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Increased Expression of Human T Lymphocyte Virus Type I (HTLV-I) Tax11-19 Peptide–Human Histocompatibility Leukocyte Antigen A*201 Complexes on $CD4^+$ $CD25^+$ T Cells Detected by Peptide-specific, Major Histocompatibility Complex–restricted Antibodies in Patients with HTLV-I–associated Neurologic Disease

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Abstract

Human T lymphocyte virus type I (HTLV-I)–associated chronic inflammatory neurological disease (HTLV-I–associated myelopathy/tropical spastic paraparesis [HAM/TSP]) is suggested to be an immunopathologically mediated disorder characterized by large numbers of HTLV-I Tax–specific $CD8^+$ T cells. The frequency of these cells in the peripheral blood and cerebrospinal fluid is proportional to the amount of HTLV-I proviral load and the levels of HTLV-I *tax* mRNA expression. As the stimulus for these virus-specific T cells are immunodominant peptide–human histocompatibility leukocyte antigen (HLA) complexes expressed on antigen-presenting cells, it was of interest to determine which cells express these complexes and at what frequency. However, until now, it has not been possible to identify and/or quantify these peptide–HLA complexes. Using a recently developed antibody that specifically recognizes Tax11-19 peptide–HLA-A*201 complexes, the level of Tax11-19–HLA-A*201 expression on T cells was demonstrated to be increased in HAM/TSP and correlated with HTLV-I proviral DNA load, HTLV-I *tax* mRNA load, and HTLV-I Tax–specific $CD8^+$ T cell frequencies. Furthermore, $CD4^+$ $CD25^+$ T cells were demonstrated to be the major reservoir of HTLV-I provirus as well as Tax11-19 peptide–HLA-A*201 complexes. These results indicate that the increased detection and visualization of peptide–HLA complexes in HAM/TSP $CD4^+$ $CD25^+$ T cell subsets that are shown to stimulate and expand HTLV-I Tax–specific $CD8^+$ T cells may play an important role in the pathogenesis of HTLV-I–associated neurological disease.

Key words: HAM/TSP • IL-2Rα chain • regulatory T cell • tetramer • quantitative PCR

Introduction

The HTLV-I is an exogenous human retrovirus that infects ~10–20 million people worldwide (1). The majority of infected individuals remain healthy lifelong asymptomatic carriers while ~0.25–3% develop an inflammatory disease of the central nervous system termed HTLV-I–associated myelopathy/tropical spastic paraparesis (HAM/TSP; 2–5). HTLV-I is also the etiologic agent in adult T cell leukemia and other inflammatory diseases including uveitis, arthritis, polymyositis, Sjögren syndrome, and alveolitis (6).

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Abbreviation used in this paper: HAM/TSP, HTLV-I–associated myelopathy/tropical spastic paraparesis.

In patients with HAM/TSP, increased HTLV-I provirus load and augmented immune responses to HTLV-I have been reported. One of the most striking features of the cellular immune response in HAM/TSP patients is the highly increased numbers of HTLV-I–specific CTLs, which are lower or absent in asymptomatic carriers (7–11). CTL activity is predominantly restricted to the HTLV-I Tax protein, where in HLA-A*201 patients the HTLV-I Tax11-19 peptide (LLFGYPVYV) is defined as an immunodominant epitope (12, 13). In HAM/TSP patients these CTLs have been shown to produce IFN-γ and TNF-α that are proinflammatory and neurotoxic cytokines (9, 14). Moreover, in some HLA-A*201 HAM/TSP patients, the frequency of Tax11-19–specific CTLs can be as high as 30% of all $CD8^+$ T cells in peripheral blood (11) and even higher in cerebrospinal fluid (8, 15, 16). Neuropathological findings in HAM/TSP have demonstrated that $CD8^+$ T cells were frequently observed in active chronic lesions (17–19). Collectively, these findings support the view that HTLV-I Tax–specific CTLs play a central role in the immunopathogenesis of HAM/TSP.

Therefore, it was of interest to address which cells are stimulating the expansion of these pathogenic HTLV-I–specific $CD8^+$ CTLs in vivo in HAM/TSP patients. It has been reported that HTLV-I infects both memory ($CD45RO^+$) $CD4^+$ T cells (20) and effector/memory ($CD27^-$ $CD45RA^-$) $CD4^+$ T cells in vivo (21), although recent work indicated that $CD8^+$ T cells were also an in vivo cellular reservoir for HTLV-I (22, 23). Because a significant positive correlation between HTLV-I proviral load and the proportion of $CD4^+$ $CD25^+$ T cells has been demonstrated in PBMCs of HTLV-I–infected individuals (24), it has been hypothesized that $CD4^+$ $CD25^+$ T cells may also be infected with HTLV-I (25).

Virus-infected cells are known to present viral peptides on HLA class I molecules that are recognized by virus-specific $CD8^+$ T cells through their antigen-specific TCR (26). Because the frequency of HTLV-I Tax–specific $CD8^+$ CTLs in peripheral blood is proportional to the amount of HTLV-I proviral DNA load and the levels of HTLV-I *tax* mRNA expression (10, 11, 27), it has been suggested that the stimulus for these HTLV-I Tax–specific $CD8^+$ CTLs are immunodominant Tax peptide–HLA complexes expressed on autologous HTLV-I–infected T cells. However, because there has never been a direct way to analyze such peptide–HLA complexes, it is unclear as to which subset of T cells dominantly express the HTLV-I Tax peptide–HLA complexes and at what frequency in HAM/TSP. Recently, using a large human Ab phage display library, novel human recombinant Fab Abs have been isolated that specifically bind to a particular peptide–HLA-A*201 complex like TCR (28, 29). The use of these Abs allowed, for the first time, the identification and/or quantification of peptide–HLA complexes on APCs. Using these newly described peptide-specific, MHC-restricted Abs, which specifically bind to Tax11-19 peptide–HLA-A*201 complexes (TaxA2-Ab; 29), we measured the level of Tax11-19 peptide–HLA-A*201 complexes in peripheral blood T cells from HAM/TSP patients. We analyzed which subset of T cells are predominantly expressing these complexes and correlated the HTLV-I proviral DNA load of different T cell subsets using the quantitative PCR technique. In addition, we tested the capability of the Tax11-19 peptide–HLA-A*201–expressing HTLV-I–infected T cells to stimulate the proliferation of HTLV-I–specific $CD8^+$ T cells. The data presented here demonstrate that $CD4^+$ $CD25^+$ T cells are the major reservoir of HTLV-I provirus and predominantly express HTLV-I *tax* mRNA as well as HTLV-I Tax peptide–HLA complexes that stimulate and expand HTLV-I Tax–specific $CD8^+$ T cells. To our knowledge, this is the first report that directly measured peptide–HLA complexes ex vivo using these unique peptide-specific, MHC-restricted Fab Abs.

Materials and Methods

Subjects and Cell Preparation. We used Ficoll-Hypaque (BioWhittaker) centrifugation to separate PBMCs from six HLA-A*201^+ HAM/TSP patients (HAM 1-6) and six HLA-A*201^+ HTLV-I–seronegative healthy donors (A2HD). HAM/TSP was diagnosed according to the World Health Organization's guidelines. Blood samples were obtained after informed consent as part of a clinical protocol reviewed and approved by the National Institutes of Health institutional review panel. HTLV-I infection was confirmed by ELISA (Abbot Laboratories) and Western blot analysis (Genelabs). $CD4^+$ T cells or $CD8^+$ T cells were negatively selected from PBMCs with magnetic beads (Miltenyi Biotec), and $CD25^+$ and $CD25^-$ T cells or $CD45RO^+$ and $CD45RO^-$ T cells were separated from the selected $CD4^+$ or $CD8^+$ T cells using magnetic beads (Miltenyi Biotec).

Flow Cytometric Analysis. The immortalized B cell line transfected with a full-length HLA-A*201 cDNA (HmyA2.1; reference 30) was used to determine the reactivity of TaxA2-Ab and a peptide-specific, MHC-restricted Ab specific for the melanoma gp100 G9-154 peptide–HLA-A*201 complex (MelanomaA2-Ab) with cell surface–expressed peptide–HLA-A*201 complexes. HmyA2.1 cells were pulsed with Tax11-19 peptide (LLFGYPVYV) or melanoma gp100 G9-154 peptide (KTWGQYWQV) at the indicated concentrations for 1 h at 37°C in DMEM medium (GIBCO BRL) supplemented with 10% fetal bovine serum (Atlanta Biological), 2 mM L-glutamine, 40 U/ml penicillin, 40 μg/ml streptomycin (all from BioWhittaker). The cells were then washed once to remove any free peptide and stained with 1 μg PE-labeled TaxA2-Ab or MelanomaA2-Ab for 1 h at 4°C, and then washed once before analysis. PBMCs from A2HD and HAM/TSP patients were also analyzed using the same procedure. For peptide pulse analysis of peripheral blood T cells, purified $CD4^+$ or $CD8^+$ T cells were incubated with peptides at the indicated concentrations for 1 h at 37°C in RPMI medium (GIBCO BRL) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 40 U/ml penicillin, 40 μg/ml streptomycin. The cells were stained with 1 μg PE-labeled TaxA2-Ab or MelanomaA2-Ab for 1 h, and then stained with Tricolor-labeled monoclonal Ab against CD4 and allophycocyanin-labeled monoclonal Ab to CD8 (all from Caltag) for the last 30 min at 4°C. For analyzing the level of HLA-A*201 expression, $CD4^+$ T cells from A2HD and HAM/TSP patients were stained with 1 μg anti–HLA-A*201 Ab (BB7.2; reference 31) for 30 min at 4°C. After washing, the cells

were stained with PE-labeled anti–mouse IgG1Ab (Southern Biotechnology Associates, Inc.) for 30 min at 4°C. For phenotypic analysis, purified $CD4^+$ T cells by magnetic beads from HAM/TSP patients were cultured for 24 h at 37°C in RPMI medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 40 U/ml penicillin, 40 μg/ml streptomycin. The cells were then stained with 1 μg PE-labeled TaxA2-Ab for 1 h, and stained with FITC-labeled monoclonal Ab against CD25, CD45RO, CD45RA (all from Caltag), or CD27 (BD Biosciences) for the last 30 min at 4°C. All cells were washed twice before analysis on a FACSCalibur™ (Becton Dickinson). Data were analyzed with CELLQuest™ software (Becton Dickinson).

Proliferation Assay. $CD4^+$ $CD25^+$ and $CD4^+$ $CD25^-$ cells were separated from HAM/TSP patients and cultured for 24 h, and the cells were irradiated (3,000 rad). 10^5 autologous $CD8^+$ T cells were then mixed with 10^5 of the irradiated $CD4^+$ $CD25^+$ or $CD4^+$ $CD25^-$ cells and cultured for 4 d in round-bottom, 96-well culture plates. Incorporation of [^{3}H]thymidine (1 μCi/well) by proliferating cells was measured during the last 16 h of culture. In addition, proliferation of HTLV-I Tax–specific T cells and CMV pp65–specific T cells was analyzed using flow cytometry. The cultured cells were stained with PE-labeled Tax11-19 peptide–loaded HLA-A*201 tetramer (provided by National Institutes of Health AIDS Research and Reference Reagent Program) or CMV pp65 peptide–loaded HLA-A*201 tetramer (Beckman Coulter) for 30 min at 4°C. The cells were stained with Tricolor-labeled anti-CD4 Ab and APC-labeled anti-CD8 Ab (Caltag) for the last 15 min at 4°C. The cells were then fixed and permeabilized with Cytofix/CytoPerm kit (BD PharMingen), and stained with FITC-conjugated anti-Ki67 Ab (BD Biosciences). All cells were washed twice before analysis on a FACSCalibur™ (Becton Dickinson).

Real-Time PCR and Real-Time RT-PCR. HTLV-I proviral DNA load and HTLV-I *tax* mRNA load were measured using an ABI PRISM 7700 Sequence Detector (Applied Biosystems) as previously described (10, 11). DNA was extracted using Puregene DNA Isolation Kit (Gentra) and 100 ng sample DNA solution per well was analyzed. The HTLV-I proviral DNA load was calculated by the following formula: copy number of HTLV-I (*pX*) per 100 cells = (copy number of *pX*) / (copy number of β actin/2) × 100. RNA was extracted using RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. cDNA was synthesized using TaqMan Gold RT-PCR Kit (Applied Biosystems). Sample cDNA from 300 ng RNA per well was applied and analyzed. We used the human housekeeping gene hypoxanthine ribosyl transferase (HPRT) primers and probe set (Applied Biosystems) for internal calibration. The relative HTLV-I *tax* mRNA load was calculated by the following formula: HTLV-I *tax* mRNA load = (value of *tax*) / (value of HPRT) × 10,000.

Results

*Specificity and Sensitivity of Anti–Tax11-19 Peptide–HLA-A*201 Ab.* To demonstrate the specificity of peptide-specific, MHC-restricted Fab Abs that bind to Tax11-19 peptide–HLA-A*201 complexes (TaxA2-Ab), a human immortalized B cell line expressing HLA-A*201 (HmyA2.1) was pulsed with 10 μM HTLV-I Tax11-19 peptide or control melanoma gp100–derived G9-154 peptides, and incubated with PE-labeled TaxA2-Ab. The ability of TaxA2-Ab to bind to Tax11-19 peptide–HLA-A*201 molecule was then monitored by flow cytometry. As shown in Fig. 1 A, TaxA2-Ab reacted only with Tax11-19 peptide–loaded

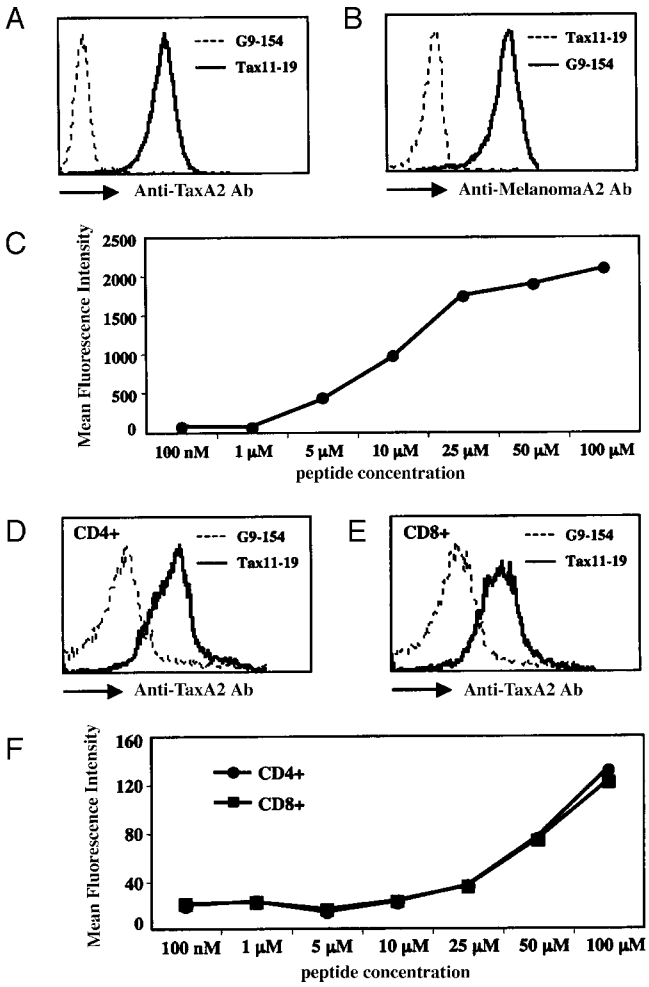


Figure 1. Peptide-specific, HLA-restricted binding of Ab. (A) Peptide-specific binding of anti–Tax11-19 peptide–HLA-A*201 Ab (TaxA2-Ab) on HmyA2.1 cells. HmyA2.1 cells were pulsed with HTLV-I Tax11-19 peptide or control melanoma gp100–derived G9-154 peptides, incubated with PE-labeled TaxA2-Ab, and then analyzed by flow cytometry. TaxA2-Ab reacted only with Tax11-19 peptide–loaded HmyA2.1 cells (solid line), but not with cells loaded with the control peptide (dotted line). (B) Peptide-specific binding of anti–melanoma gp100 G9-154 peptide–HLA-A*201 Ab (MelanomaA2-Ab) on HmyA2.1 cells. HmyA2.1 cells were pulsed with melanoma gp100–derived G9-154 peptides or control HTLV-I Tax11-19 peptide, incubated with PE-labeled MelanomaA2-Ab, and then analyzed by flow cytometry. MelanomaA2-Ab reacted only with G9-154 peptide–loaded HmyA2.1 cells (solid line), but not with cells loaded with the control peptide (dotted line). (C) Peptide titration study of TaxA2-Ab staining on HmyA2.1 cells. HmyA2.1 cells were pulsed with the indicated concentration of HTLV-I Tax11-19 peptide, incubated with PE-labeled TaxA2-Ab, and then analyzed by flow cytometry. The levels of Tax11-19 peptide–HLA-A*201 complexes are expressed by mean fluorescence intensity. (D and E) Peptide-specific binding of TaxA2-Ab on $CD4^+$ T cells (D) and $CD8^+$ T cells (E) from an HLA-A*201^+ healthy donor (A2HD). $CD4^+$ and $CD8^+$ T cells from A2HD were pulsed with 100 μM HTLV-I Tax11-19 peptide or control melanoma gp100–derived G9-154 peptides, incubated with PE-labeled TaxA2-Ab, and then analyzed by flow cytometry. TaxA2-Ab reacted only with Tax11-19 peptide–loaded HmyA2.1 cells (solid line), but not with cells loaded with the control peptide (dotted line). (F) Peptide titration study of TaxA2-Ab staining on $CD4^+$ and $CD8^+$ T cells from A2HD. $CD4^+$ (●) and $CD8^+$ (■) T cells from A2HD were pulsed with the indicated concentration of HTLV-I Tax11-19 peptide, incubated with PE-labeled TaxA2-Ab, and then analyzed by flow cytometry. The levels of Tax11-19 peptide–HLA-A*201 complexes are expressed by mean fluorescence intensity.

HmyA2.1 cells, but not with cells loaded with the control peptide. Conversely, a peptide-specific, MHC-restricted Fab Ab specific for the melanoma gp100 G9-154 peptide–HLA-A*201 complex (MelanomaA2-Ab) specifically bound HmyA2.1 cells pulsed with the melanoma gp100 peptide, but not Tax11-19 (Fig. 1 B). Peptide titration studies demonstrated that the level of TaxA2-Ab staining on HmyA2.1 cells correlated with the concentration of loaded Tax11-19 peptide (Fig. 1 C).

As these peptide-specific, MHC-restricted Fab Abs have never been used for the staining of ex vivo human T lymphocyte samples, we validated the ability of these Abs to detect peptide–HLA complexes using ex vivo T cells from HLA-A*201 HTLV-I–seronegative healthy donors (A2HD) pulsed with Tax11-19 peptide. $CD4^+$ and $CD8^+$ T cells were purified using magnetic beads from A2HD PBMCs, pulsed with or without Tax11-19 peptide, and stained with TaxA2-Ab followed by flow cytometry analysis. As shown

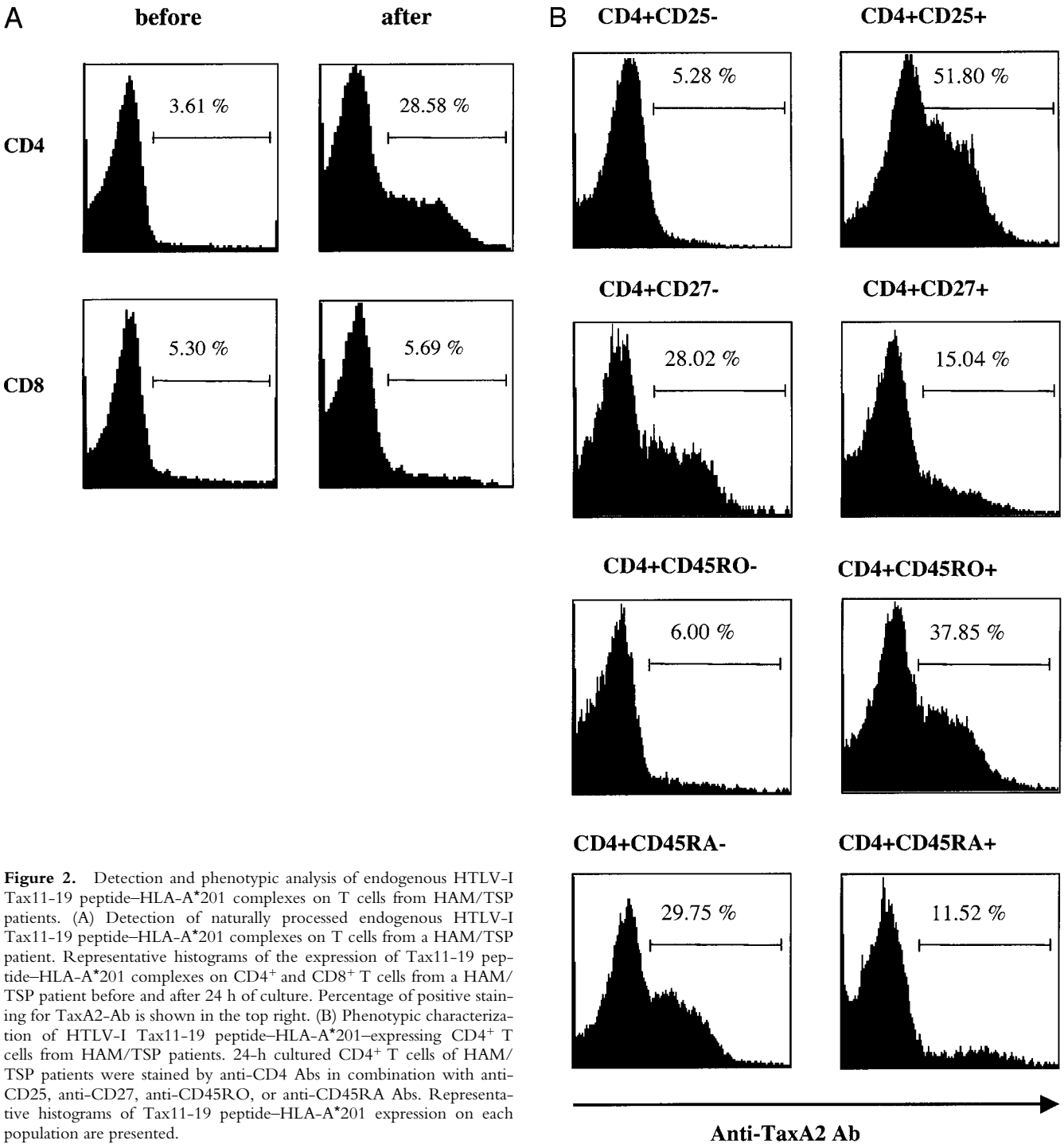


Figure 2. Detection and phenotypic analysis of endogenous HTLV-I Tax11-19 peptide–HLA-A*201 complexes on T cells from HAM/TSP patients. (A) Detection of naturally processed endogenous HTLV-I Tax11-19 peptide–HLA-A*201 complexes on T cells from a HAM/TSP patient. Representative histograms of the expression of Tax11-19 peptide–HLA-A*201 complexes on $CD4^+$ and $CD8^+$ T cells from a HAM/TSP patient before and after 24 h of culture. Percentage of positive staining for TaxA2-Ab is shown in the top right. (B) Phenotypic characterization of HTLV-I Tax11-19 peptide–HLA-A*201–expressing $CD4^+$ T cells from HAM/TSP patients. 24-h cultured $CD4^+$ T cells of HAM/TSP patients were stained by anti-CD4 Abs in combination with anti-CD25, anti-CD27, anti-CD45RO, or anti-CD45RA Abs. Representative histograms of Tax11-19 peptide–HLA-A*201 expression on each population are presented.

in Fig. 1, D and E, TaxA2-Ab could detect the Tax11-19 peptide–HLA-A*201 complexes on A2HD $CD4^+$ T cells (Fig. 1 D) as well as on $CD8^+$ T cells (Fig. 1 E) when pulsed with 100 μM Tax11-19 peptide, but not with control G9-154 peptide. The binding of TaxA2-Ab to Tax11-19 peptide pulsed A2HD T cells were also peptide dose dependent (Fig. 1 F).

*Detection of Tax11-19 Peptide–HLA-A*201 Complexes on T Cells from HAM/TSP Patients.* Using this TaxA2-Ab, the expression of Tax11-19 peptide–HLA-A*201 complexes was analyzed on T cells from HAM/TSP patients. It has been reported that HTLV-I infects both $CD4^+$ and $CD8^+$ T cells (20, 22). Although HTLV-I antigen expression ex vivo is negligible, infected cells can start to express HTLV-I antigen after short-term culture (32). Therefore, $CD4^+$ and $CD8^+$ T cells were separated using MACS beads and the expression of Tax11-19 peptide–HLA-A*201 complexes on each T cell subset before and after culture was investigated using the TaxA2-Ab. Before culture, the Tax11-19 peptide–HLA-A*201 complexes could not be detected both on ex vivo $CD4^+$ and $CD8^+$ T cells (Fig. 2 A). However, after 24 h of culture, the expression of Tax11-19 peptide–HLA-A*201 complexes was demonstrated on $CD4^+$ T cells, but not on $CD8^+$ T cells (Fig. 2 A). These results indicate that the peptide-specific, MHC-restricted Fab Abs are capable of detecting the specific peptide–HLA complexes after natural endogenous intracellular antigen processing as previously reported (29), and in HAM/TSP patients $CD4^+$ T cells present more Tax11-19 peptide–HLA-A*201 complexes than $CD8^+$ T cells.

As there are no direct methods for the enumeration and phenotyping of individual cells bearing physiological levels of peptide–HLA complexes in mixed cell populations, the peptide-specific, MHC-restricted Fab Abs are ideally suited to conduct such an analysis. Therefore, to investigate which subset of $CD4^+$ T cells preferentially express endogenous Tax11-19 peptide–HLA-A*201 complexes, the TaxA2-Ab staining on 24-h cultured $CD4^+$ T cells was analyzed in combination with flow cytometric staining of cell surface antigens (CD25, CD27, CD45RO, and CD45RA). As shown in Fig. 2 B, Tax11-19 peptide–HLA-A*201 complexes were preferentially expressed on $CD25^+$, $CD27^-$, $CD45RO^+$, $CD45RA^-$ cells (i.e., phenotypically defining an activated, effector/memory population). Among these cell surface markers, CD25 was shown to be the best marker to discriminate Tax11-19 peptide–HLA-A*201 complex–positive cells from –negative cells. Identical results were obtained from all six HAM/TSP patients tested (not depicted).

$CD4^+$ $CD25^+$ T Cells Are the Main Reservoir for HTLV-I and Express Virus. Increased detection of Tax11-19 peptide–HLA-A*201 complexes on $CD4^+$ $CD25^+$ T cells from HAM/TSP patients suggested two possibilities: HTLV-I preferentially infects $CD4^+$ $CD25^+$ T cells, or HTLV-I–infected T cells expressed CD25 during short-term culture as previously reported (33). Although these two possibilities are not mutually exclusive, to address whether $CD4^+$ $CD25^+$ T cells are preferentially infected with HTLV-I, we separated peripheral blood T cells from HAM/TSP patients into $CD4^+$ $CD25^+$, $CD4^+$ $CD25^-$, $CD4^+$ $CD45RO^+$, $CD4^+$ $CD45RO^-$, $CD8^+$ $CD25^+$, $CD8^+$ $CD25^-$ T cells using MACS beads, and measured HTLV-I proviral DNA load within each population using a real-time quantitative PCR method (TaqMan). As shown in Fig. 3 A (means of six HAM/TSP patients), HTLV-I proviral DNA was highest in $CD4^+$ $CD25^+$ T cells, although HTLV-I also showed a tropism for memory $CD4^+$ T cells ($CD4^+$ $CD45RO^+$) and is consistent with a previous report (20). This preferen-

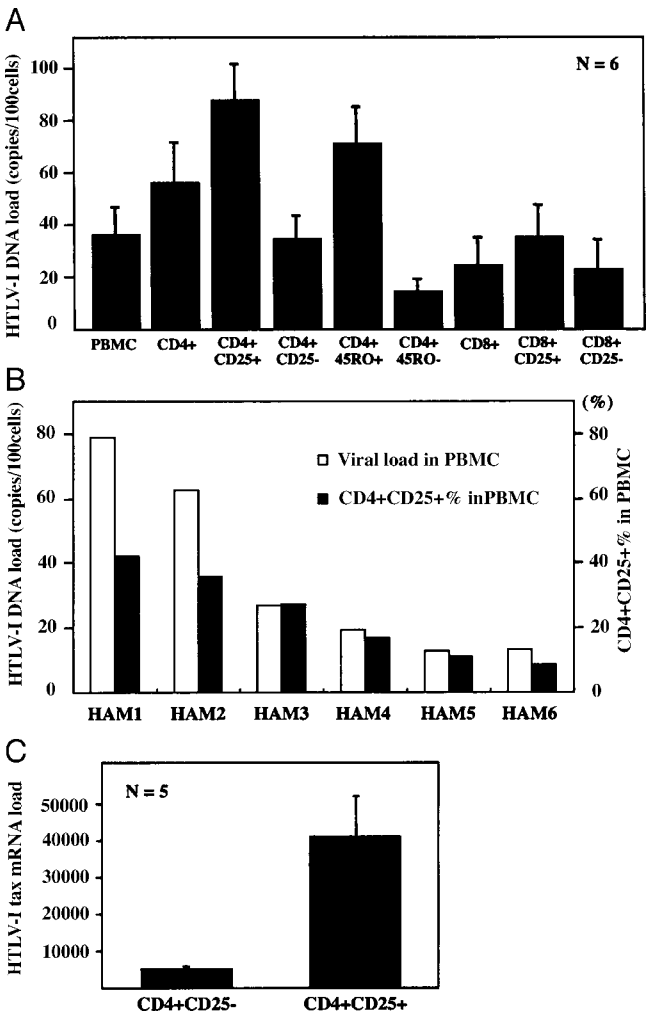
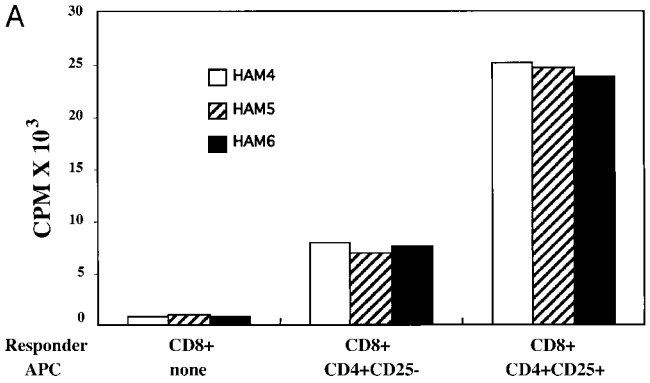


Figure 3. HTLV-I tropism and preferential virus expression in $CD4^+$ $CD25^+$ T cells in HAM/TSP patients. (A) HTLV-I tropism in $CD4^+$ $CD25^+$ T cells in HAM/TSP patients. HTLV-I proviral DNA load of $CD4^+$ $CD25^+$, $CD4^+$ $CD25^-$, $CD4^+$ $CD45RO^+$, $CD4^+$ $CD45RO^-$, $CD8^+$ $CD25^+$, $CD8^+$ $CD25^-$ T cells were assessed using quantitative PCR method in six HAM/TSP patients. Data are mean ± SE. (B) HTLV-I virus load and the percentages of $CD4^+$ $CD25^+$ T cells in PBMCs of HAM/TSP patients. HTLV-I proviral DNA load in PBMCs and the percentages of $CD4^+$ $CD25^+$ T cells in PBMCs of six HAM/TSP patients were plotted. (C) Increased HTLV-I *tax* mRNA expression of $CD4^+$ $CD25^+$ than $CD4^+$ $CD25^-$ T cells in HAM/TSP patients. HTLV-I *tax* mRNA load of 24-h cultured $CD4^+$ $CD25^+$ and $CD4^+$ $CD25^-$ T cells were assessed using quantitative RT-PCR method in five HAM/TSP patients. Data are mean ± SE.

tial infection for $CD25^+$ population was also observed in $CD8^+$ T cells (Fig. 3 A).

These results directly demonstrated that $CD4^+$ $CD25^+$ T cells are an important virus reservoir. To investigate how much of the virus load of PBMCs was accounted for by $CD4^+$ $CD25^+$ T cells, HTLV-I proviral DNA load in PBMCs and the percentage of $CD4^+$ $CD25^+$ T cells in PBMCs in each HAM/TSP patient were analyzed (Fig. 3 B). Surprisingly, virus load and percentage of $CD4^+$ $CD25^+$ T cells were similar in HAM/TSP patients with medium to low virus load (HAM 3–6). Furthermore, in the patients with high virus load (HAM 1 and 2), the number of virus-infected cells (virus load) was demonstrated to be higher than the percentage of $CD4^+$ $CD25^+$ T cells, suggesting that other T cell populations were also infected with HTLV-I in the patients with high virus load. As previously reported (24), a positive correlation between HTLV-I proviral load and the proportion of $CD4^+$ $CD25^+$ T cells has also been observed.

Although both $CD4^+$ $CD25^+$ and $CD4^+$ $CD25^-$ T cells were infected with HTLV-I (Fig. 3 A), it was shown that $CD4^+$ $CD25^+$ T cells preferentially express Tax11-19 peptide–HLA-A*201 complexes (Fig. 2 B), suggesting that HTLV-I–infected $CD4^+$ $CD25^+$ T cells inherently express virus relative to HTLV-I–infected $CD4^+$ $CD25^-$ cells. To test this possibility, $CD4^+$ $CD25^+$ and $CD4^+$ $CD25^-$ T cells were isolated from peripheral blood of five HAM/TSP patients, cultured for 24 h, and the level of HTLV-I



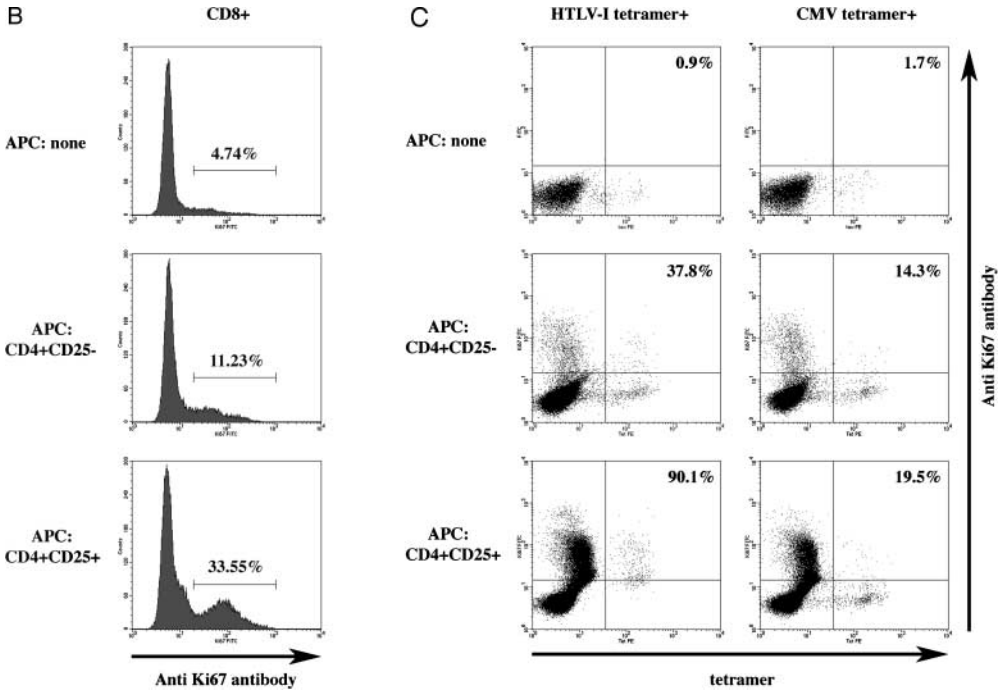


Figure 4. Proliferation of HTLV-I Tax–specific $CD8^+$ T cells are stimulated by $CD4^+$ $CD25^+$ T cells in HAM/TSP patients. (A) Proliferation of $CD8^+$ T cells stimulated by autologous $CD4^+$ $CD25^+$ T cells in HAM/TSP patients. Purified $CD4^+$ $CD25^+$ and $CD4^+$ $CD25^-$ T cells from three HAM/TSP patients (HAM 4–6; HTLV-I Tax11-19–HLA-A*201 tetramer–specific T cell frequency in total CD8 was 13.24, 5.70, and 1.78%, respectively) were cultured for 24 h and irradiated. Autologous $CD8^+$ T cells were then incubated with the $CD4^+$ $CD25^+$ or $CD4^+$ $CD25^-$ T cells for 4 d and proliferation of $CD8^+$ T cells was assessed by the incorporation of [^{3}H]thymidine (1 μCi/well) during the last 16 h of culture. In these experiments, background counts in the wells containing APCs were <300 cpm. (B) Proliferation of $CD8^+$ T cells stimulated by autologous $CD4^+$ $CD25^+$ T cells in a HAM/TSP patient. Histograms of intracellular Ki-67 staining in $CD8^+$ T cells when stimulated by irradiated, cultured, autologous $CD4^+$ $CD25^+$ or $CD4^+$ $CD25^-$ T cells are presented. Percentage of positive staining for Ki-67 is shown in the center. (C) Proliferation of HTLV-I Tax11-19–specific T cells stimulated by autologous $CD4^+$ $CD25^+$ T cells in a HAM/TSP patient. Dot plots of intracellular Ki-67 staining in $CD8^+$ T cells, Tax11-19–HLA-A*201 tetramer$^+$ $CD8^+$ T cells, and CMV pp65–HLA-A*201 tetramer$^+$ $CD8^+$ T cells when stimulated by irradiated, cultured, autologous $CD4^+$ $CD25^+$ or $CD4^+$ $CD25^-$ T cells are presented. Percentage of positive staining for Ki-67 is shown in the top right.

tax mRNA expression in each population was compared using real-time quantitative RT-PCR (Fig. 3 C). Interestingly, $CD4^+$ $CD25^+$ T cells expressed extremely high HTLV-I *tax* mRNA levels (approximately eight times) compared with $CD4^+$ $CD25^-$ T cells. These results are consistent with the observations that in HAM/TSP, $CD4^+$ $CD25^+$ T cells are the main reservoir for HTLV-I and express endogenous virus mRNA and Tax11-19 peptide–HLA-A*201 complexes.

$CD4^+$ $CD25^+$ T Cells Stimulate the Expansion of HTLV-I Tax–specific $CD8^+$ T Cells in HAM/TSP Patients. Having observed that $CD4^+$ $CD25^+$ T cells are preferentially infected with HTLV-I and express Tax11-19 peptide–HLA-A*201 complexes, we tested whether these cells can stimulate the proliferation of autologous $CD8^+$ T cells (and more specifically, HTLV-I–specific $CD8^+$ T cells) in HAM/TSP patients. Peripheral blood T cells from three HAM/TSP patients were separated into $CD4^+$ $CD25^+$ and $CD4^+$ $CD25^-$ cells and cultured for 24 h. After culture, both populations were irradiated. Autologous $CD8^+$ T cells were then added at a 1:1 ratio where proliferation of $CD8^+$ T cells was analyzed after 4 d of culture. As shown in Fig. 4 A, $CD4^+$ $CD25^+$ T cells from HAM/TSP patients preferentially stimulated the proliferation of autologous $CD8^+$ cells.

To determine whether proliferation of HTLV-I–specific $CD8^+$ T cells were MHC restricted and HTLV-I specific, intracellular Ki-67 staining combined with HTLV-I Tax11-19–HLA-A*201 tetramer or CMV pp65–HLA-A*201 tetramer staining was performed in a HAM/TSP patient who harbored detectable levels of both HTLV-I and CMV $tetramer^+$ $CD8^+$ T cells. As shown in Fig. 4 C, when HAM/TSP patient $CD8^+$ T cells were cultured with autologous $CD4^+$ $CD25^+$, the majority of HTLV-I Tax11-19–specific $CD8^+$ T cells (90.1%) was shown to be proliferating ($Ki67^+$). By comparison, stimulation with autologous $CD4^+$ $CD25^-$ cells resulted in the proliferation of only 37.4% of the HTLV-I Tax11-19–specific $CD8^+$ T cells. Surprisingly, CMV pp65–specific $CD8^+$ T cells showed a similar degree of proliferation when stimulated with autologous $CD4^+$ $CD25^+$ or $CD4^+$ $CD25^-$ T cells. These results directly demonstrate that HTLV-I–infected $CD4^+$ $CD25^+$ T cells can preferentially stimulate the expansion of HTLV-I–specific $CD8^+$ T cells through peptide–HLA complexes in HAM/TSP patients.

*Increased Detection of Tax11-19 Peptide–HLA-A*201 Complexes on $CD4^+$ T Cells in HAM/TSP Patients.* The inability to detect Tax11-19 peptide–HLA-A*201 complexes directly from ex vivo T cells of HAM/TSP patients (Fig. 2 A) could be reflective of a low concentration of Tax11-19 peptide bound to endogenous HLA-A*201 molecules. As we had shown that the staining intensity of the TaxA2-Ab was dependent on the concentration of the peptide used for pulsing T cells in A2HD (Fig. 1 F), it was of interest to determine whether this TaxA2-Ab staining of peptide-pulsed ex vivo T cells was different between A2HD and HLA-A*201 HAM/TSP patients. $CD4^+$ T cells were separated from ex vivo peripheral blood T cells of six A2HD and six HLA-A*201 HAM/TSP patients.

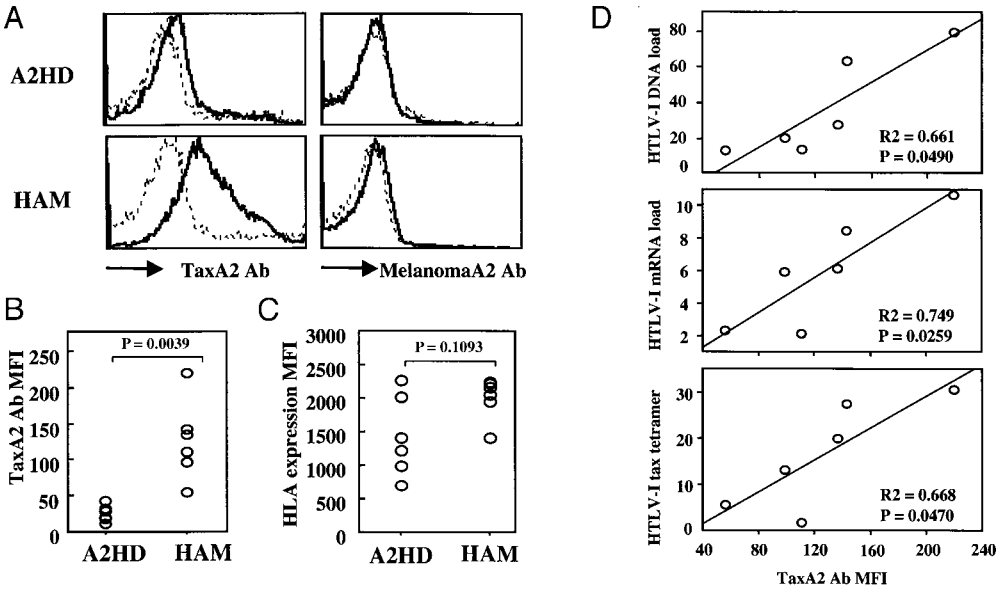


Figure 5. Increased detection of Tax11-19 peptide–HLA-A*201 complexes on $CD4^+$ T cells in HAM/TSP patients. (A) Representative histograms of increased sensitivity of $CD4^+$ T cells from HAM/TSP patients for TaxA2-Ab staining. Fluorescence intensity for TaxA2-Ab staining of $CD4^+$ T cells from a HAM/TSP patient (bottom left) was stronger than $CD4^+$ T cells from A2HD (top left) when pulsed with 10 μM Tax11-19 peptide (solid line) compared with 10 μM control melanoma gp100 G9-154 peptide (dotted line). However, fluorescence intensity for MelanomaA2-Ab staining of $CD4^+$ T cells from a HAM/TSP patient (bottom right) was similar to that of A2HD $CD4^+$ T cells (top right) when pulsed with 10 μM melanoma gp100 G9-154 peptide (solid line) compared with 10 μM control Tax11-19 peptide (dotted line). A2HD, HLA-A*201^+ healthy donor. (B) Fluorescence intensity for TaxA2-Ab staining of $CD4^+$ T cells from six HAM/TSP patients was significantly stronger ($P = 0.0039$) than $CD4^+$ T cells from six A2HD patients when pulsed with 10 μM Tax11-19 peptide. (C) No significant difference ($P = 0.1093$) of the fluorescence intensity of anti–HLA-A*201 staining between $CD4^+$ T cells from six HAM/TSP patients and $CD4^+$ T cells from six A2HD patients. (D) Correlation between the level of Tax11-19 peptide–HLA-A*201 expression and HTLV-I DNA load, RNA load, and HTLV-I Tax–specific $CD8^+$ T cell frequency. The levels of Tax11-19 peptide–HLA-A*201 expression on $CD4^+$ T cells were significantly correlated with HTLV-I proviral DNA load ($P = 0.0490$, $r^2 = 0.661$), HTLV-I mRNA load ($P = 0.0259$, $r^2 = 0.749$), and HTLV-I Tax11-19 tetramer–specific $CD8^+$ T cell frequencies ($P = 0.0470$, $r^2 = 0.668$) in six HAM/TSP patients.

These ex vivo T cells were pulsed at a concentration of 10 μM Tax11-19 peptide, previously determined to be in a range where the TaxA2-Ab was unable to detect Tax11-19 peptide–HLA-A*201 complexes (Fig. 1 F). In contrast to HTLV-I Tax11-19 peptide–pulsed A2HD T cells (Figs. 5 A and 1 F), staining intensity of the TaxA2-Ab on comparably peptide-pulsed HAM/TSP T cells was significantly higher (Fig. 5, A and B). To determine if this difference between A2HD and HAM/TSP patients was specific for the amount of Tax11-19 peptide–HLA-A*201 complexes as defined by the TaxA2-Ab, $CD4^+$ T cells from the same individuals were pulsed with the same concentration of control melanoma gp100 G9-154 peptide, and the level of G9-154 peptide–HLA-A*201 complexes was analyzed using the MelanomaA2-Ab. As shown in Fig. 5 A, staining intensity with the MelanomaA2-Ab in HAM/TSP patients was similar to that in A2HD. These observations were confirmed using a wider range of peptide concentrations (Fig. 6, A and B). As the staining sensitivity of the TaxA2-Ab might be affected by the amount of expressed HLA-A*201 complexes, we compared the amount of HLA-A*201 expression on $CD4^+$ T cells between A2HD and HAM/TSP using anti–HLA-A*201–specific Ab (BB7.2). There was no statistically significant difference in the amount of HLA-A*201 expression between A2HD and HAM/TSP (Fig. 5 C), suggesting that differential levels of HLA-A*201 expression do not contribute to the TaxA2-Ab staining intensity between A2HD and HAM/TSP patients. In addition, we were able to identify two HLA-A*0201 asymptomatic carriers from which sufficient cells were available to analyze with the TaxA2-Ab. TaxA2-Ab staining intensities on $CD4^+$ T cells of these two HLA-A*0201 asymptomatic carriers were similar with those on A2HD (unpublished data).

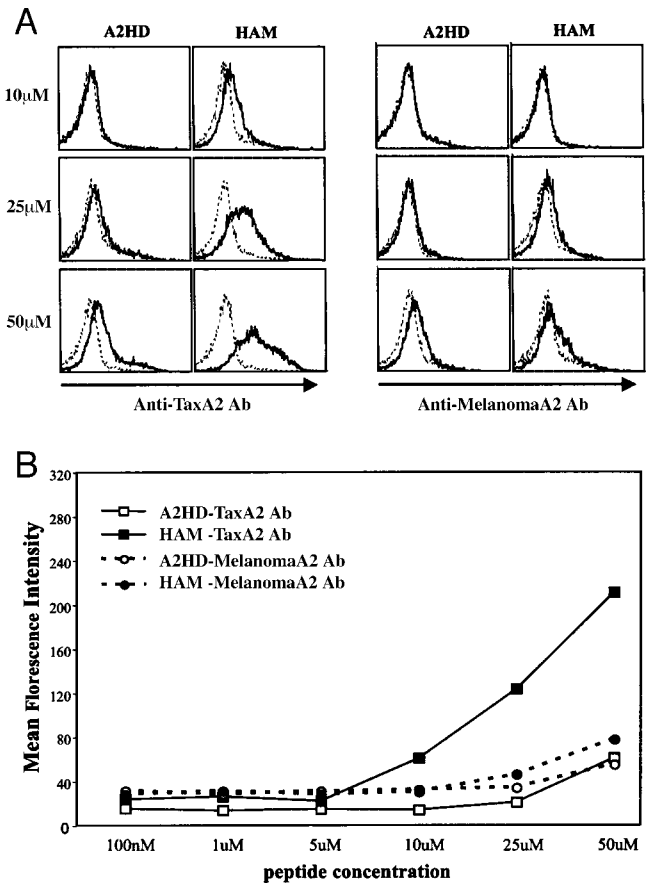


Figure 6. Peptide titration studies using T cells from HAM/TSP patients and A2HD. Peptide–HLA*A201-specific binding of TaxA2-Ab or MelanomaA2-Ab on T cells from HAM/TSP patients or A2HD. These cells were pulsed with various concentrations of HTLV-I Tax11-19 peptide or melanoma gp100–derived G9–154 peptides, incubated with PE-labeled TaxA2-Ab or MelanomaA2-Ab, and then analyzed by flow cytometry. (A) Representative histograms for TaxA2-Ab and MelanomaA2-Ab analysis. Fluorescence intensity for TaxA2-Ab staining of T cells from a HAM/TSP patient was significantly higher than T cells from A2HD when pulsed with Tax11-19 peptide at concentrations >10 μM (solid line). Melanoma gp100 G9-154 peptide was used as control peptide (dotted line). There was no change in fluorescence intensity between a HAM/TSP patient and A2HD when pulsed with melanoma gp100 G9-154 peptide (solid line) or Tax11-19 peptide (dotted line) and stained with MelanomaA2-Ab. (B) Mean fluorescence intensity of Tax11-19 peptide–HLA-A*201 complexes and Melanoma G9-154–HLA-A*201 peptide complexes from A and B. T cells from A2HD were pulsed with the indicated concentrations of HTLV-I Tax11-19 peptide and incubated with PE-labeled TaxA2-Ab (□), or pulsed with the indicated concentrations of melanoma gp100 G9-154 peptide and incubated with PE-labeled MelanomeA2-Ab (○). T cells from HAM/TSP patients were pulsed with the indicated concentrations of HTLV-I Tax11-19 peptide and incubated with PE-labeled TaxA2-Ab (■), or pulsed with the indicated concentrations of melanoma gp100 G9-154 peptide and incubated with PE-labeled MelanomeA2-Ab (●).

Because the level of staining intensity for Tax11-19 peptide–HLA-A*201 complexes was different between HAM/TSP patients, controls, and two HLA-A*0201 asymptomatic carriers, it was likely that increased detection of these complexes on ex vivo $CD4^+$ T cells from HAM/TSP patients might reflect the amount of endogenously processed Tax11-19 peptide. In support of this hypothesis, we determined if there was a correlation between the level of Tax11-19 peptide–HLA-A*201 complexes and HTLV-I proviral DNA load, HTLV-I *tax* mRNA load, and HTLV-I Tax11-19 peptide–specific CTL frequencies. As shown in Fig. 5 D, all three parameters significantly correlated with the level of expression of Tax11-19 peptide–HLA-A*201 complexes in HAM/TSP patients. However, the level of TaxA2-Ab staining did not correlate with the level of HLA-A*201 expression (unpublished data), again suggesting that the differential levels of Tax11-19 peptide–HLA-A*201 complex expression is not reflective of the amount of HLA expression. Collectively, these results suggest the existence of endogenously processed Tax11-19 peptide–HLA-A*201 complexes on ex vivo $CD4^+$ T cells from HAM/TSP patients, which are stimulating HTLV-I Tax11-19–specific $CD8^+$ T cells.

Discussion

Virus-specific T cells recognize antigens by engaging the antigen-specific TCR with peptide–HLA complexes displayed on the surface of APCs. A major advance in immu-

nology has been the description and use of polyvalent, soluble peptide–HLA complexes that specifically bind the TCR (tetramers; 34). Quantitative detection of antigen-specific T cell populations by such peptide–HLA tetramers has been useful for monitoring virus-specific T cell immunity in laboratory and clinical settings (35). However, there has been a tremendous shortage of reagents to study and visualize the ligand for the TCR, the peptide–HLA complex. Recently, by using large human Ab phage libraries, unique Abs with peptide-specific, HLA-restricted recognition pattern, termed peptide-specific, MHC-restricted Abs, have been isolated (28, 29). In this study we used these Abs, which specifically recognize HTLV-I Tax11-19 peptide–HLA-A*201 complexes (TaxA2-Ab), in analyzing ex vivo T cells from HLA-A*201 healthy individuals and HTLV-I–infected HAM/TSP patients. We have demonstrated specific binding of this Ab to exogenous peptide-pulsed as well as naturally processed endogenous HTLV-I Tax11-19 peptide–HLA-A*201 complexes on peripheral T cells from HAM/TSP patients, but not from HLA-A*201 healthy donors or two HLA-A*201 asymptomatic carriers. We feel this peptide-specific, MHC-restricted Ab will be useful in the analysis of other infectious agents, tumors, and autoantigens, and will further enhance our understanding of antigen–host cell immunological interactions.

This study also has defined the in vivo cellular tropism of HTLV-I using a reliable and accurate real-time quantitative PCR technique (TaqMan). HTLV-I was originally isolated from cultured $CD4^+$ T cells of a patient with cutaneous T cell lymphoma (36). Soon after, HTLV-III/lymphadenopathy–associated virus was identified (37) and subsequently renamed HIV-I. These retroviruses predominantly infect $CD4^+$ cells (38, 39), a seminal observation that directly led to defining CD4 as a receptor component for HIV-I (40, 41). Although the receptor for HTLV-I has not yet been identified, it must clearly be ubiquitous because HTLV-I infects a wide range of cells in vitro (42). Since 1990, HTLV-I has been thought to preferentially infect memory $CD4^+$ ($CD4^+$ $CD45RO^+$) T cells in vivo (20). A recently established real-time quantitative PCR technique (TaqMan) has demonstrated that $CD8^+$ T cells were also infected with HTLV-I (22). Because HTLV-I Tax has been known to transactivate the expression of IL-2Rα chain (CD25; 33), and a significant positive correlation between HTLV-I provirus load and the proportion of $CD4^+$ $CD25^+$ T cells has been demonstrated in PBMCs of HTLV-I–infected individuals (24), it has been hypothesized that $CD4^+$ $CD25^+$ T cells might be a reservoir for HTLV-I (25). This study, again using real-time quantitative PCR, supported this hypothesis but suggested two possibilities: HTLV-I preferentially infects $CD4^+$ $CD25^+$ T cells, or HTLV-I–infected T cells could induce CD25 in vivo. Although these possibilities are not mutually exclusive, the report that the HTLV-I receptor is an early T cell activation marker (43) may support the former possibility because CD25 is also a T cell activation marker, whereas a study demonstrating that HTLV-I Tax protein transactivates the promoters of IL-2 and CD25 (33) may support the latter.

Here we demonstrate that a high HTLV-I proviral load in $CD4^+$ $CD25^+$ T cells from HAM/TSP patients was associated with an increased ability to express HTLV-I mRNA and process HTLV-I Tax11-19–HLA-A*201 complexes on their cell surface. Of importance is the observation that HTLV-I–infected $CD4^+$ $CD25^+$ T cells were directly shown to stimulate the proliferation of HTLV-I–specific $CD8^+$ T cells where the level of Tax11-19–HLA-A*201 expression correlated with HTLV-I Tax–specific $CD8^+$ CTL frequency ex vivo. These increased antigen-presenting abilities of HTLV-I–infected $CD4^+$ $CD25^+$ T cells may also be associated with HTLV-I–induced overexpression of adhesion molecules and down-regulation of immunosuppressive cytokine as previously reported (44–46). These results support previous reports demonstrating that the frequency of HTLV-I Tax–specific $CD8^+$ CTLs in the peripheral blood is proportional to the amount of HTLV-I proviral DNA load and the levels of HTLV-I *tax* mRNA expression (10, 11, 27). These results suggest the existence of continuous in vivo HTLV-I antigen presentation, which may serve to drive the increased numbers of HTLV-I–specific $CD8^+$ CTLs in HAM/TSP patients. Moreover, the existence of such continuous HTLV-I antigen presentation in the presence of very high frequencies of HTLV-I–specific $CD8^+$ CTLs (as high as 30% of total $CD8^+$ cells in some patients), also supports the hypothesis that HTLV-I–specific CTLs might be insufficient to control persistent HTLV-I infection (47, 48).

Recently, $CD4^+$ $CD25^+$ T cells have been reported to include regulatory T cell populations that are engaged in the maintenance of immunologic self-tolerance by actively suppressing the activation and expansion of self-reactive T cells that may cause autoimmune disease (49, 50). This normal $CD4^+$ $CD25^+$ regulatory T cell population constitutes 5–10% of peripheral $CD4^+$ T cells in mice and humans (49, 50). Removal of this population from normal rodents leads to the spontaneous development of various autoimmune diseases (49, 50). Importantly, $CD4^+$ $CD25^+$ T cells have been reported to suppress the proliferation of $CD4^+$ $CD25^-$ T cells as well as $CD8^+$ T cells induced either by polyclonal or antigen-specific stimuli (49, 50). It has not escaped our attention that a proportion of HTLV-I–infected $CD4^+$ $CD25^+$ T cells in HAM/TSP may also be regulatory and infection with HTLV-I may alter function of these cell populations. It is clear from the results in this study that HTLV-I–infected $CD4^+$ $CD25^+$ cells are not suppressive but are stimulatory for HTLV-I Tax–specific proliferation of $CD8^+$ T cells. These results suggest that $CD4^+$ $CD25^+$ cells infected with HTLV-I may lack regulatory T cell function in HAM/TSP patients. This hypothesis is currently being examined.

In conclusion, using a recently developed peptide-specific, MHC-restricted Ab that specifically recognizes Tax11-19 peptide–HLA-A*201 complexes coupled with a real-time quantitative PCR methodology, $CD4^+$ $CD25^+$ T cells from HAM/TSP patients were demonstrated to be a

major reservoir of HTLV-I proviral DNA. These cells preferentially express HTLV-I mRNA and can present Tax11-19 peptide–HLA-A*201 complexes that can stimulate the proliferation of HTLV-I–specific $CD8^+$ T cells. Moreover, higher sensitivity to HTLV-I Tax11-19 peptide–pulsed $CD4^+$ T cells in HAM/TSP patients suggest the existence of endogenously processed Tax11-19 peptide–HLA-A*201 complexes on ex vivo $CD4^+$ T cells. This novel Ab demonstrated a significant correlation between HTLV-I DNA, RNA, peptide–HLA complexes, and HTLV-I–specific T cell immune responses. These results indicate that the increased detection of HTLV-I proviral DNA and Tax11-19 peptide–HLA-A*201 complexes in HAM/TSP $CD4^+$ $CD25^+$ T cell subsets may serve to stimulate and expand HTLV-I Tax–specific $CD8^+$ T cells that are important in the pathogenesis of HTLV-I–associated neurological disease.

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