The Journal of Immunology

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J Immunol 2006; 177:5718-5726; ;
doi: 10.4049/jimmunol.177.8.5718
http://www.jimmunol.org/content/177/8/5718

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Reduced Frequency, Diversity, and Function of Human T Cell Leukemia Virus Type 1-Specific CD8$^+$ T Cell in Adult T Cell Leukemia Patients[1]

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Human T cell lymphotropic virus type 1 (HTLV-1)-specific CTL are thought to be immune effectors that reduce the risk of adult T cell leukemia (ATL). However, in vivo conditions of anti-HTLV-1 CTL before and after ATL development have yet to be determined. To characterize anti-HTLV-1 CTL in asymptomatic HTLV-1 carriers (AC) and ATL patients, we analyzed the frequency and diversity of HTLV-1-specific CD8$^+$ T cells in PBMC of 35 AC and 32 ATL patients using 16 distinct epitopes of HTLV-1 Tax or Env/HLA tetramers along with intracellular cytolytic effector molecules (IFN-γ, perforin, and granzyme B). Overall frequency of subjects possessing Tax-specific CD8$^+$ T cells was significantly lower in ATL than AC (53 vs 90%; $p = 0.001$), whereas the difference in Env-specific CD8$^+$ T cells was not statistically significant. AC possessed $Tax_{11–19}$/HLA-A*0201-specific tetramer$^+$ cells by 90% and $Tax_{301–309}$/HLA-A*2402-specific tetramer$^+$ cells by 92%. Some AC recognized more than one epitope. In contrast, ATL recognized only $Tax_{11–19}$ with HLA-A*0201 and $Tax_{301–309}$ with HLA-A*2402 at frequencies of 30 and 55%. There were also significant differences in percentage of cells binding $Tax_{11–19}$/HLA-A*0201 and $Tax_{301–309}$/HLA-A*2402 tetramers between AC and ATL. Anti-HTLV-1 Tax CD8$^+$ T cells in AC and ATL produced IFN-γ in response to Tax. In contrast, perforin and granzyme B expression in anti-HTLV-1 CD8$^+$ T cells of ATL was significant lower than that of AC. Frequency of Tax-specific CD8$^+$ T cells in AC was related to proviral load in HLA-A*0201. These results suggest that decreased frequency, diversity, and function of anti-HTLV-1 Tax CD8$^+$ T cell clones may be one of the risks of ATL development. *The Journal of Immunology,* **2006, 177: 5718–5726.**

Adult T cell leukemia (ATL)[3] is caused by infection with human T cell lymphotropic virus type 1 (HTLV-1) (1–3), a retrovirus infecting ~10–15 million people worldwide, in southern Japan, the Caribbean basin, South America, Melanesia, and equatorial Africa (4). More than 800 cases of ATL are diagnosed each year in Japan (5). Although there has been recent progress in chemotherapy for ATL, with the LSG15 protocol showing an overall 5-year survival rate of 17.5% (6), the prognosis of ATL is still poor. The recent report of successful, sustained complete remission of ATL by hemopoietic stem cell transplantation led to the hypothesis that immunocompetency reconstructed by stem cell transplantation may regenerate cytotoxic immune effectors against HTLV-1-tranformed T cell and related tumor Ags, and then induce a graft-vs-leukemia reaction in ATL patients (7, 8).

HTLV-1-specific CTL plays an important role in suppressing proliferation of HTLV-1-infected or -transformed T cells in vitro (9–12) and thus may prevent development of ATL (13, 14). Because ATL develops in ~2% of people infected with HTLV-1 after a long latent period (15), it is possible that CTL fails in only a fraction of HTLV-1 carriers with a specific immunogenetic background (16–18). Previously, we identified HTLV-1 Tax epitopes recognized by HLA class I molecules using PMBC of asymptomatic HTLV-1 carriers (AC) in vitro and reported that the frequencies of HLA alleles lacking epitope anchor motifs, HLA-A*26, HLA-B*4002, HL-B*4006, and HLA-B*4801, were higher in ATL patients than in AC. These findings suggested that insufficient generation of CTL allowed outgrowth of HTLV-1-transformed cells in the host (19). Indeed, ATL patients produced anti-Tax CD8$^+$ T cell in short-term cultivation, although their IFN-γ production was insufficient (14). These findings suggested a key role of anti-HTLV-1 Tax CTL in prevention of ATL leukemogenesis.

The chromium-51 (^{51}Cr) release assay and the calcein acetoxymethyl fluorescence assay are the most widely used methods for estimating CD8$^+$ CTL (19–23). However, these assays are not suitable for mass screening of clinical samples because they are time-consuming. In addition, these assays do not reflect the in vivo status because they require short-term culture. Infected or leukemic cells easily produce HTLV-1 Tax protein as rapidly as within

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Received for publication April 19, 2006. Accepted for publication July 28, 2006.

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[1] This study was supported in part by Grants-in-Aid for Scientific Research on Priority Area for Cancer Research (122198232 and 12218104) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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[3] Abbreviations used in this paper: ATL, adult T cell leukemia; HTLV-1, human T cell lymphotropic virus type 1; AC, asymptomatic HTLV-1 carrier; 7-AAD, 7-aminoactinomycin D.

0022-1767/06/$02.00

a few hours in culture (12, 24–26). Flow cytometric assay with HLA tetramers in combination with intracellular IFN-γ, perforin, and granzyme B, which play important and diverse roles in controlling many viral infections (27, 28), is useful because of its simplicity, specificity, and sensitivity in detection of Ag-specific CTL in PMBC in vivo or in vitro (29–31).

In the present study, we developed 14 HTLV-1/HLA tetramers to detect anti-HTLV-1 $CD8^+$ CTL clones. To characterize the in vivo status of anti-HTLV-1 $CD8^+$ CTL in AC and ATL patients, we analyzed clonal frequency, diversity, and function of anti-HTLV-1 $CD8^+$ CTL in freshly isolated PBMC by examining the 16 distinct HTLV-1/HLA tetramers with HLA-A*0201 and HLA-A*2402 in combination with functional CTL assay for intracellular molecules relevant to cytolytic effector function. We found that frequency, diversity, and function of HTLV-1-specific $CD8^+$ T cell are significantly reduced in ATL.

Materials and Methods

Subjects

The study subjects included 35 AC (22–82 years old; mean, 60.0; male: female, 15:20) and 32 ATL (27–85 years old; mean, 61.2; male:female, 14:18), all of whom were recruited from Kagoshima University Hospital located in southern Kyushu, Japan. All subjects came to the hospital for examination of HTLV-1 infection and clinical checkup. They were examined by standard serological testing for HTLV-1 and hematological examinations for ATL. Those patients positive for HTLV-1 serology but with no clinical symptoms of ATL (32) or other HTLV-1-related diseases (33) were designated as AC. Diagnosis of ATL was made according to Shimoyama's criteria (32). We tested PBMC of all ATL patients before treatment. All subjects were inhabitants of Kagoshima prefecture, southern Kyushu, Japan, where ATL is endemic. All subjects signed an informed consent to participate in the present study and to allow review of their medical records, and gave a sample of peripheral blood for HLA typing and HLA tetramer assay. The study protocol was reviewed and approved by the Medical Ethical Committee of Kagoshima University. By HLA typing, 88% of the HTLV-1-infected subjects (AC and ATL combined) were shown to have HLA-A*02 or HLA-A*24. HLA allele types were representative of the population of Southern Kyushu (34). Of the 35 AC, 10 had HLA-A*0201, 24 had HLA-A*2402, 3 had both alleles in heterozygous combination, and 4 included as negative controls had neither HLA-A*0201 nor HLA-A*2402. Of the 32 ATL patients, 10 had HLA-A*0201, 22 had HLA-A*2402, 4 had both alleles in heterozygous combination, and 4 included as negative controls had neither HLA-A*0201 nor HLA-A*2402.

Preparation of PBMC

PBMC were obtained from peripheral blood by separating on Ficoll Hypaque (Amersham Biosciences) density gradient centrifugation at $400 \times g$ for 30 min, followed by washing three times with 1% FCS RPMI 1640 at $200 \times g$ centrifugation for 10 min to remove residual platelets. The fresh PBMC were used for tetramer assay and in vitro expansion of anti-HTLV-1 $CD8^+$ CTL. The remaining PBMC were cryopreserved in liquid nitrogen until examination as described previously (35).

HLA typing of PBMC

Subjects positive for HLA-A*02 and A*24 were screened by serological staining with mAbs for HLA-A*02 supertype (clone BB7.2) and HLA-A*24 supertype (clone 17A10) (Medical and Biological Laboratories), followed by secondary staining with goat anti-mouse IgG-FITC (Immunotech) according the manufacturer's instruction and subjected to flow cytometry on a FACScan (BD Biosciences). HLA allele types were determined by the PCR-sequence-specific oligonucleotide probes protocols with the Luminex 100 xMAP flow cytometry dual-laser system method using high m.w. DNA isolated from the cryopreserved PBMC as described (G&G Science) (36). Briefly, amplification was as follows: target DNA was PCR-amplified using 5′-biotin-labeled primers that are highly specific to certain sequences of HLA genes. Hybridization was as follows: after denaturation at 95°C, amplified DNA was allowed to hybridize to complementary DNA probes coupled to microbeads. Streptavidin-PE reaction was as follows: the hybridized PCR product on the oligobeads was labeled with streptavidin-PE. Measurement was as follows: Luminex apparatus identified the fluorescent intensity of PE on each coded oligobead that has hybridized with the biotin-labeled PCR product. Genosearch typing software (G&G Science) assisted in determining the HLA genotype (alleles) of the sample DNA.

Preparation of HTLV-1 Tax, Env/HLA tetramers

A total of 16 distinct HTLV-1/HLA tetramers were used in the present study (Table I). We originally developed 14 distinct PE-conjugated HLA-A*0201 and HLA-A*2402 tetramers for four Tax and three Env peptides. These Tax and Env peptides were selected based on our own published data of CTL epitope mapping for HTLV-1 Tax and Env peptides in vitro (19). Two additional HTLV-1/HLA tetramers for Tax peptides were purchased from Beckman Coulter. We and Beckman Coulter developed tetramers by the same procedure. HLA tetramers were produced as described previously (37, 38). Briefly, recombinant β_2-microglobulin and the extracellular portion of the HLA class I H chain containing the BirA recognition sequence in frame at its C terminus were expressed in *Escherichia coli* as insoluble aggregates that formed inclusion bodies. Purified inclusion bodies were solubilized in urea, and monomeric peptide-HLA class I complexes were refolded around peptides by dilution denaturing conditions. After buffer exchange, a specific lysine residue in the H chain C-terminal tag was biotinylated with the BirA enzyme. Monomeric complexes were purified by monomeric avidin gel chromatography (Pierce). Tetrameric arrays of biotinylated peptide-HLA class I complexes were formed by the addition of PE-labeled streptavidin (Prozyme) at a molar ratio of 4:1. The purity of each 16 HLA tetramer was tested by HPLC and its binding affinity was evaluated from the BIMAS score at Medical and Biological Laboratories.

Detection of anti-HTLV-1 $CD8^+$ T cells in PBMC using tetramer

The procedure was slightly changed from Haanen et al. (39) and Skinner et al. (40). We used Alexa Fluor 488-labeled anti-CD8 mAbs and Alexa Fluor 647-labeled mouse-anti-PE mAbs following PE-labeled HTLV-1/HLA tetramer because these dyes provide resistance to photobleaching. Briefly, the PBMC were incubated with PE-labeled HTLV-1/HLA tetramer (20 μg/ml) with normal goat serum for 50 min at 4°C in the dark. The PBMC were washed with PBS, and then incubated with Alexa Fluor 488-labeled anti-CD8 mAbs (6 μg/ml; Caltag Laboratories) and Alexa Fluor 647-labeled mouse-anti-PE mAbs (20 μg/ml; Sigma-Aldrich), which was produced using Alexa Fluor 647 mAbs Labeling kit (Molecular Probes) according to the manufacturer's manuals, for 50 min at 4°C in the dark. Finally, the PBMC were washed three times, and then mounted to slides with 10 μl of ProLong Gold (Molecular Probes). Stained PBMC were visualized using an OYMPAS IX81 confocal microscope.

HTLV-1 Tax, Env/HLA tetramer assay

Aliquots of 1×10^6 freshly isolated PBMC or cultured cells were incubated with the HTLV-1/HLA tetramers with each of the 16 distinct HTLV-1 Tax or Env peptides for 45 min at 4°C, followed by staining with FITC-conjugated murine anti-human CD8 mAbs (Beckman Coulter) and anti-CD45-PerCP (BD Biosciences) for 45 min at 4°C according to the manufacturer's instructions (Medical and Biological Laboratories). Aliquots of 1×10^5 fresh CD45-positive lymphocytes were performed using

Table I. *HTLV-1 Tax, Env/HLA tetramers*[a]

Tetramers	HLA Allele	HTLV-1 Peptide	Epitopes
T11	A*0201	$Tax_{11–19}$	**LL**FGYPVY**V**
T123	A*0201	$Tax_{123–131}$	**TL**GQHLPT**L**
T155	A*0201	$Tax_{155–163}$	**YL**YQLSPP**I**
T178	A*0201	$Tax_{178–186}$	**QL**GAFLTN**V**
T307	A*0201	$Tax_{307–315}$	**LL**FEEYTN**I**
E175	A*0201	$Env_{175–183}$	**FL**NTEPSQ**L**
E239	A*0201	$Env_{239–247}$	**VL**YSPNVS**V**
E442	A*0201	$Env_{442–450}$	**AL**QTGITL**V**
T12	A*2402	$Tax_{12–20}$	L**F**GYPVYV**F**
T187	A*2402	$Tax_{187–195}$	P**Y**KRIEEL**L**
T289	A*2402	$Tax_{289–297}$	S**F**LLSHGL**I**
T301	A*2402	$Tax_{301–309}$	S**F**HSLHLL**F**
T311	A*2402	$Tax_{311–319}$	E**Y**TNIPIS**L**
E11	A*2402	$Env_{11–19}$	**FF**QFCPLI**F**
E21	A*2402	$Env_{21–29}$	D**Y**SPSCCT**L**
E153	A*2402	$Env_{153–161}$	H**F**SKCGFP**F**

[a] HLA binding peptide anchor motifs are shown in bold.

FIGURE 1. Ex vivo detection of anti-HTLV-1 $CD8^+$ T cell. Freshly isolated PBMC were stained with FITC-labeled anti-CD8 mAbs (*A*) and Alexa 647-labeled anti-PE mAbs in combination with PE-labeled HTLV-1/HLA tetramer (*B*). *C*, Colored overlay of *A* and *B*.

FACScan (BD Biosciences) (41) and analyzed with FlowJo software (Tree Star) (28). In the cultured PBMC samples, apoptotic or necrotic cells in the cultures were stained with 7-amino-actinomycin D (7-AAD; Beckman Coulter) (42), and the 7-AAD-positive cells were excluded by FACScan analysis. HIV/HLA tetramer (Medical and Biological Laboratories) for negative and CMV/HLA tetramer (Beckman Coulter) were also stained.

Real-time PCR quantification of HTLV-1 proviral load in PBMC

DNA was extracted from 1×10^6 cells of PBMC using SMI TEST EX-R&D (G&G Science). The HTLV-1 proviral load in PBMC was assayed in 64 of 67 subjects by methods of quantitative PCR using a Light Cycler System (Roche Diagnostics) by intraassay using a series of duplicate measurements of 12 test samples with standard DNA of four different dilutions for each assay as described previously (43). In brief, the duplicate intraassay for HTLV-1 provirus load in PBMC was run by simultaneous measurements of β-globin DNA and HTLV-1 DNA using the standard DNA, β-globin DNA from Roche Diagnostics, and HTLV-1 provirus DNA from MT-2 cells. One PBMC has two copies of β-globin gene (equivalent to 6 pg of β-globin DNA) and one MT-2 cell has eight copies of HTLV-1 provirus DNA (equivalent to 6 pg of HTLV-1 in DNA). The β-globin PCR primer and probe sets were commercial kits (Roche Diagnostics). The HTLV-1 primer set corresponded to the highly conserved HTLV-1 pX region, SK43 and SK44. The HTLV-1 pX probe set was designed by ourselves for the two adjacent parts of the pX region, which were labeled with different fluorophores according to the manufacturer's instructions. The HTLV-1 provirus load was expressed as number of copies per 10^3 cells using the following formula: HTLV-1 provirus load = ((HTLV-1 pX copy number)/(β-globin copy number/2)) $\times 10^3$. The detection limit of this method was 0.2 copies of HTLV-1 provirus/10^3 cells.

Induction of HTLV-1 Tax, Env-specific CTL

Aliquots of PBMC (1×10^6 cells) were used for in vitro expansion of $CD8^+$ CTL clones in cultures with 2×10^{-6} M of distinct HTLV-1 Tax and Env peptides in RPMI 1640 medium supplemented with the following reagents: 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.1 mM nonessential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.05 mM 2-ME, 50 U/ml recombinant human IL-2, and 10% heat-inactivated FCS (RPMI 1640-CM). All culture conditions were the same as described elsewhere (41). The cultured PBMC were examined using the HTLV-1/HLA tetramer assay described above.

Intracellular IFN-γ, perforin, and granzyme B assay

PBMC (1×10^6) for IFN-γ analysis were cultured for 16 h with or without 0.02 μM HTLV-1 Tax peptide in combination with brefeldin A (BD Biosciences) in RPMI 1640-CM. Harvested cells for IFN-γ and freshly isolated PBMC for perforin and granzyme B were labeled with HTLV-1/HLA-tetramer-PE and anti-CD8-allophycocyanin Ab (BD Biosciences) for cell surface Ags (44). The cells were further treated with Permeabilizing Solution (BD Biosciences) for 10 min. After washing with buffer containing 0.1% saponin, the cells were incubated with anti-human IFN-γ-FITC, perforin-FITC, or granzyme B-FITC Ab (BD Biosciences) in buffer containing saponin. FastImmune Control γ2aFITC/γ1PE was stained as negative control (BD Biosciences). Aliquots of 1×10^4 $CD8^+$ T lymphocytes were performed using FACSCalibur (BD Biosciences) and analyzed with FlowJo software.

CD107a mobilization assay

PBMC (1×10^6) were cultured for 6 h with or without 0.02 μM HTLV-1 Tax peptide in combination with anti-CD107a mAbs-FITC (Southern Biotech) and the secretion inhibitor monensin (BD Biosciences) in RPMI 1640-CM (45, 46). Following incubation, cell suspensions were washed with cold PBS. The cells were further stained with tetramer-PE and anti-CD8 mAb-PE-Cy5 (Beckman Coulter) as described above. Aliquots of 1×10^4 $CD8^+$ T lymphocytes were performed using FACSCalibur (BD Biosciences) and analyzed with FlowJo software.

Statistical analysis

Differences in HTLV-1-specific $CD8^+$ T cell frequency between AC and ATL patients were evaluated by χ^2 test or exact test. The Mann-Whitney *U* test was used to compare the percentages of cells binding $tetramer^+$ or $tetramer^+$intracellular $cytokine^+$ in $CD8^+$ lymphocytes between subjects with AC and ATL, between the proviral load of Tax-tetramer positive AC

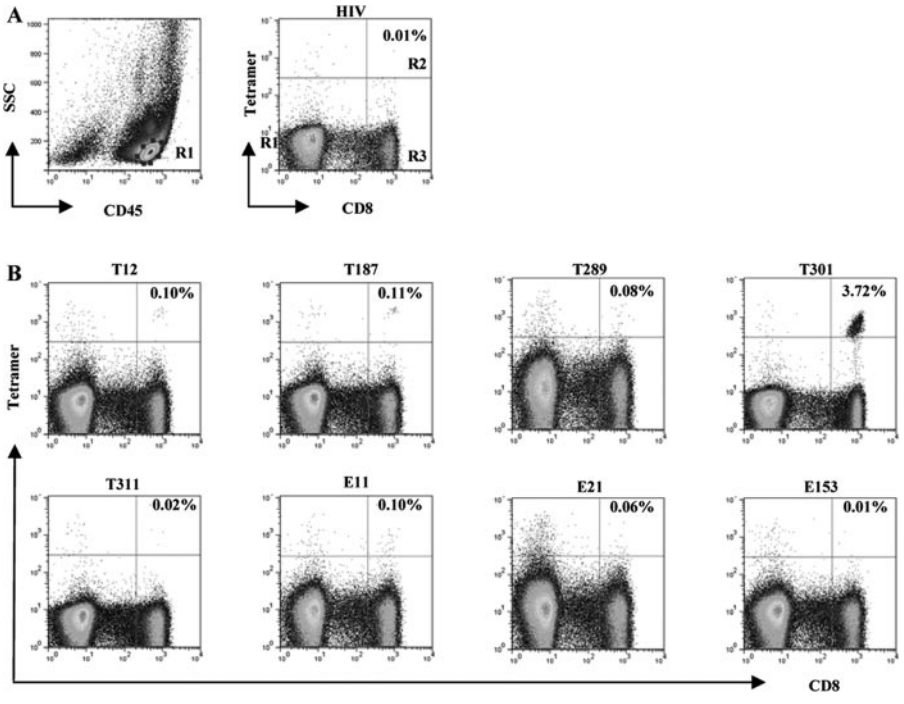

FIGURE 2. Variety of anti-HTLV-1 $CD8^+$ T cells in fresh PBMC. *A*, $Tetramer^+CD8^+$ T cells were estimated in $CD45^+$ T lymphocyte region (R1). We observed HIV-$tetramer^+CD8^+$ T cells as negative control. *B*, Fresh PBMC isolated from AC were stained with eight distinct HTLV-1/HLA-A*2402-tetramers (T12, T187, T289, T301, T311, E11, E21, and E153). Numbers in the *upper right quadrants* represent the percentages of $tetramer^+CD8^+$ T cells in $CD8^+$ $CD45^+$ T lymphocytes (R2/R2+R3).

Table II. *Frequencies of HTLV-1-specific tetramer binding cells in PBMC of AC and ATL patients*

HLA Allele	Tetramers	Positive Epitopes Detected by HTLV-1/HLA Tetramers[a] AC (%)	ATL (%)
A*0201	T11	90 (9/10)[b]	30 (3/10)*
A*0201	T123	0 (0/10)	0 (0/10)
A*0201	T153	0 (0/10)	0 (0/10)
A*0201	T178	10 (1/10)	0 (0/10)
A*0201	T307	10 (1/10)	0 (0/10)
A*0201	E175	0 (0/10)	0 (0/10)
A*0201	E239	0 (0/10)	0 (0/10)
A*0201	E442	0 (0/10)	0 (0/10)
A*2402	T12	17 (4/24)	0 (0/22)
A*2402	T187	13 (3/24)	0 (0/22)
A*2402	T289	0 (0/24)	0 (0/22)
A*2402	T301	92 (22/24)	55 (12/22)*
A*2402	T311	0 (0/24)	0 (0/22)
A*2402	E11	4 (1/24)	0 (0/22)
A*2402	E21	4 (1/24)	0 (0/22)
A*2402	E153	8 (2/24)	0 (0/22)
Tax CTL positives		24 (40/170)	9 (15/160)**
Env CTL positives		4 (4/102)	0 (0/96)
Total CTL positives		16 (44/272)	6 (15/256)**

[a] The percentages of HTLV-1/HLA tetramer$^+$CD8$^+$ T cells in the CD8$^+$CD45$^+$ T lymphocytes that are ≥0.1% are counted as positives, whereas those 0–0.09% are counted as negatives.

[b] Epitopes detected by HTLV-1/HLA tetramers/number of tetramers tested. Eight tetramers were used for testing in subjects carrying either HLA-A*0201 or HLA-A*2402, whereas those carrying both HLA-A*0201 and HLA-A*2402 were tested with 16 tetramers.

*, $p < 0.01$; **, $p < 0.001$, significant differences between AC and ATL by χ^2 test.

and negative AC. Statistical significance was two-sided at α of 0.05. Values of p were corrected for multiple comparisons using StatView software version 5.0 (SAS Institute).

Results

Specificity and sensitivity of HTLV-1/HLA tetramer assay for anti-HTLV-1 CD8$^+$ T cells

The feasibility of the developed HTLV-1/HLA tetramer assay was tested by detection of anti-HTLV-1 CD8$^+$ T cells in freshly isolated PBMC from 35 AC and 32 ATL patients. As shown in Figs. 1–4, anti-HTLV-1 CD8$^+$ T cells were detected. We could visually detect anti-HTLV-1 CD8$^+$ T cells in combination staining with tetramer and CD8 using confocal microscopy (Fig. 1). For specificity test, we observed HIV-tetramer$^+$CD8$^+$ cells (R2; Fig. 2*A*) as negative control in CD8$^+$CD45$^+$ T lymphocytes (R2+R3; Fig. 2*A*) (mean, 0.04; SD, 0.05; 95% confidential interval, 0.06). We also observed 0–0.02% tetramer$^+$CD8$^+$ cells in CD8$^+$CD45$^+$ T lymphocytes of negative control subjects who had neither HLA-A*0201 nor HLA-A*2402. These HLA tetramers could detect anti-HTLV-1 CD8$^+$ T cells possessing HLA-A*0201 or HLA-A*2402 in circulating PBMC.

Fig. 2 shows representative data of anti-HTLV-1 CD8$^+$ T cells in AC possessing HLA-A*2402. Tetramer$^+$CD8$^+$ cells were estimated in CD45$^+$ T lymphocytes (R1). This subject showed a wide spectrum of HLA tetramer staining with CD8$^+$ T cells ranging from 0.01 to 3.72% (R2/R2+R3) in which >0.1% of the positive staining showed a definitely clustered pattern of CD8$^+$ T cells (Fig. 2*B*, T12, T187, T301, and E11), but the subject with <0.1% staining did not show the clustered pattern of CD8$^+$ T cells. Based on negative control, we adopted a tentative cut-off point of 0.1% for the HTLV-1/HLA tetramer assay, which was the lower limit of tetramer staining with the HLA-compatible CD8$^+$ T cells. Four samples of AC were positive (Fig. 2*B*, T12, T187, T301, and E11) and the other four were negative (Fig. 2*B*, T289, T311, E21, and E153) using this cut-off point.

Frequency of anti-HTLV-1 CD8$^+$ T cells in PBMC of AC and ATL patients

We assessed a total of 59 subjects consisting of 31 AC patients and 28 ATL patients using eight epitope-specific tetramers per subject possessing HLA-A*0201 or HLA-A*2402. In the case of subjects possessing both HLA alleles (three AC and four ATL), epitope-specific CD8$^+$ T cells were analyzed using 16 distinct tetramers per subject.

Frequency of HTLV-1/HLA tetramer positivity varied by HTLV-1 epitope and HLA allele in AC: 90% in $Tax_{11–19}$ with HLA-A*0201, 92% in $Tax_{301–309}$ with HLA-A*2402, and 4–17% in other combinations of $Tax_{12–20}$, $Tax_{178–185}$, $Tax_{187–195}$, $Tax_{307–315}$, $Env_{11–19}$, $Env_{21–29}$, and $Env_{153–161}$ with respective HLA alleles (Table II). In contrast, ATL recognized only $Tax_{11–19}$ with HLA-A*0201 and $Tax_{301–309}$ with HLA-A*2402 at frequencies of 30 and 55%, respectively. Among the individual HTLV-1/HLA tetramers, two ($Tax_{11–19}$, $p = 0.0042$; and $Tax_{301–309}$, $p = 0.0031$; Table II) were significantly more frequent in AC than ATL. Among AC, 24% of Tax epitopes were positive, whereas significantly fewer epitopes were positive among ATL (9%, $p = 0.0004$; Table II). In contrast, Env epitopes were not statistically significant.

Frequency of subjects detected anti-HTLV-1 CD8$^+$ T cells in ATL (15 of 28; 54%) was significantly lower than that in AC (29 of 31; 94%; $p = 0.0003$). In particular, the frequency of subjects possessing Tax-specific CD8$^+$ T cells in ATL (15 of 28; 54%) was significantly lower than that in AC (28 of 31; 90%; $p = 0.001$), but differences in frequency possessing Env-specific CD8$^+$ T cells were not significant (Table III).

With regard to $Tax_{11–19}$-specific tetramer binding cells in individual subjects with HLA-A*0201, the frequency of percentage of CD8$^+$ T cells binding $Tax_{11–19}$/HLA-A*0201 tetramer in

Table III. *Summary of HTLV-1-specific tetramer and HTLV-1 proviral load in AC and ATL patients*

	AC	ATL
Subjects positive for Tax tetramer[a]	28 ($n = 31$)	15 ($n = 28$)*
Subjects positive for Env tetramer[a]	4 ($n = 31$)	0 ($n = 28$)
$Tax_{11–19}$ tetramer$^+$ CD8$^+$ T cells[b]	0.91 ± 0.37 ($n = 10$)	0.74 ± 0.49 ($n = 10$)**
$Tax_{301–309}$ tetramer$^+$ CD8$^+$ T cells[b]	2.46 ± 0.71 ($n = 24$)	0.21 ± 0.05 ($n = 22$)*
HTLV-1 proviral load	65.4 ± 7.4 ($n = 35$)	1095.4 ± 194.1 ($n = 29$)

[a] The number of subjects positive for tetramers; the percentages of HTLV-1/HLA tetramer$^+$ CD8$^+$ T cells in the CD8$^+$ CD45$^+$ T lymphocytes ≥0.1% are counted as subjects positives for tetramer.

[b] The Tax-specific tetramer-positive CD8$^+$ T cells in the CD8$^+$ CD45$^+$ T lymphocytes is shown as the mean ± SE percentage.

[c] The HTLV-1 proviral load is shown as the ±SE copies/10^3 PBMC.

*, $p < 0.0001$; **, $p < 0.05$.

Table IV. *Relationship between HTLV-1 proviral load and HTLV-1 Tax-specific CD8$^+$ T cell*

	HTLV-1 Tax-Specific CD8$^+$ T Cell			
	Tax_{11-19} Tetramer*		$Tax_{301-309}$ Tetramer	
	Positive ($n = 9$)[b]	Negative ($n = 26$)	Positive ($n = 22$)[b]	Negative ($n = 13$)
Proviral load[a]	38.1 ± 13.8	74.8 ± 8.0	72.4 ± 9.4	53.5 ± 11.6

[a] The HTLV-1 proviral load is shown as the mean ± SE copies/10^3 PBMC.
[b] The percentages of HTLV-1/HLA tetramer$^+$ CD8$^+$ T cells in the CD8$^+$ CD45$^+$ T lymphocytes that are ≥0.1% are counted as positives.
*, $p < 0.05$, significant differences between positive group and negative group by Mann-Whitney *U* test.

CD8$^+$CD45$^+$ T lymphocytes ranged from 0.03 to 3.77% in AC and 0 to 4.43% in ATL patients. There were significant differences in percentage of cells binding Tax_{11-19}/HLA-A*0201 tetramer between AC and ATL ($p = 0.037$; Table III) as well as the frequencies of epitopes found on anti-HTLV-1 CD8$^+$ T cells mentioned above. There were also significant differences in percentage of CD8$^+$ T cells binding CMV/HLA-A*0201 tetramer in CD8$^+$/CD45 T lymphocytes between AC and ATL ($p = 0.028$). With regard to the ratio of $Tax_{301-309}$-specific CD8$^+$ T cells in individual subjects with HLA-A*2402, the frequency of percentage of CD8$^+$ T cells binding $Tax_{301-309}$/HLA-A*2402 tetramer in CD8/CD45$^+$ T lymphocytes ranged from 0 to 15.6% in AC and 0 to 0.79% in ATL patients. There was a significant difference in the ratio of cells binding $Tax_{301-309}$/HLA-A*2402 tetramer between AC and ATL ($p < 0.0001$; Table III) as well as the frequencies of epitopes.

HTLV-1 proviral load in AC and ATL patients

The proviral load of AC and ATL patients ranged from 4.6 to 225.8 and from 44.3 to 2838.3 (copies/10^3 PBMC) (Table III), respectively. Because the ATL patients should contain leukemic cells, we assessed the proviral load of only AC in terms of the relationship with Tax-specific tetramer$^+$ cells. The proviral load of Tax_{11-19} tetramer-positive AC was significantly lower than that of Tax_{11-19} tetramer-negative AC (mean ± SE, 38.1 ± 13.8 vs 74.8 ± 8.0; $p = 0.043$; Table IV). These findings are consistent with Bangham's report (47) and suggest that Tax_{11-19} CTL works as a strong down-regulator of the proviral load. In contrast, with regard to $Tax_{301-309}$ tetramer, there was no significant difference between the proviral load of $Tax_{301-309}$ tetramer-positive AC and negative AC (mean ± SE, 72.4 ± 9.4 vs 53.5 ± 11.6; Table IV). The reason why the proviral load of $Tax_{301-309}$ tetramer-positive AC was lower than that of negative AC can be explained by the negative group containing many Tax_{11-19} tetramer-positive AC (7 of 13). Therefore, we omitted HLA-A*02-positive (including HLA-A*0201 and HLA-A*0206) subjects in analysis of $Tax_{301-309}$ tetramer. Under this condition, the proviral load of $Tax_{301-309}$ tetramer-positive AC and negative AC was 74.3 ± 10.1 ($n = 18$) and 97.6 ± 11.5 ($n = 5$), respectively ($p = 0.09$).

Expansion of anti-HTLV-1 CD8$^+$ T cells by in vitro cultivation

Thirty of 67 subjects (18 AC and 12 ATL patients) were cultured for further analysis of anti-HTLV-1 CD8$^+$ T cells in vitro. The cultured cells were morphologically activated T cells and clustered in colony formation. We observed increases in the numbers of positive cells corresponding to Tax_{11-19} in ATL patient and AC (Fig. 3, *A* and *B*). Two specificities of HTLV-1 Tax-specific CD8$^+$ T cells for $Tax_{289-295}$ and $Tax_{311-319}$ with HLA-A*2402 were newly identified in the cultured PBMC from AC but not from ATL patients (Fig. 3).

Diversity of anti-HTLV-1 CD8$^+$ T cells in PBMC of AC and ATL patients

Anti-HTLV-1 CD8$^+$ T cells in AC recognized a wide spectrum of Tax and Env epitopes, HLA-A*0201-restricted Tax_{11-19}, $Tax_{178-196}$, $Tax_{307-315}$, and HLA-A*2402-restricted Tax_{12-20}, $Tax_{187-195}$, $Tax_{289-297}$, $Tax_{301-309}$, $Tax_{311-319}$, Env_{11-19}, Env_{21-29}, and $Env_{153-161}$, in vivo or in vitro. In contrast, anti-HTLV-1 CD8$^+$ T cells in ATL patients recognized only two epitopes (Tax_{11-19} with HLA-A*0201 and $Tax_{301-309}$ with HLA-A*2402). The number of epitope repertoires found on anti-HTLV-1 CD8$^+$ T cells in ATL patients was considerably lower than that in AC (2 of 18 and 11 of 18; $p < 0.05$) as shown in Table III and Fig. 3.

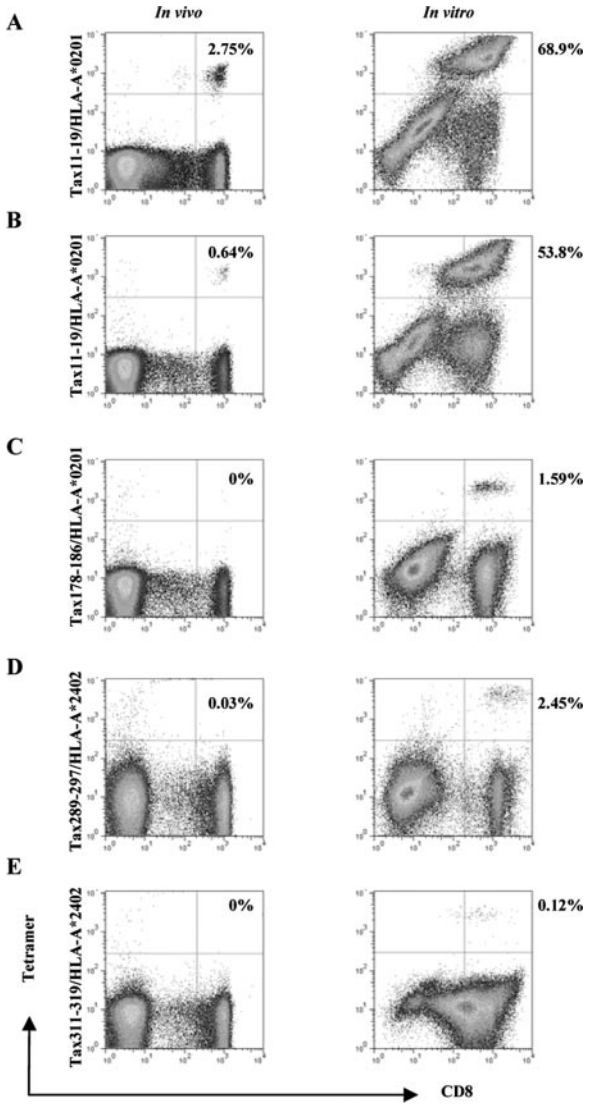

FIGURE 3. In vitro expansion of anti-HTLV-1 CD8$^+$ T cells. Freshly isolated PBMC were stained with Tax_{11-19} (*A* and *B*), $Tax_{178-186}$ (*C*), $Tax_{289-297}$ (*D*), and $Tax_{311-319}$ (*E*) tetramers, respectively, shown in the *left panels*. Following cultivation with 2×10^{-6} M of each epitope peptide for 14 (*A* and *B*), 31 (*C*), 46 (*D*), and 22 (*E*) days, these cells were stained with the respective tetramers, indicated in the *right panels*. Samples of *C* and *D* were restimulated after 14 days. Numbers in the *upper right quadrants* represent the percentages of tetramer$^+$CD8$^+$ T cells in 7-AAD-negative CD8$^+$ T lymphocytes. We observed increases in number of positive cells corresponding to peptides.

Intracellular IFN-γ produced in response to HTLV-1 Tax peptide, and expression of perforin and granzyme B

Representative results regarding intracellular cytokines are shown in Fig. 4. Intracellular cytokine$^+$tetramer$^+$ cell were estimated in CD8$^+$ T lymphocytes (R4). None of anti-HTLV-1 CD8$^+$ T cells produced IFN-γ in short-term culture without Tax peptide (Fig. 4*B*, *left*). IFN-γ production in AC increased from 0 to 52% in the tetramer$^+$CD8$^+$ T cells (R6/R5+R6) after Tax peptide stimulation (Fig. 4*B*, *upper quadrants* of AC, 0/(5.8 + 0) vs 1.1/(1.0 + 1.1)). Similarly, that in ATL patient increased from 0 to 40% in the tetramer$^+$CD8$^+$ T cells after Tax peptide stimulation (Fig. 2*B*, *upper quadrants* of ATL, 0/(1.0 + 0) vs 0.4/(0.6 + 0.4)). Intracellular IFN-γ production was also detected in PBMC of all subjects examined (5 of both AC and ATL patients; average ratios of positive cells were 21.8 and 13.1%, respectively). Thus, production of IFN-γ demonstrated that the tetramer$^+$CD8$^+$ T cells in AC and ATL patients are functional CD8$^+$ T cells targeting HTLV-1 Tax epitopes. Tax peptide stimulation caused decrease of tetramer$^+$CD8$^+$ T cell. After peptide stimulation, CD8$^+$7-AAD$^+$ cells were increased more than untreated culture. Expression of the degranulation marker CD107a, which allows measurement of cytolytic cell activation (28, 45, 46), was significantly increased in tetramer$^+$CD8$^+$ lymphocytes treated with the peptide (Fig. 4*C*). These findings suggest that the tetramer$^+$CD8$^+$ cells were decreased as a result of cytotoxicity.

Intracellular perforin and granzyme B were detected in HTLV-1 Tax tetramer$^+$CD8$^+$ T lymphocytes of all subjects examined (10 of both AC and ATL patients, respectively; representative data are shown in Fig. 4, *D* and *E*). AC subject had 57% (R6/R5+R6) of perforin$^+$ cells and 40% of granzyme B$^+$ cells in HTLV-1 Tax tetramer$^+$CD8$^+$ T lymphocytes (shown in *upper column* of Fig. 4, *D* and *E*, respectively), whereas ATL subject had 30% of perforin$^+$ cells and 36% of granzyme B$^+$ cells (shown in *upper column* of Fig. 4, *D* and *E*, respectively).

Interestingly, notable insufficiency of perforin (Fig. 5*A*) and granzyme B (Fig. 5*B*) expression in HTLV-1 Tax tetramer$^+$CD8$^+$

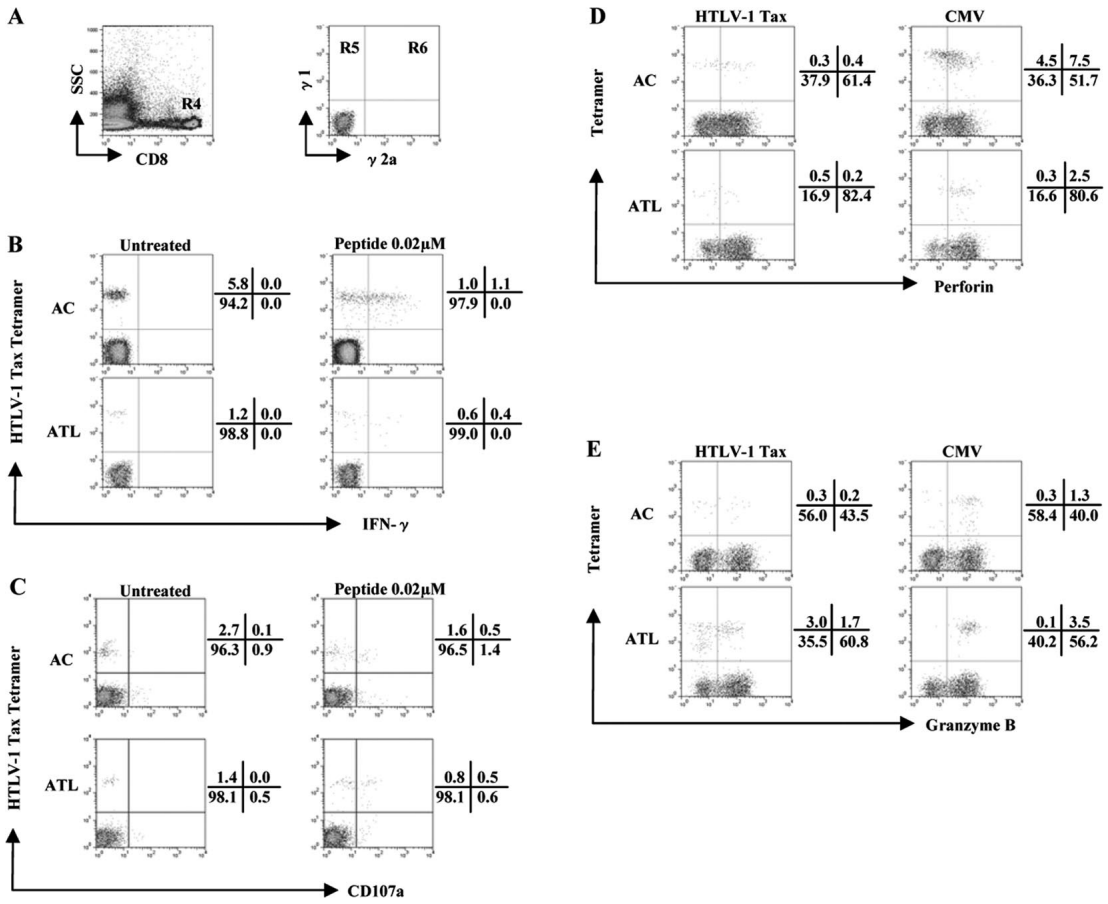

FIGURE 4. Intracellular production of IFN-γ, CD107a mobilization, and expression of perforin and granzyme B in HTLV-1-specific CD8$^+$ T lymphocytes. *A*, Intracellular cytokine$^+$tetramer$^+$ cell were estimated in CD8$^+$ T lymphocytes (R4). The *right panel*, which was extended in the R4 region, showed negative control for surface and intracellular immunofluorescence. *B*, Intracellular IFN-γ production in anti-HTLV-1 CD8$^+$ T cells are shown on the *top* (AC) and *bottom* (ATL), respectively. The *left panel* showed untreated condition, whereas the *right panel* showed <0.02 μM peptide concentration. Increases of IFN-γ were observed corresponding to peptide pulsing in AC and ATL patient. Numbers indicate the percentages in CD8$^+$ T lymphocytes. Figure shows one representative result of IFN-γ production in five of both AC and ATL patients. *C*, CD107a mobilization in anti-HTLV-1 CD8$^+$ T cells are shown on the *top* (AC) and *bottom* (ATL), respectively. The *left panel* showed untreated condition, whereas the *right panel* showed <0.02 μM peptide concentration. Increases of CD107a$^+$tetramer$^+$ cells were observed corresponding to peptide pulsing in AC and ATL patient. Figure shows one representative result of CD107a mobilization assay in three of both AC and ATL patients, respectively. *D*, Intracellular perforin expression in tetramer$^+$CD8$^+$ T cells are shown on the *top* (AC) and *bottom* (ATL), respectively. The *left panel* showed anti-HTLV-1 Tax CD8$^+$ T cells, whereas the *right panel* showed anti-CMV CD8$^+$ T cells. Figure shows 1 representative result of granzyme B expression in 10 of both AC and ATL patients, respectively. *E*, Intracellular granzyme B expression in tetramer$^+$CD8$^+$ T cells are shown on the *top* (AC) and *bottom* (ATL), respectively. The *left panel* showed anti-HTLV-1 Tax CD8$^+$ T cells, whereas the *right panel* showed anti-CMV CD8$^+$ T cells. Figure shows 1 representative result of granzyme B expression in 10 of both AC and ATL patients, respectively.

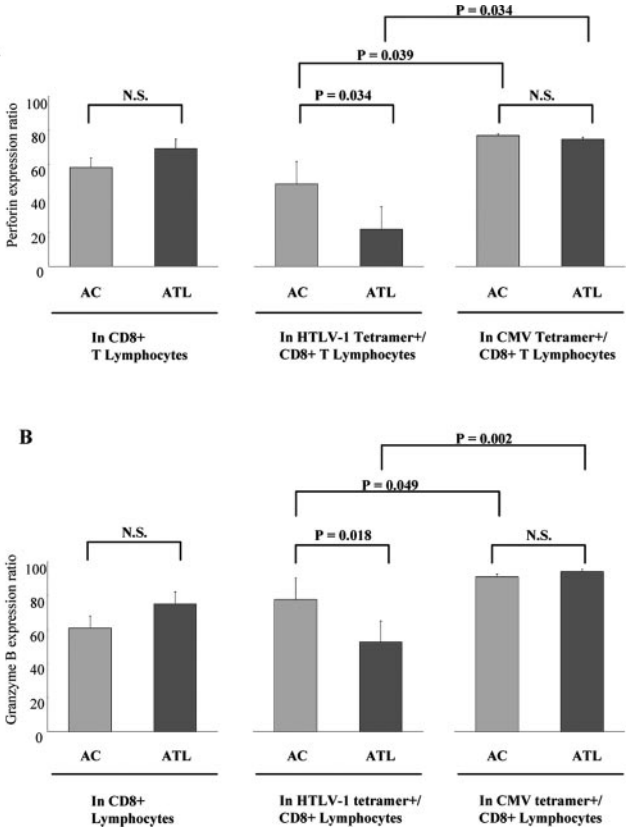

FIGURE 5. Differential expression of perforin and granzyme B in anti-HTLV-1 and anti-CMV $CD8^+$ T lymphocytes between AC and ATL. *A*, Expression of perforin in $CD8^+$, HTLV-1 Tax tetramer$^+CD8^+$, and CMV tetramer$^+CD8^+$ T lymphocytes (mean of 10 independent experiments). *B*, Expression of granzyme B in $CD8^+$, HTLV-1 Tax tetramer$^+CD8^+$ and CMV tetramer$^+CD8^+$ T lymphocytes (mean of 10 independent experiments).

T lymphocytes were observed in ATL in comparison with AC, but not in CMV tetramer$^+CD8^+$ T lymphocytes. Regarding perforin, expression in anti-Tax CTL of ATL were significantly lower than AC (average ratios of positive cells were 21.7 and 48.2%, respectively; $p = 0.034$), but not in $CD8^+$ T lymphocytes. Regarding granzyme B, expression in anti-Tax CTL of ATL were also significantly lower than AC (average ratios of positive cells were 52.5 and 77.6%, respectively; $p = 0.018$), but not in $CD8^+$ T lymphocytes.

In addition, expression of perforin and granzyme B in HTLV-1 Tax tetramer$^+CD8^+$ T lymphocytes were diminished in comparison with those in CMV tetramer$^+CD8^+$ T lymphocytes in both AC (anti-HTLV-1 vs anti-CMV with perforin and granzyme B; $p = 0.039$ and $p = 0.049$, respectively) and ATL (anti-HTLV-1 vs anti-CMV with perforin and granzyme B; $p = 0.034$ and $p = 0.002$, respectively).

Discussion

HTLV-1-specific CTL are thought to be important immune effectors that suppress the outgrowth of HTLV-1-transformed T cell and thus reduce the risk of ATL development (9–14, 21). To confirm the correlation between a deficiency of anti-HTLV-1 CTL and increased risk of ATL, we compared the frequency of anti-HTLV-1 CTL and diversity of epitope in freshly isolated PBMC from AC and ATL patients using the tetramers. Our results demonstrated that the frequency and diversity of anti-HTLV-1 Tax $CD8^+$ T cells in ATL patients was significantly reduced compared with those in AC. These observations suggest that the lower frequency and diversity of anti-Tax $CD8^+$ T cells is risk for ATL development. A recent report demonstrated insufficient expression of HTLV-1 Tax in vivo in ATL patients and suspected a role of Tax-specific CTL for therapy or prevention of ATL (48). However, other researchers, as well as our group, demonstrated clear expression of HTLV-1 Tax in short-term cultures of fresh ATL cells (24–26). It is likely that cell-to-cell interactions in short-term culture mimic the cellular interactions in lymphoid tissues in vivo. Therefore, it is possible that ATL cells in lymphoid tissues produce HTLV-1 Tax protein. In fact, Marin et al. (49) recently demonstrated HTLV-1 Tax expression by immunohistochemistry in lymphoid tissues in ATL patients. Regarding AC, Hanon et al. (12) also demonstrated HTLV-1 Tax expression in cultured PBMC from HTLV-1-infected carriers. Although direct evidence that infected cells in lymphoid tissue produce HTLV-1 Tax in vivo has not been reported, it is likely that the cells produce Tax protein similar to primary ATL cells. A few previous studies that demonstrated CTL activity stimulated by Tax peptide have been reported (14, 50). However they did not indicate quantitative anti-Tax $CD8^+$ T cells. The present studies examined directly for anti-Tax $CD8^+$ T cells in vivo of AC and ATL patients by tetramer assay for quantitative analysis.

We identified 11 of 16 distinct clones of anti-HTLV-1 $CD8^+$ T cells in the PBMC of AC and 2 clones of $CD8^+$ T cells in ATL patients (Table III and Fig. 3). The $CD8^+$ T cells for $Tax_{11–19}$ and $Tax_{301–309}$ were commonly detected in AC and ATL patients carrying HLA-A*0201 or HLA-A*2402, although the detection rate in ATL patients was much lower than that in AC. Therefore, $Tax_{11–19}$ and $Tax_{301–309}$ were thought to be the major epitopes for Tax-specific $CD8^+$ T cell in both AC and ATL patients. In fact, $Tax_{301–309}$ was found to be the immunodominant epitope for anti-Tax CTL generated in ATL patients with HLA-A*2402 who underwent allogeneic hemopoietic stem cell transplantation (20). Nine other epitopes, $Tax_{12–20}$, $Tax_{178–186}$, $Tax_{187–195}$, $Tax_{289–297}$, $Tax_{309–315}$, $Tax_{311–319}$, $Env_{11–19}$, $Env_{21–29}$, and $Env_{153–161}$, were likely to be minor epitopes for generation of anti-HTLV-1 $CD8^+$ T cells. It is possible that even minor epitopes have significant immune function in vivo, although we have no direct evidence.

In the present study, we showed that anti-Tax $CD8^+$ T cells are significantly more abundant in patients with AC compared with ATL patients, but anti-Env is not in both HLA phenotypes. These findings suggest strongly that anti-Tax $CD8^+$ T cells are more significant for prevention of the development of ATL than anti-Env $CD8^+$ T cells. High levels of Tax-specific $CD8^+$ T cells are advantageous for suppression of outgrowth of HTLV-1-infected or -transformed T cell and thus reduce the risk of ATL (9–14, 21). The present study demonstrated that the $Tax_{11–19}$-specific tetramer$^+CD8^+$ T cells works as a strong down-regulator of the proviral load (47). The $Tax_{301–309}$-specific tetramer$^+CD8^+$ T cells show the tendency of decreasing the viral load. This interpretation is supported by the results of our previous study in which risk of ATL was shown to be associated with the number of HLA anchor motifs that recognized HTLV-1 Tax epitopes but not HTLV-1 Env epitopes (19). If deficient anti-Tax $CD8^+$ T cells are responsible for the development of ATL, AC with low frequency or diversity of anti-Tax $CD8^+$ T cells may belong to a group at high risk of developing ATL. Further follow-up studies are needed to clarify the significance of anti-Tax $CD8^+$ T cells for prevention of this disease.

ATL patients usually show immune dysfunction, and this may explain the lower frequency of CTL in these subjects. In fact, dysfunction of cellular immunity has been reported since the discovery of this disease (32). Therefore, the low frequency of

anti-Tax CTL may be just one of the general immune dysfunctions present in ATL patients. Conversely, the low frequency of anti-HTLV-1 CTL may be an Ag-specific phenomenon, and not representative of whole immune dysfunction. Our observation of low anti-Tax $CD8^+$ T cells frequency despite high numbers of $CD8^+$ cells in ATL patients (T11, 0.05%; T301, 0.01%, 663 cells/μl; T301, 0.12%, 550 cells/μl) and high anti-Tax $CD8^+$ T cells frequency despite low numbers of $CD8^+$ T cells in AC (T11, 0.64%, 356 cells/μl; T301, 1.16%, 309 cells/μl) may support the latter suggestion (normal values are between 400 and 800 $CD8^+$ T cells/μl). In such cases, the low frequency of anti-Tax $CD8^+$ T cells is likely to be Ag-specific immune dysfunction rather than general immune dysfunction. The low frequency of anti-Tax $CD8^+$ T cells in ATL patients may be involved in progression from HTLV-1 carrier to ATL, and also contribute to the aggressiveness of this disease, which is refractory to treatment.

The present study demonstrated that HTLV-1 Tax tetramer$^+CD8^+$ T cells in AC and ATL patients produce intracellular IFN-γ, and possess perforin and granzyme B, which are molecular markers of functional CTL in response to the corresponding peptides. Interestingly, perforin and granzyme B expressions in Tax-specific tetramer-positive cells are significantly lower in ATL than AC, but there was no difference in CMV-specific tetramer-positive cells. These findings suggest that reduction of Tax-specific tetramer positive cells in not only frequency and diversity but also the function work as risk for ATL development. In contrast, the present study demonstrated that the reduction in CMV-specific tetramer-positive cells CTL is observed only in the frequency but not in the function. Therefore, frequent CMV infection during clinical course of ATL patient may be caused by this mechanism. In contrast, the present study demonstrated that the two functional molecules expression is reduced in anti-Tax CTL but not in anti-CMV CTL in either AC subjects or ATL patients. Although the mechanism why such differential regulation of CTL function with Ag specificity works in AC or ATL is unclear at present, these findings suggest that dysfunction of anti-Tax CTL in the present study reflect Ag specificity but not general immune function.

In conclusion, our HTLV-1/HLA tetramer assay enabled analysis of anti-HTLV-1 $CD8^+$ T cells in PBMC of AC and ATL patients and demonstrated deletion of anti-Tax $CD8^+$ T cells in ATL patients. Intracellular cytokine expression in anti-HTLV-1 $CD8^+$ T cells had significant difference between AC and ATL, but not in anti-CMV $CD8^+$ T cells. The reduced frequency, diversity, and function of anti-HTLV-1 Tax $CD8^+$ T cell clones may be related to the development of ATL. This HLA tetramer assay can be used for monitoring the in vivo status of CTL, and it may be possible to identify the high risk group in AC of developing to ATL. Furthermore, the successful expansion of anti-Tax CTL clones in the present study may facilitate the development of novel approaches for immunoadaptive therapy against ATL.

Acknowledgments

We thank Drs. Atsuo Ozaki, Hideaki Kawada, Makoto Yoshimitsu, and Noriko Aoki for collection of clinical samples, Dr. Koriyama for statistical analysis, Dr. Shimada and Ms. Ariyoshi for HLA typing, and Drs. J. Goedert and M. Hisada for critical reading of this manuscript.

Disclosures

The authors have no financial conflict of interest.

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