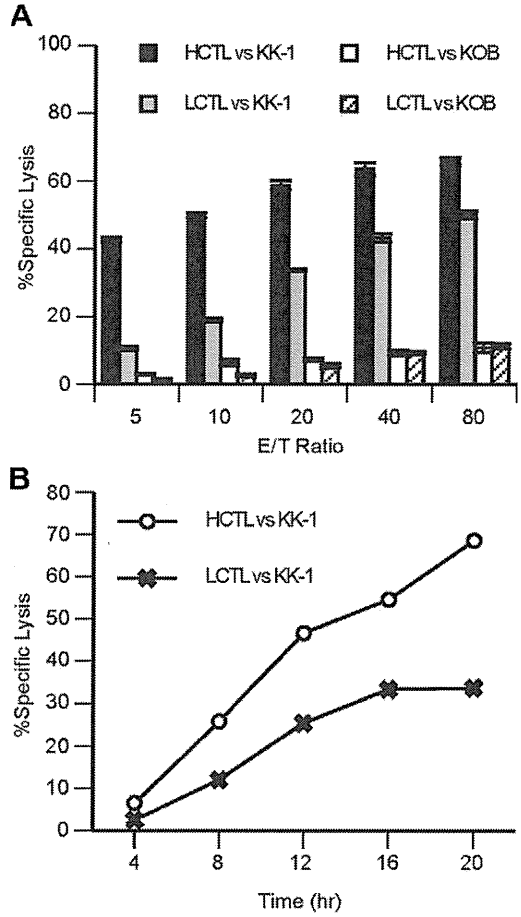

Fig. 4. Recognition pattern of human ATL targets by Tax-specific CTLs. (A) Comparison of cytotoxicity for human ATL targets (KK1, HLA-A2; KOB, HLA-A30) between HCTLs and LCTLs. (12 h ^{51}Cr release assay) (B) Comparison of kinetics of Tax-specific CTL-mediated cytotoxicity (E:T ratio = 40:1) between HCTLs and LCTLs. Similar results were obtained in three different experiments.

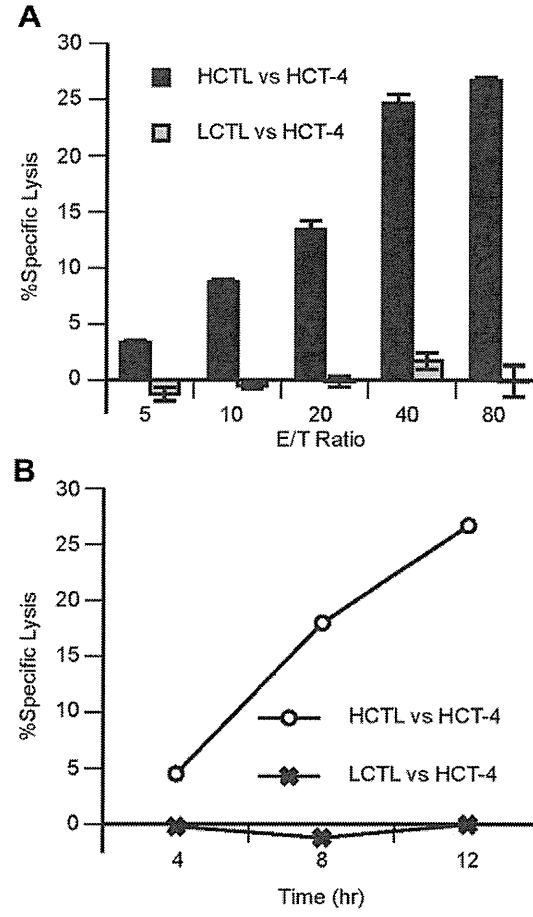

Fig. 5. Recognition pattern of HTLV-1 infected human T cell line by Tax-specific CTLs. (A) Comparison of cytotoxicity for human IL-2 dependent HTLV-1 infected cell, HCT-4 (HLA-A2), between HCTLs and LCTLs. (12 h ^{51}Cr release assay). (B) Comparison of kinetics of Tax-specific CTL-mediated cytotoxicity (E:T = 40:1) between HCTLs and LCTLs. Similar results were obtained in four different experiments.

striking effect in the case of recognizing normal cells infected with the virus.

3.6. Expression of Tax product in human ATL tumors and HTLV-1 infected T cell target

The cytotoxicity data against human targets indicated that higher functional avidity in CTLs is critical for efficient cytotoxicity against tumor or infected normal cell targets in humans. However, the amount of Tax antigen expressed in target cells that could be recognized by higher avidity CTLs was unclear. Therefore, we investigated how much Tax products could be yielded in these human ATL and HTLV-1 infected target cells. Using western blotting (Fig. 6A), Tax protein was detected in KOB and HCT-4 target cells, but not in KK-1. Since KK-1 cells were recognized by HCTLs more strongly than by LCTLs, we further evaluated the level of Tax mRNA produced in KK-1 using real-time PCR. The expression levels of Tax mRNA in KK-1 were around one thousand-fold lower than that in KOB (Fig. 6B). These results demonstrated that Tax11-19-specific higher avidity CTLs showed more efficient cytotoxicity against ATL by recognizing very small amount of Tax product detected only with real-time PCR.

4. Discussion

HTLV-1 infection elicits a strong CTL response, with Tax protein being the major target of HTLV-1-specific CTLs [10,11]. In the field of anti-tumor immunity, the in vivo relevance of differences in functional avidity has been established by demonstrating that high-avidity CTLs clear tumor antigens more efficiently than low-avidity CTL [34–38]. In HTLV-1 infection, however, while there is increasing body of evidence that CTL quality from the aspect of functional avidity of CTL might be crucial for the efficient control of HTLV-1 infection [17,39], little is known about how the functional avidity of HTLV-1 virus-specific CTLs is related to the control of HTLV-1-infected cells and tumors. Furthermore, the virus is latent in the tumor cells and it is difficult to detect expression of viral proteins [40–42]. This is the reason why there has not been direct evidence on whether Tax11-19 works as a definitive CTL antigen in HLA-A2-restricted patients with HTLV-1 infection and ATLs. The present study provides clear evidence regarding the notion that high avidity CTLs specific for Tax protein play a greater role in the specific destruction of ATL and HTLV-1-infected cells using Tax-specific CTLs with different functional avidity generated from HLA-A2 transgenic HHD mice, with human ATL lines and HTLV-1 infected cells acting as targets. As Tax11-19 peptide antigen binds HLA-A2 with almost as high affinity as FMP58-66 in influenza A virus (Fig. 1), which has one of the highest affinity peptides among HLA-A2 restricted peptide antigens [27,28], we developed CTL lines specific for Tax11-19, HCTL and LCTL, for which we found the optimum antigen-presenting conditions for the induction and maintenance of the CTL lines were 10 nM- and

10 μM-peptide pulsing APCs, respectively. The 1000-fold difference of such antigenic concentration resulted in the CTL lines with differences of functional avidity in antigen-specific cytotoxicity and IFN-γ production (Fig. 2). These different avidity CTLs also had different repertoires of TCRVβ, suggesting the structure of TCR in the major repertoire of two lines were distinct (Fig. 3A). In order to compare TCR affinity for the human Tax-tetramer, the mismatch of which to murine CD8 could permit assessment of the strength of TCR ligation to peptide/MHC complex more closely without the influence of CD8 binding [43], we titrated the tetramer and evaluated the effect of the number of TCR molecules expressed at the same time. Higher avidity Tax-specific CTLs showed higher fluorescene on both Tax-tetramer (~5-fold) and anti-CD3Ab (~3-fold) staining (Fig. 3B), thus suggesting that CTL might acquire higher avidity state by possessing the different structure of the TCR as well as by increasing the number of TCR molecules expressed although other factors could also play a role for determining the avidity of CTLs [15].

HTLV-1 Tax, a critical viral protein for HTLV-1 leukemogenesis, is the most likely target for HTLV-1 specific CTL in HTLV-1-infected individuals [10,11]. In HTLV-1-infected patients with HLA-A2, the Tax11-19-specific CTL response is predominantly detected in culture [44]. However, few details are known about the recognition mechanism by Tax-specific CTLs because of the difficulty of developing CTL lines specific for Tax11-19 antigen [9]. Although both HCTLs and LCTLs developed from HLA-A2 transgenic mice were not able to induce cytotoxicity against the human HLA-A2-restricted ATL line, KK-1, on 4 h assay because of the mismatch between the murine CD8 and human α3 domain [22], HCTLs clearly showed more efficient cytotoxicity than LCTLs with longer-term assay of more than 4 h (Fig. 4). Furthermore, the use of the human IL-2-dependent HTLV-1-infected non-tumor cell, HCT-4, clearly brought out the difference in cytotoxic efficacy between HCTL and LCTL (Fig. 5). These findings could be direct evidence not only that Tax11-19 might be naturally processed for presentation as a CTL antigen in both ATL tumor cells and virus-infected cells but also that the higher avidity CTL for Tax11-19 could be more critical in clearing HTLV-1-infected cells as well as ATL tumors in HLA-A2-restricted patients. In addition, HCTLs could more strongly recognize a latent level of Tax product detected only with a real-time PCR, not detectable with western blotting in the ATL target (Fig. 6). Furthermore, HCTLs also possessed higher elimination potential against HTLV-1 infected non-tumor targets when compared with LCTLs (Figs. 4 and 5).

The present findings are consistent with previous reports showing that the lytic efficiency of $CD8^+$ T cell response was inversely correlated with the proviral load and the rate of proviral expression in patients with HTLV-1 infection [17]. These data also strongly support the notion that induction of high avidity CTLs is critical for development of more effective vaccines against cancer and chronic viral infection such as HTLV-1and HIV. In addition, based on the observation that the high-avidity CTLs expressed a greater number of TCR molecules when compared with the low-avidity CTLs (Fig. 3B), such more multivalent TCR display might be one of the critical factors in establishing functional high avidity, leading to more efficient TCR cell therapy in the future [45].

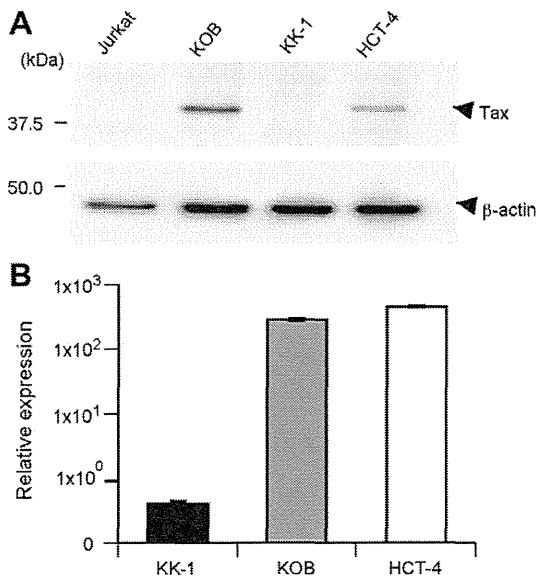

Fig. 6. Expression of Tax product in human ATL tumors and HTLV-1 infected T cell targets. (A) Tax protein is detected in KOB and HCT-4 by western blotting, but not in KK-1. Jurkat cells were used as a negative control. (B) Comparison of mRNA production of Tax by real-time PCR among KOB, KK-1 and HCT-4. Tax production in KK-1 was detected by real-time PCR, but not in a western blotting. Jurkat cells as a negative control gave no detectable signal with the Tax-primer.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Functional impairment of Tax-specific but not cytomegalovirus-specific CD8$^+$ T lymphocytes in a minor population of asymptomatic human T-cell leukemia virus type 1-carriers

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Abstract

Background: Human T-cell leukemia virus type 1 (HTLV-1) causes adult T-cell leukemia (ATL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) in a small percentage of infected individuals. ATL is often associated with general immune suppression and an impaired HTLV-1-specific T-cell response, an important host defense system. We previously found that a small fraction of asymptomatic HTLV-1-carriers (AC) already showed impaired T-cell responses against the major target antigen, Tax. However, it is unclear whether the impaired HTLV-1 Tax-specific T-cell response in these individuals is an HTLV-1-specific phenomenon, or merely reflects general immune suppression. In this study, in order to characterize the impaired HTLV-1-specific T-cell response, we investigated the function of Tax-specific CD8$^+$ T-cells in various clinical status of HTLV-1 infection.

Results: By using tetramers consisting of HLA-A*0201, -A*2402, or -A*1101, and corresponding Tax epitope peptides, we detected Tax-specific CD8$^+$ T-cells in the peripheral blood from 87.0% of ACs (n = 20/23) and 100% of HAM/TSP patients (n = 18/18) tested. We also detected Tax-specific CD8$^+$ T-cells in 38.1% of chronic type ATL (cATL) patients (n = 8/21), although its frequencies in peripheral blood CD8$^+$ T cells were significantly lower than those of ACs or HAM/TSP patients. Tax-specific CD8$^+$ T-cells detected in HAM/TSP patients proliferated well in culture and produced IFN-γ when stimulated with Tax peptides. However, such functions were severely impaired in the Tax-specific CD8$^+$ T-cells detected in cATL patients. In ACs, the responses of Tax-specific CD8$^+$ T-cells were retained in most cases. However, we found one AC sample whose Tax-specific CD8$^+$ T-cells hardly produced IFN-γ, and failed to proliferate and express activation (CD69) and degranulation (CD107a) markers in response to Tax peptide. Importantly, the same AC sample contained cytomegalovirus (CMV) pp65-specific CD8$^+$ T-cells that possessed functions upon CMV pp65 peptide stimulation. We further examined additional samples of two smoldering type ATL patients and found that they also showed dysfunctions of Tax-specific but not CMV-specific CD8$^+$ T-cells.

Conclusions: These findings indicated that Tax-specific CD8$^+$ T-cells were scarce and dysfunctional not only in ATL patients but also in a limited AC population, and that the dysfunction was selective for HTLV-1-specifc CD8$^+$ T-cells in early stages.

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Background

Human T-cells leukemia virus type 1 (HTLV-1) is the causative agent of a highly aggressive $CD4^+$ T-cell malignancy, adult T-cell leukemia (ATL)[1,2]. As many as 10 million individuals are thought to be infected worldwide, in southern Japan, the Caribbean basin, South America, Melanesia, and equatorial Africa[3]. Unlike human immunodeficiency virus (HIV), the majority of HTLV-1-infected individuals are clinically asymptomatic during their lifetime. However, approximately 5% develop ATL, and another 2-3% develop a variety of chronic inflammatory diseases such as HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP)[4-8].

HTLV-1-specific cytotoxic T-lymphocytes (CTLs) are thought to play a pivotal role in containing the proliferation of HTLV-1-infected T-cells[9,10]. Tax is known to be the dominant target antigen for HTLV-1-specific CTLs[10-13], and a high frequency of Tax-specific CTLs can be detected in HAM/TSP patients and some asymptomatic HTLV-1 carriers (ACs)[10-14]. However, ATL patients show general immune suppression[15], reduced frequency and dysfunction of Tax-specific CTLs[16,17]. Regulatory T cell (Treg)-like function of $FoxP3^+$ ATL cells and diminished function of dendritic cells may be involved in the immune suppression in ATL patients [18,19], but the precise mechanism is not yet clarified. We previously demonstrated that a fraction of ACs also exhibit reduced T-cell responses against Tax protein [20]. These observations suggest that the reduced HTLV-1-specific T-cell response might be an underlying risk of ATL development, but not the result of ATL. However, it is unknown how the function of HTLV-1-specific $CD8^+$ T-cells becomes impaired in a small percentage of ACs and whether its dysfunction is specific for HTLV-1 antigen or due to general immune suppression.

During chronic stage of infection with several viruses, such as HIV and hepatitis C virus (HCV), virus-specific CTLs gradually lose their cytotoxic activity, the ability to proliferate and secrete a diverse profile of cytokines, ultimately leading to exhaustion, anergy or even deletion of these cells[21-26]. Programmed death-1 (PD-1), a negative regulator in the CD28 superfamily, has recently been shown to be highly expressed on virus-specific T-cells during many chronic viral infections[27-29]. It has also been reported that the interaction of PD-1 with PD-ligand 1 (PD-L1) negatively regulates cytokine production and proliferation of T-cells[30,31]. A previous report indicates that PD-1 is up-regulated on the dominant Tax-specific CTLs in ATL patients and ACs and that immune regulation through the PD-1/PD-L1 pathway may be involved in the dysfunction of HTLV-1-specific CTLs in ATL patients[32].

Studies on memory T-cell differentiation have shown that phenotype, function, and homeostasis of memory T-cells vary for different persistent virus infections[33]. Central memory T-cells (T_{CM}; $CD45RA^-CCR7^+$) are elicited by non-persisting virus that provide transient antigen stimulation, such as in Influenza virus infection. In contrast, effector memory T-cells (T_{EM}; $CD45RA^-CCR7^-$) predominate when relatively high levels of antigen persist, such as in HIV infection. Terminally differentiated memory (T_{Diff}; $CD45RA^+CCR7^-$) can be seen when antigen persists at a low level, such as in cytomegalovirus (CMV) infection. In HTLV-1 infection, it has been reported that dominant Tax-specific CTLs in HAM/TSP patients consist of T_{EM} and T_{Diff} compartments[34].

We previously identified some major epitopes recognized by HTLV-1-specific CTLs in infected individuals carrying HLA-A2, -A11, or -A24[12,35,36]. These allowed us to monitor HTLV-1-specific CTLs and analyze their functions *ex vivo*, by using antigen/HLA tetrameric complexes. In this study, we demonstrate that IFN-γ production and proliferative capacity of tetramer-binding Tax-specific $CD8^+$ T-cells were severely impaired not only in ATL patients but also in a minor population of asymptomatic HTLV-1 carriers (ACs). Importantly, the T-cell dysfunction at the asymptomatic stage was selective for HTLV-1 but not for CMV antigen. In addition, severely impaired HTLV-1-specific but not CMV-specific $CD8^+$ T-cells responses were also observed in patients diagnosed as smoldering ATL, the clinical condition of which is close to that of AC. The dysfunction of HTLV-1-specific $CD8^+$ T-cells in an early clinical stage implies HTLV-1-specific immune suppressive mechanism might be an underlying risk for ATL.

Results

Incidence and frequency of Tax-specific $CD8^+$ T-cells in ACs, and HAM/TSP and cATL patients

In 23 ACs and 18 HAM/TSP and 21 cATL patients carrying HLA-A2, -A11 and/or -A24 alleles, we evaluated the frequencies of Tax-specific $CD8^+$ T-cells by using cognate Tax/HLA tetramers (Figure 1 and Table 1). Tax-specific $CD8^+$ T-cells were detected in 87.0% of ACs and all HAM/TSP patients tested. In contrast, only 38.1% of cATL patients have detectable frequencies of Tax-specific $CD8^+$ T-cells (Table 1). Figure 1B shows that the average frequency of Tax-specific $CD8^+$ T-cells in the $CD8^+$ T-cells of cATL patients (n = 21, 0.90% range: 0%-9.45%) was significantly lower than that in ACs (n = 23, 2.37%, range: 0%-8.23%, $P = 0.0023$). HAM/TSP patients had the highest average frequency of Tax-specific $CD8^+$ T-cells among the three groups (n = 18, 8.88%, range: 1.86%-29.9%, $P = 0.0001$; vs. AC, $P < 0.0001$; vs. cATL patients), which is consistent with

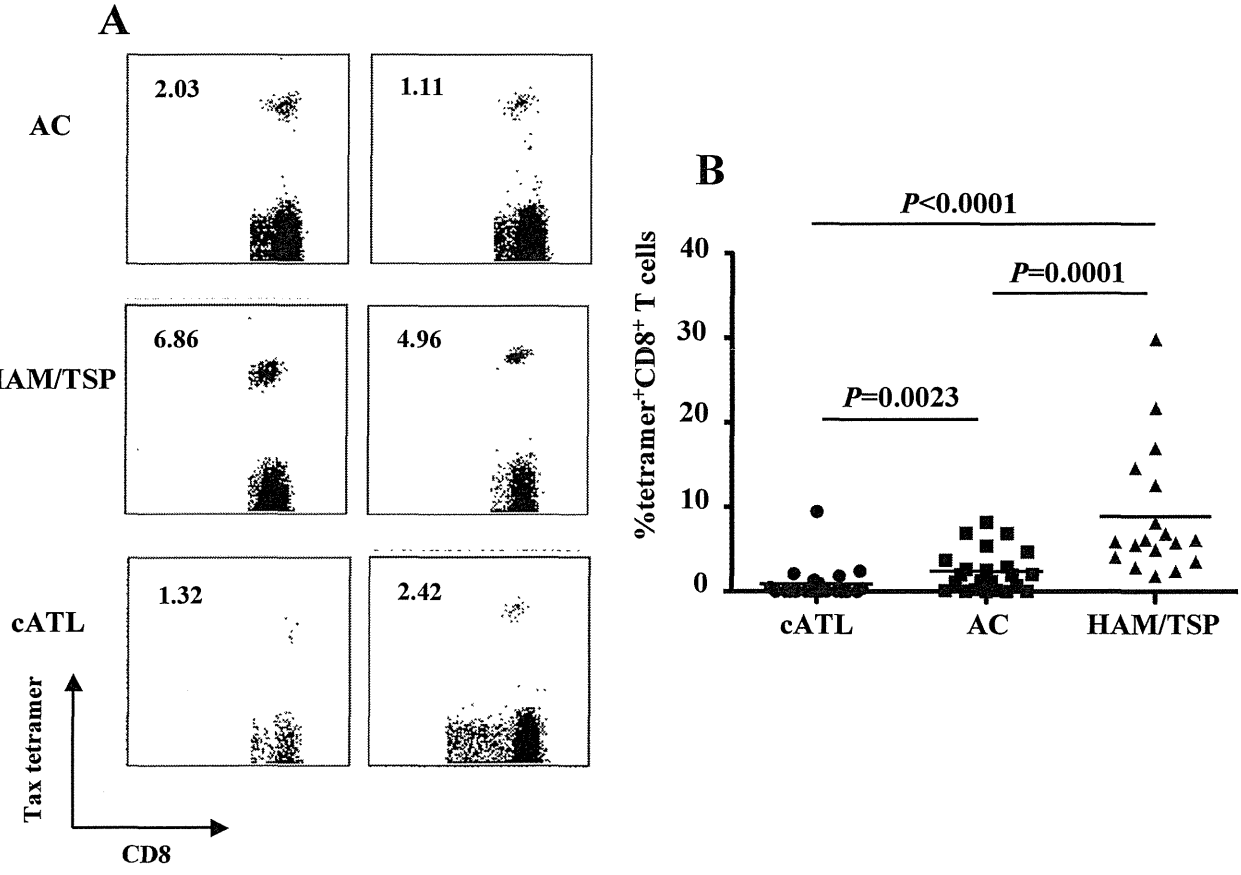

Figure 1 Incidence and frequency of Tax-specific CD8$^+$ T-cells in ACs, and HAM/TSP and cATL patients. (A) Whole blood or PBMCs from AC (top), and HAM/TSP (middle) and cATL (bottom) patients were stained with Tax/HLA tetramer. Number indicates the percentage of tetramer$^+$ cells in CD8$^+$ T-cells. (B) The percentage of Tax-tetramer$^+$ CD8$^+$ T-cells in AC (n = 23), and HAM/TSP (n = 18) and cATL (n = 21) patients. *P* value was determined by the Mann-Whitney *U* test. Horizontal bars indicate the average percentage of Tax-tetramer$^+$ CD8$^+$ T-cells for the group.

previous reports [10,17,37]. It is of note that Tax-specific CD8$^+$ T-cells are detectable even in cATL patients, although the frequency is very low.

Impaired cell proliferation and IFN-γ production of Tax-specific CD8$^+$ T-cells in cATL but not HAM/TSP patients

We next examined IFN-γ production and cell proliferation of Tax-specific CD8$^+$ T-cells in HAM/TSP and cATL patients (Figure 2A). Intracellular IFN-γ staining showed that Tax-specific CD8$^+$ T-cells in all HAM/TSP patients tested produced IFN-γ when stimulated with Tax peptide (Figure 2A). Tax-specific CD8$^+$ T-cells in those HAM/TSP patients proliferated regardless of stimulation with Tax peptide (Figure 2B). In contrast to HAM/TSP patients, IFN-γ production from Tax-specific CD8$^+$ T-cells in a cATL patient was hardly detectable even when stimulated with Tax peptide (4.8%, Figure 2A). In the same donor, Tax-specific CD8$^+$ T-cells

Table 1 The number of blood samples with detectable Tax-specific CD8$^+$ T-cells in all samples tested in this study

Tax/HLA tetramers used in this study	Disease Status		
	AC	HAM/TSP	cATL
HLA-A*0201/Tax11-19	12/14[1]	7/7	2/11
HLA-A*1101/Tax88-96	4/4	4/4	3/5
HLA-A*2402/Tax301-309	13/15	13/13	5/16
No. of tetramer$^+$ samples/total no. of blood samples[2]	20/23 (87.0%)	18/18 (100%)	8/21 (38.1%)

[1] No. of samples with detectable Tax-specific CD8$^+$ T-cells/total no. of samples carrying each HLA allele. When the frequency of tetramer$^+$ cells was more than 0.04% of CD8$^+$ T-cells, the sample was regarded as detectable.

[2] In case Tax-specific CD8$^+$ T-cells was detectable by either tetramer in a sample carrying two of three HLA-A alleles above, the sample was regarded as positive.

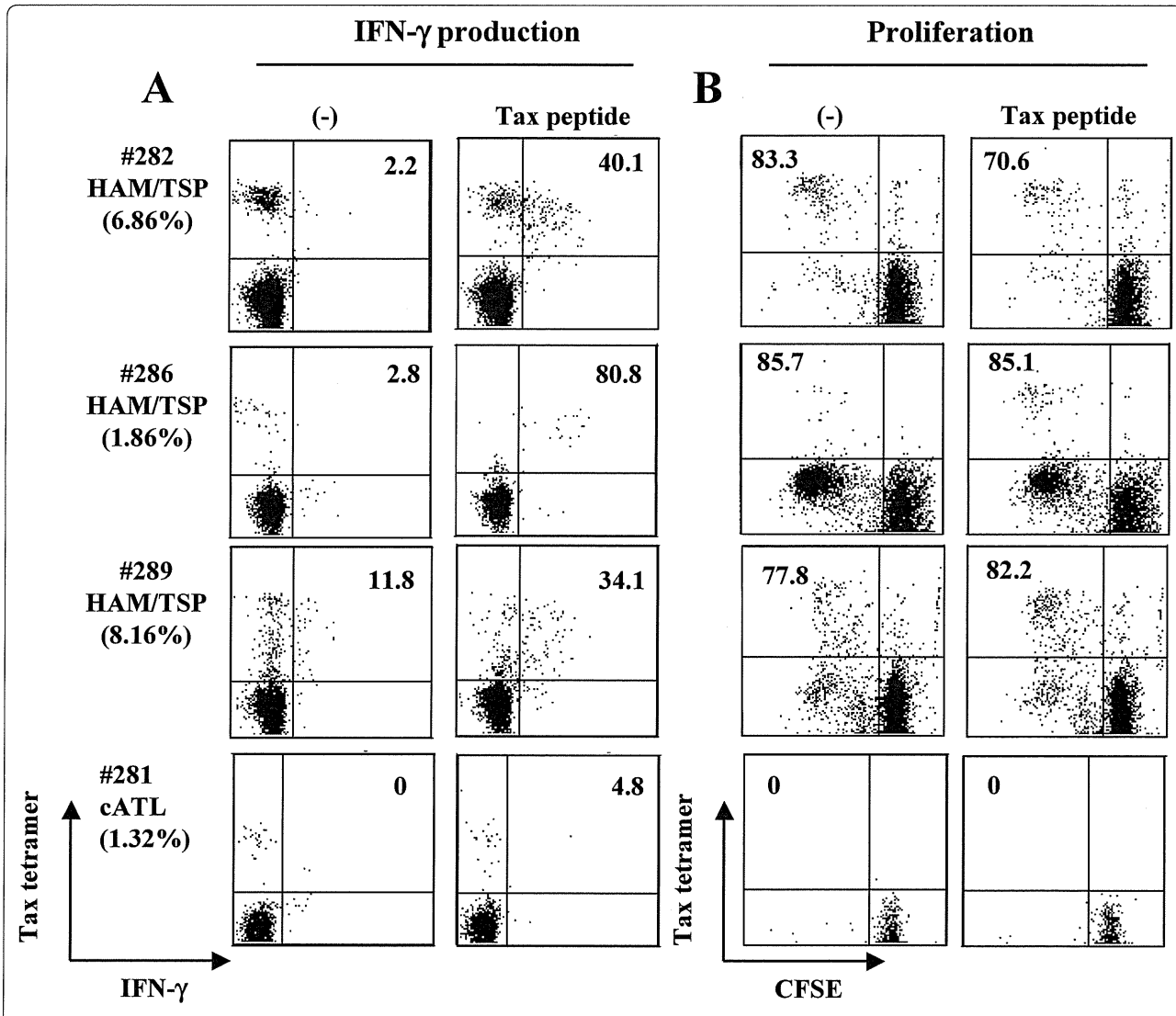

Figure 2 IFN-γ production and proliferation of Tax-specific CD8$^+$ T-cells in HAM/TSP and cATL patients. (A) PBMCs from HAM/TSP and cATL patients were stimulated with or without 10 μM Tax peptide for 6 hrs. The number indicates the percentage of IFN-γ-producing cells in tetramer$^+$ cells. (B) For CFSE-based T-cell proliferation, CFSE-labeled PBMCs from HAM/TSP and cATL patients were cultured in the presence or absence of 100 nM Tax peptide for 6 days. The number indicates the percentage of dividing (CFSE^{low}) cells in tetramer$^+$ cells. The percentage of tetramer$^+$ cells among CD8$^+$ T cells in fresh blood is indicated in parenthesis under the patient ID.

could be detected in fresh blood (1.32%) and after 6 hrs incubation as shown in Figure 2A, but not after 6 day-culture, suggesting that Tax-specific CD8$^+$ T-cells in this cATL patient had no proliferative capacity (Figure 2B). We tested PBMC from four other cATL patients who had detectable Tax-specific CD8$^+$ T-cells, but none of them showed proliferation of Tax-specific CD8$^+$ T-cells by either the CFSE-based proliferation assay or 13-day culture (Additional file 1). Collectively, these results indicate that Tax-specific CD8$^+$ T-cells from most cATL patients are impaired in their capacities to proliferate and produce IFN-γ.

Diversity in the IFN-γ production and cell proliferation of Tax-specific CD8$^+$ T-cells in ACs

Our recent studies using the GST-Tax protein-based assay demonstrated that the extent of Tax-specific T-cell responses varied widely in ACs[20]. We then evaluated proliferation and/or IFN-γ production of tetramer-binding Tax-specific CD8$^+$ T-cells in 14 ACs (Table 2). Representative data on 4 of 14 ACs are shown in Figures 3A and 3B. In 3 ACs (#251, #313, and #360), Tax-specific CD8$^+$ T-cells produced IFN-γ and proliferated in response to Tax peptide (Figures 3A and 3B). Similarly to HAM/TSP samples, a large proportion of Tax-

Table 2 Clinical information and summary for Tax-specific CD8$^+$ T cells in 14ACs

ID	Age	Sex	WBC (/μl)	CD4 (%)[1]	CD8 (%)[1]	HLA	Tetramer (%)[2]	Functions and phenotype of Tax-specific CD8$^+$ T-cells[3]			Ably (%)[7]	PVL[8]
								IFN-γ$^+$ (%)[4]	CFSE^{low} (%)[5]	PD-1$^+$ (%)[6]		
#217	70s	F	6800	ND[9]	5.72	A24	1.94	27.7	78.9	78.7	0	14
#236	30 s	F	6500	ND	11.9	A24	2.54	31.1	0	54.1	0	22
#238	60 s	F	5700	ND	12.7	A11	1.29	36.4	100	0	0	2
#243	50 s	F	4100	ND	24.6	A2/24	0.39/3.67	11.3	27.6	93.8	0	3
#245	40 s	F	5000	ND	22.6	A2	0.73	62.5	75	ND	1	58
#251	60 s	M	4800	ND	11.9	A2/11	0.70/8.23	35.8	84.4	36.7	0	2
#279	40 s	M	6200	34.1	11.6	A2/24	4.70/0.18	12.9	30.8	70.2	1	48
#287	70 s	M	4800	72.5	10.0	A2/24	1.17/0.23	11.1	0	55.6	2	81
#309	60 s	F	4600	37.5	24.8	A11/24	6.88/4.26	51.7	76.2	85.3	1.5	29
#311	60 s	F	3200	30.6	14.8	A2/24	1.02/1.94	51.3	ND	ND	0	6
#312	50 s	F	2700	27.3	36.4	A24	2.03	77.8	ND	ND	ND	UN[10]
#313	60 s	M	7300	25.4	31.0	A24	1.11	55.7	60	90.6	ND	4
#315	50 s	F	7500	26.5	7.9	A2/24	6.88/0	24.5	84.7	20	0.6	17
#360	50 s	M	6200	37.7	29.9	A2	2.6	63.1	68.4	10.2	0	UN

[1]The number indicates percentage of CD4$^+$ or CD8$^+$ T cells in lymphocytes.

[2]The number indicates percentages of tetramer$^+$ cells in CD8$^+$ T-cells. Two numbers divided by a slash represent those detected by two different tetramers corresponding to two HLA alleles shown in the HLA column.

[3]In case of a sample carrying two of three HLA-A alleles (A2, A11, or A24), Tax-specific CTLs predominantly detected by a tetramer were used. The number represents percentage of indicated cells in the tetramer-binding CD8$^+$ T cells.

[4]Evaluated by intracellular IFN-γ staining following 6 hours stimulation with corresponding Tax peptide.

[5]Evaluated by CFSE intensities in labeled PBMC after 6 days incubation with corresponding Tax peptide stimulation.

[6]The number represents percentage of indicated PD-1$^+$Tax-specific CD8$^+$ T cells without culture.

[7]Ably; abnormal lymphocytes

[8]PVL; proviral load. The number represents copy number per 1000 PBMCs.

[9]ND; not determined

[10]UN; undetectable

specific CD8$^+$ T-cells in these ACs spontaneously proliferated without stimulation with Tax peptide, probably due to viral reactivation in HTLV-1-infected cells *in vitro*[38,39]. IFN-γ production was specifically detected for peptide stimulation, and 35.8-55.7% of Tax-specific CD8$^+$ T-cells produced a good amount of IFN-γ (mean fluorescence intensity, MFI: 63.7-195.3) upon stimulation in the samples of #251, #313, and #360. In contrast, Tax-specific CD8$^+$ T-cells in one AC (#287) did not proliferate in response to Tax peptide and showed a very weak IFN-γ response with low amounts of IFN-γ (MFI: 37.5) in a low percentage (11.1%) of Tax-specific CD8$^+$ T-cells (Figures 3A and 3B). In other ACs (#243 and #279), low frequency of IFN-γ$^+$ Tax-specific CD8$^+$ T-cells was observed, but the levels of IFN-γ production (MFI: #243; 58.8, #279; 77.6) and the proliferative responses were comparable to other ACs (Table 2). Tax-specific CD8$^+$ T-cells in #236 failed to proliferate but showed favorable IFN-γ production (MFI: 80.1) in 31.1% of the cells.

Among AC samples tested, AC#287 carried higher proviral load (81 copies in 1000 PBMCs) than any other ACs (Table 2). Since Tax-specific CD8$^+$ T-cells in #287 had severely impaired IFN-γ production and proliferative potential, we examined the relationship of the function of these T-cells with proviral loads. Both percentages of IFN-γ$^+$ and dividing Tax-specific CD8$^+$ T-cells among CD8$^+$ T-cells were likely to be inversely correlated with proviral loads although they were not statistically significant (Figure 3C and 3D). Because of the limited availability of the samples, we focused mainly on two ACs (#287 and #313) in the studies hereafter.

Dysfunction of Tax-specific CD8$^+$ T-cells and inefficient CD8$^+$ cell-mediated HTLV-1 control in AC #287

To examine whether Tax-specific CD8$^+$ T-cell responses were influenced by activation of antigen-presenting cells (APCs), PBMC from #313 (responder) and #287 (low responder) were stimulated with Tax peptide in the presence or absence of LPS, a potent activator of APCs such as dendritic cells (DCs) and monocytes/macrophages. In #313, the frequency of Tax-specific CD8$^+$ T-cells increased from 1.11% to 6.47% or 4.07% at day 13, after stimulation with or without Tax peptide, respectively. The frequency of Tax-specific CD8$^+$ T-cells in #313 further increased in the presence of Tax peptide and LPS (15.81%). In contrast to #313, the frequency of Tax-specific CD8$^+$ T-cells in #287 decreased from 1.17%