

including autoimmune disorders and viral infection [25–29]. To more precisely analyze phenotypic changes in HTLV-I-infected cells, markers that indicate HTLV-I infection should be incorporated in future studies.

A summary of this study is shown in Figure 5. In the CD3 versus CD7 profile, most AC cases were included in Group 1, in which the D and L subpopulations were relatively small. Consistent with disease progression to smoldering- and chronic-type ATL, a decrease in the H subpopulation and increases in the D and L subpopulations occur (Group 2). In this step, increases in the sizes of clones in the D and L subpopulations are observed. Further expansion of the leukemic clone results in progression to acute-type ATL in which the L subpopulation has expanded (Group 3). According to a study by Yamaguchi *et al.*, the natural course of ATL is to progress from the HTLV-I carrier state through the intermediate state, smoldering ATL, and chronic ATL, and finally to the acute ATL, indicating a process of multistage leukemogenesis [19]. We consider this study to successfully link the progressive clinical status and phenotypic changes in HTLV-I-infected cells. However, the way in which this profile reflects multistep oncogenesis in HTLV-I infection at the molecular level remains unclear. Further molecular analyses of the three subpopulations will help in understanding the mechanism(s).

Supporting Information

Figure S1 Representative flow cytometric analysis of an HTLV-I asymptomatic carrier (patient no. 32). The CD3 versus CD7 plot of CD4⁺ cells was constructed according to the gating procedure shown in this figure. In the plot, we designated three subpopulations: H (CD3^{high}CD7^{high}), D (CD3^{dim}CD7^{dim}), and L (CD3^{dim}CD7^{low}). (PPTX)

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Figure S2 A two-dimensional plot of 10 normal controls showing the percentage of the D and L subpopulations. (PPTX)

Figure S3 Southern blot analysis of clonal integration of the HTLV-I provirus. Representative data (AC, No. 34) are shown. In *EcoRI* or *PstI* digestion, a band indicated by a red arrow represents the monoclonal integration of the provirus. The band pattern indicates that two major clones coexist. This analysis was performed by a commercial laboratory (SRL, Tokyo, Japan). (PPTX)

Table S1 Clinical data in a case of chronic-type ATL (No. 53). Proportion of abnormal lymphocytes in the peripheral blood WBC were evaluated by morphological examination. LDH: Lactate dehydrogenase (normal range, 120–240 U/L) sIL-2R: soluble interleukin-2 receptor (normal range, 122–496 U/ml). (XLSX)

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Author Contributions

Conceived and designed the experiments: KT AT KU. Performed the experiments: SK YT. Analyzed the data: EW NW TI NO. Contributed reagents/materials/analysis tools: MI MT KU NO. Wrote the paper: SK KU.

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CSF CXCL10, CXCL9, and Neopterin as Candidate Prognostic Biomarkers for HTLV-1-Associated Myelopathy/Tropical Spastic Paraparesis

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Abstract

Background: Human T-lymphotropic virus type 1 (HTLV-1)-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is a rare chronic neuroinflammatory disease. Since the disease course of HAM/TSP varies among patients, there is a dire need for biomarkers capable of predicting the rate of disease progression. However, there have been no studies to date that have compared the prognostic values of multiple potential biomarkers for HAM/TSP.

Methodology/Principal Findings: Peripheral blood and cerebrospinal fluid (CSF) samples from HAM/TSP patients and HTLV-1-infected control subjects were obtained and tested retrospectively for several potential biomarkers, including chemokines and other cytokines, and nine optimal candidates were selected based on receiver operating characteristic (ROC) analysis. Next, we evaluated the relationship between these candidates and the rate of disease progression in HAM/TSP patients, beginning with a first cohort of 30 patients (Training Set) and proceeding to a second cohort of 23 patients (Test Set). We defined "deteriorating HAM/TSP" as distinctly worsening function (≥ 3 grades on Osame's Motor Disability Score (OMDS)) over four years and "stable HAM/TSP" as unchanged or only slightly worsened function (1 grade on OMDS) over four years, and we compared the levels of the candidate biomarkers in patients divided into these two groups. The CSF levels of chemokine (C-X-C motif) ligand 10 (CXCL10), CXCL9, and neopterin were well-correlated with disease progression, better even than HTLV-1 proviral load in PBMCs. Importantly, these results were validated using the Test Set.

Conclusions/Significance: As the CSF levels of CXCL10, CXCL9, and neopterin were the most strongly correlated with rate of disease progression, they represent the most viable candidates for HAM/TSP prognostic biomarkers. The identification of effective prognostic biomarkers could lead to earlier detection of high-risk patients, more patient-specific treatment options, and more productive clinical trials.

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Introduction

Human T-lymphotropic virus type 1 (HTLV-1) is a human retrovirus associated with persistent infection of T-cells [1]. While the majority of HTLV-1-infected individuals remain asymptomatic, approximately 2.5–5% develop an aggressive T-cell malignancy, termed adult T-cell leukemia (ATL) [2,3] and 0.3–3.8% develop a serious chronic neuroinflammatory disease, termed HTLV-1-associated myelopathy/tropical spastic paraparesis

(HAM/TSP) [4–6]. Aside from Japan, endemic areas for this virus and the associated disorders are mostly located in developing countries in the Caribbean, South America, Africa, the Middle East, and Melanesia [7,8], which may explain why these conditions have remained ill-defined and virtually untreatable for so long [9].

HAM/TSP is characterized by unremitting myelopathic symptoms such as spastic paraparesis, lower limb sensory disturbance, and bladder/bowel dysfunction [10,11]. Although

Author Summary

HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is a rare neurodegenerative disease caused by infection with human T-lymphotropic virus type 1 (HTLV-1). HTLV-1 infects 10–20 million people worldwide, and, depending on the region, 0.25–3.8% of infected individuals develop HAM/TSP. As the disease progresses, chronic inflammation damages the spinal cord and lower limb and bladder function gradually decline. In the worst cases, even middle-aged patients can become perpetually bedridden. Today, there are treatments that may alleviate the symptoms to a certain degree, but there is no cure that can halt disease progression, and there are no known biomarkers to indicate the level and speed of disease progression. In this study, we successfully identified three promising candidate biomarkers. We believe that the use of these biomarkers could lead to more accurate prognoses and more prudent, patient-specific treatment plans. We not only hope that these biomarkers are sensitive enough to use as selection criteria for clinical trials, but also that measurements of these biomarkers can be used to accurately evaluate drug effectiveness. In short, the biomarkers we identified have the potential to help more effectively treat current HAM/TSP patients and to pave the way for new drugs to potentially cure future HAM/TSP patients.

the symptoms of HAM/TSP have been well documented for quite some time, the rate at which these symptoms progress has only recently become a point of interest. The clinical course of HAM/TSP has classically been described very simply as insidious onset and continuous progression [12], but recent reports have hinted at a more complex, heterogeneous pool of patients with differing clinical needs. Recent studies have shown that although HAM/TSP usually progresses slowly and without remission as per the classical description, there is a subgroup of patients whose conditions decline unusually quickly and who may be unable to walk within two years of onset and another subgroup whose conditions decline unusually slowly and who may only display very mild symptoms [13–15]. It is only logical that these patients should receive treatments tailored to suit their individual needs rather than identically aggressive treatments. Unfortunately, clinicians are currently only able to distinguish between these different groups by observing the way a patient's disease progresses over time, usually years; clinicians often decide to treat the patients immediately and identically rather than wait and allow the disease to progress further. Therein lies the dire need for biomarkers with the power to forecast the rate and extent of disease progression and enable clinicians to make more accurate prognoses and prescribe the most appropriate and effective treatments in a timely manner.

Several candidate prognostic biomarkers with elevated levels in HAM/TSP patients have already been identified in the peripheral blood and cerebrospinal fluid (CSF). In the peripheral blood, such candidates include the HTLV-1 proviral load in peripheral blood mononuclear cells (PBMCs) and serum levels of the soluble IL-2 receptor (sIL-2R) [16,17]. The level of neopterin in the CSF has been reported to be a useful parameter for detecting cell-mediated immune responses in the spinal cord of HAM/TSP patients and the CSF anti-HTLV-1 antibody titer has been shown to be associated both with CSF neopterin levels and the severity of clinical symptoms [18–20]. In addition, several cytokines have been detected in the CSF and/or spinal cord of HAM/TSP patients, including interleukin (IL)-1 β , granulocyte-macrophage

colony-stimulating factor (GM-CSF), interferon (IFN)- γ , and tumor necrosis factor (TNF)- α [21–24]. Some chemokines, such as chemokine (C-X-C motif) ligand (CXCL) 9, CXCL10, and chemokine (C-C motif) ligand (CCL) 5, have been shown to be substantially elevated in both the blood and the CSF with respect to asymptomatic carriers (ACs) or patients with other neurological diseases such as multiple sclerosis [25–28]. This is the first study to compare the adequacies of several of these candidate biomarkers for forecasting the rate of disease progression.

We hypothesized the existence of biomarkers capable of differentiating stable and deteriorating HAM/TSP patients. In this retrospective study, a preliminary experiment was first conducted to select the most promising candidate biomarkers by comparing blood and CSF levels in HAM/TSP patients and control subjects (Figure S1). Four candidate blood markers (sIL-2R, CXCL9, CXCL10, and proviral load) and five candidate CSF markers (CXCL9, CXCL10, neopterin, cell count, and anti-HTLV-1 antibody titer) were selected. To evaluate the relative effectiveness of these candidate biomarkers for predicting rate of disease progression, a classification system was created and HAM/TSP patients were designated as either deteriorating or relatively stable. The levels of candidate biomarkers were then compared between the two patient groups. In the current study, we identified three viable candidates for HAM/TSP prognostic biomarkers that could lead to more accurate prognoses and more prudent, patient-specific treatment plans.

Materials and Methods

Ethical considerations

The study was designed and conducted in accordance with the tenets of the Declaration of Helsinki. The protocol in this study was approved by the Ethics Review Committee of St. Marianna University School of Medicine (No. 1646). Prior to the collection of blood or CSF samples, all subjects gave written informed consent permitting the analysis of their samples for research purposes as part of their clinical care.

Subjects

Between April 2007 and February 2013, we enrolled 53 HAM/TSP patients according to the inclusion and exclusion criteria shown in Table 1, and divided them into two cohorts based on the chronological order of their doctor's visits: a 30-patient Training set and a 23-patient Test set. Demographics and clinical characteristics of the Training set and Test set are shown in Table 2 and Table 3, respectively. Between April 2007 and December 2009, we enrolled 22 HTLV-1-infected ACs as control subjects for blood analysis and eight HTLV-1-infected subjects (seven ACs, one patient with smoldering ATL) as control subjects for CSF analysis according to the inclusion and exclusion criteria shown in Table 1. These two groups were not mutually exclusive; some ACs donated both blood and CSF to this study. Demographics of control subjects as compared to the HAM/TSP patients are shown in Table S1.

Sample preparation

Blood and/or CSF samples were obtained within a one-hour window for each subject. Peripheral blood samples were collected in heparin-containing blood collection tubes and serum-separating tubes. Plasma and PBMCs were obtained from the former tubes and serum was obtained from the latter. PBMCs were isolated with standard procedures using Pancoll[®] density gradient centrifugation (density 1.077 g/mL; PAN-Biotech GmbH, Aidenbach, Germany). Plasma and serum samples were stored at -80°C until

Table 1. Inclusion and exclusion criteria for this study.

	HAM/TSP	Control for Blood	Control for CSF
Inclusion Criteria	Willing and able to give informed consent HTLV-1 seropositive individuals conformed by CLEIA and Western blot Diagnosed with HAM/TSP as defined by WHO criteria		Choose to provide CSF for the purposes of differential diagnosis
Exclusion Criteria	History of treatment with corticosteroids or other immunomodulating drugs (interferon, cyclosporin, methotrexate, etc.) Diagnosed with an autoimmune disease or other chronic inflammatory disorder aside from HAM/TSP Diagnosed with additional disease affecting gait disturbance (e.g. parkinsonism, rheumatoid arthritis, cervical spondylosis, brain infarction, etc.) History of severe urinary infection, decubitus scars, pneumonia, deep venous thrombosis, or other condition potentially affecting disease course within the last four years Diagnosed with adult T-cell leukemia (ATL)	Diagnosed with HAM/TSP as defined by WHO criteria	

CLEIA = chemiluminescent enzyme immunoassay.
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use. CSF was collected in polypropylene tubes. A small amount of CSF was used for routine laboratory tests, which included total protein, cell count, and IgG level. The remaining CSF was aliquoted into cryotubes and stored at -80°C until undergoing further analysis. All tests in this study were performed on samples from these frozen stocks.

Measurement of blood candidate markers

The serum concentration of sIL-2R was determined using an ELISA (Cell Free N IL-2R; Kyowa Medex Ltd., Tokyo, Japan). HTLV-1 proviral load was measured using real-time PCR, following DNA extraction from PBMCs, as previously described [29–31]. Plasma levels of IL-1 β , TNF- α , and IFN- γ were measured using a cytometric bead array (CBA) (BD Biosciences, Franklin Lakes, NJ USA), which was used according to the manufacturer's instructions. Plasma concentrations of CXCL9, CXCL10, CXCL11, and CCL5 were also measured using a CBA (BD Biosciences).

Measurement of CSF candidate markers

CSF cell count was determined using the Fuchs–Rosenthal chamber (Hausser Scientific Company, Horsham PA USA). Total protein and IgG levels in the CSF were measured using a pyrogallol red assay and a turbidimetric immunoassay, respectively. The anti-HTLV-1 antibody titer was determined using the gelatin particle agglutination test (Serodia-HTLV-1; Fujirebio, Tokyo, Japan). CSF concentration of sIL-2R was determined using an ELISA (Cell Free N IL-2R; Kyowa Medex). CSF neopterin level was measured using high-performance liquid chromatography. IFN- γ and six chemokines (CXCL9, CXCL10, CXCL11, CCL3, CCL4, and CCL5) were measured using a CBA (BD Biosciences). The CSF concentrations of three chemokines (CCL17, CCL20, and CCL22) and IL-17A were measured using commercially available ELISA kits (CCL17, CCL20, and CCL22: TECHNE/R&D Systems, Minneapolis, MN USA; IL-17A: Gen-Probe, San Diego, CA USA). All assays were conducted according to the respective manufacturers' instructions.

Table 2. Demographics and clinical characteristics of HAM/TSP patients (Training Set).

	Total n = 30	Stable HAM/TSP n = 14	Deteriorating HAM/TSP n = 11	p-value*
Demographics				
Age, y**	58 [37–75]	54.5 [39–75]	62 [53–72]	0.0183 [†]
Female sex	80.0%	64.3%	90.9%	0.1696 [‡]
Clinical characteristics				
Age of onset, y**	48 [20–70]	33 [20–58]	57 [40–70]	0.0021 [†]
Disease duration, y**	12.5 [1–33]	19 [7–33]	9 [1–13]	0.0021 [†]
OMDS**	6 [2–11]	5 [2–9]	8 [5–11]	0.0065 [†]

In the Training set, deteriorating patients were significantly older, experienced disease onset later in life, had been living with the disease for shorter periods, and were more severely disabled (OMDS).

*Stable HAM/TSP vs Deteriorating HAM/TSP.

**Data are expressed as median [range].

[†]By Mann-Whitney test.

[‡]By Fisher's exact test.

OMDS = Osame's Motor Disability Score.

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Table 3. Demographics and clinical characteristics of HAM/TSP patients (Test Set).

	Total n = 23	Stable HAM/TSP n = 11	Deteriorating HAM/TSP n = 9	p-value*
Demographics				
Age, y**	58 [22–75]	61 [22–75]	59 [48–68]	0.8491 [†]
Female sex	78.3%	81.8%	77.8%	1.000 [‡]
Clinical characteristics				
Age of onset, y**	43 [12–70]	40 [14–70]	51 [39–63]	0.0184 [†]
Disease duration, y**	9 [2–41]	19 [5–41]	6 [2–14]	0.0148 [†]
OMDS**	5 [2–8]	5 [4–8]	5 [4–8]	0.4526 [†]

In the Test set, deteriorating patients experienced disease onset later in life and had been living with the disease for shorter periods, but there were no significant differences in current age or OMDS.

*Stable HAM/TSP vs Deteriorating HAM/TSP.

**Data are expressed as median [range].

[†]By Mann-Whitney test.

[‡]By Fisher's exact test.

OMDS = Osame's Motor Disability Score.

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Classification system based on the natural history of HAM/TSP

The 53 total HAM/TSP patients without any history of HAM/TSP-targeting treatments were interviewed using a questionnaire (Figure S2) to determine the changes in Osame's Motor Disability Score (OMDS) over time (Figure S3). OMDS is a standardized neurological rating scale as a measure of disability [10] (Figure S1). Based on the changes in OMDS, "deteriorating cases" and "stable cases" were identified in both the Training set and Test set patient cohorts. Patients with deteriorating HAM/TSP were defined as those whose OMDS worsened ≥ 3 grades over four years and patients with stable HAM/TSP were defined as those whose OMDS remained unchanged or worsened 1 grade over four years. Patients whose OMDS worsened 2 grades over four years were excluded from the patient cohort in order to create a larger gap between the deteriorating and stable patient groups.

Statistical analysis

GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA USA) was used to plot graphs and perform statistical analyses. Differences between the two subject groups were tested using the Mann-Whitney U-test. Receiver operating characteristic (ROC) analysis was performed to examine the sensitivity and specificity of individual biomarkers. For the ROC analyses, an area under the ROC curve (AUC) of 1.0 was used to represent a perfect test with 100% sensitivity and 100% specificity, whereas an area of 0.5 was used to represent random discrimination. Spearman's rank correlation test was employed to investigate the correlation between the four CSF markers (CXCL10, CXCL9, neopterin, and cell count) and the proviral load in PBMCs. To compare the four CSF markers between three groups (HTLV-1-infected control, n = 8; stable HAM/TSP, n = 25; and deteriorating HAM/TSP, n = 20), we used the Kruskal-Wallis test followed by Dunn's post-hoc tests. P-values < 0.05 were considered statistically significant.

Results

Identification of biomarkers elevated in the blood of HAM/TSP patients

In order to identify candidate blood markers for HAM/TSP, the concentrations of IL-1 β , TNF- α , and IFN- γ were measured in

plasma samples from four ACs and four HAM/TSP patients. Plasma levels of IL-1 β and TNF- α were below the detection limits (<2.3 pg/mL and <1.2 pg/mL, respectively) except in one patient with HAM/TSP. Plasma IFN- γ levels showed no significant differences between ACs and HAM/TSP patients (median 10.4 pg/mL and 13.9 pg/mL, respectively). Therefore, these quantities were not measured in additional samples (Figure S1). The proviral DNA load in PBMCs, serum sIL-2R, and plasma levels of the chemokines CXCL9, CXCL10, CXCL11, and CCL5 were also measured in 22 ACs and 30 HAM/TSP patients without any history of immunomodulating treatments, including corticosteroids, IFN- α , and immunosuppressive drugs. The results revealed that serum levels of sIL-2R, plasma levels of CXCL10 and CXCL9, and proviral DNA load in PBMCs were markedly higher in HAM/TSP patients compared to ACs ($p \leq 0.0001$, Figure 1A). These quantities were then compared using ROC analysis to determine which parameters were superior markers for HAM/TSP. From the results of the ROC analysis, we determined that serum sIL-2R and plasma CXCL10 had the highest potential for distinguishing HAM/TSP patients from ACs with high sensitivity and specificity (area under the ROC curve [AUC] > 0.9), followed by plasma CXCL9 and HTLV-1 proviral load in PBMCs ($0.8 < \text{AUC} < 0.9$) (Figure 1B). Thus, four candidate blood biomarkers were selected for further investigation: serum sIL-2R, plasma CXCL10, plasma CXCL9, and HTLV-1 proviral load in PBMCs.

Identification of biomarkers elevated in the CSF of HAM/TSP patients

In order to identify candidate CSF markers for HAM/TSP, elevated levels of various potential markers were screened for in CSF samples from HAM/TSP patients. CSF IL-17A was detectable (>3.0 pg/mL) in only one of eight HAM/TSP patients screened (including six deteriorating-type patients), and the level in this one patient (deteriorating-type) was negligible (4.0 pg/mL). CSF IFN- γ was detectable (>1.8 pg/mL) in only 3 of 10 HAM/TSP patients screened (six deteriorating patients), and the levels in all three were negligible (range 3.3–4.2 pg/mL). Therefore, these cytokines were not measured in additional patients. Total protein, cell count, IgG, neopterin, sIL-2R, and nine chemokines (CXCR3 ligands: CXCL9, CXCL10, and CXCL11; CCR5 ligands: CCL3, CCL4, and CCL5; CCR4 ligands: CCL17 and CCL22; CCR6

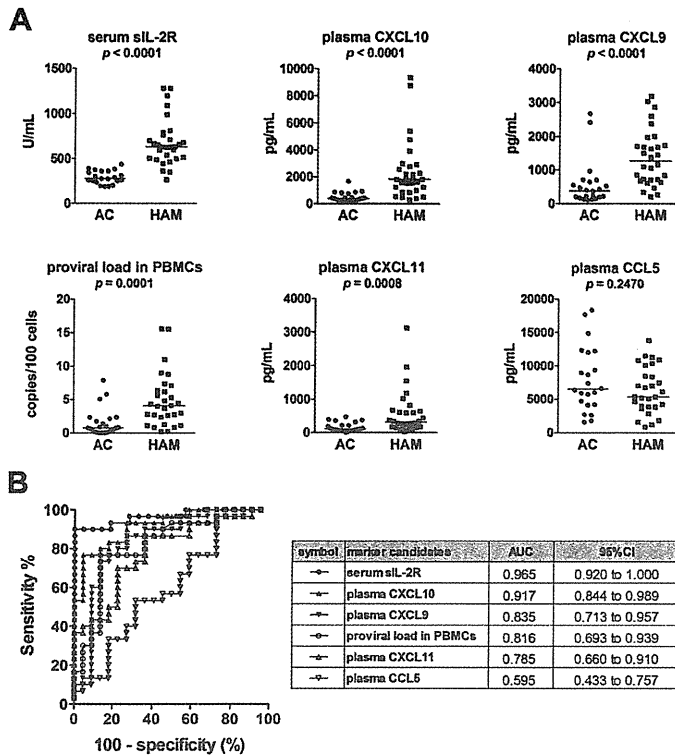


Figure 1. Selection of candidate biomarkers in the blood by comparing HAM/TSP patients and asymptomatic carriers. (A) Serum levels of soluble IL-2 receptor (sIL-2R), proviral loads in peripheral blood mononuclear cells (PBMCs), and plasma levels of four chemokines (chemokine (C-X-C motif) ligand (CXCL) 9, CXCL10, CXCL11, and chemokine (C-C motif) ligand (CCL) 5) were compared between HAM/TSP patients (HAM; $n = 30$) and asymptomatic carriers (AC; $n = 22$). Horizontal bars indicate the median values. The Mann-Whitney U -test was used for statistical analysis. (B) Receiver operating characteristic (ROC) analysis was employed to assess the sensitivities and specificities of the six markers exhibited in part (A) for discriminating HAM/TSP patients from ACs: greater proximity of the ROC curve to the upper left corner indicates higher sensitivity and specificity of the marker. AUC = area under the ROC curve; 95% CI = 95% confidence interval. doi:10.1371/journal.pntd.0002479.g001

ligand: CCL20) were also measured in the CSF of 30 untreated HAM/TSP patients and in eight HTLV-1-infected control subjects (seven ACs and one patient with smoldering ATL). The results indicated that CSF levels of CXCL10, neopterin, and CXCL9 were remarkably higher in HAM/TSP patients compared to control subjects ($p < 0.0001$ overall, Figures 2A and S4) and that CSF levels of cell count and CCL5 were less so but still significantly higher ($p = 0.0019$ and $p = 0.0119$, respectively; Figure 2A). By contrast, there were no differences in the CSF levels of IgG and total protein between HAM/TSP patients and control subjects, and CSF sIL-2R levels were only detectable in a single HAM/TSP patient (data not shown). ROC analysis showed that the CSF levels of CXCL10, neopterin, CXCL9, and CSF cell count could be used to relatively accurately distinguish HAM/TSP patients from control subjects (AUC > 0.8) (Figure 2B). Therefore, these four CSF markers were selected as candidates for further investigation. It should be noted that the sensitivity of CSF cell count was very low (36.7%) when compared to the other three: CXCL10 (83.3%), CXCL9 (86.7%), and neopterin (76.7%) (Figure S5).

Identification of biomarkers correlated with rate of HAM/TSP disease progression

In short, we selected nine markers: eight markers chosen based on the analyses described above and CSF anti-HTLV-1 antibody

titer, which is a known diagnostic marker for HAM/TSP. To determine which biomarkers were associated with HAM/TSP disease progression, the levels of these nine markers were compared between the deteriorating and stable HAM/TSP patient groups (see Methods for definitions of deteriorating and stable). The results revealed that all five CSF markers were significantly higher in the deteriorating group compared to the stable group (Figure 3A), but that none of the four blood markers, including proviral load, were significantly different between the two groups. The deteriorating group included three patients with particularly rapidly progressive HAM/TSP, defined as those who had been confined to wheelchairs (OMDS: \geq grade 6) within two years after the onset of symptoms [13,14] (black circles in Figures 3A and S3B). These rapid progressors exhibited high levels of the CSF markers and high proviral loads. ROC analysis revealed that the levels of the CSF markers (CXCL10, CXCL9, neopterin, and cell count), but not anti-HTLV-1 antibody titer, distinguished clearly between patients with deteriorating HAM/TSP and stable HAM/TSP (AUC > 0.8, Figure 3B).

Validation of nine candidate biomarkers using the Test Set

To validate the results obtained using the Training Set, the same nine markers were compared between deteriorating and stable patients using the Test Set (a second cohort of 23 HAM/

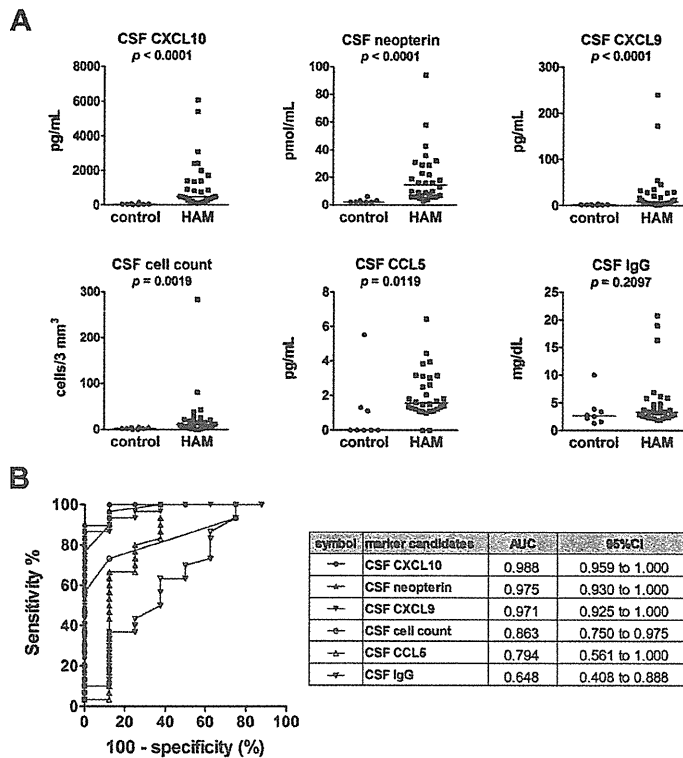


Figure 2. Selection of candidate biomarkers in the cerebrospinal fluid (CSF) by comparing HAM/TSP patients and control subjects. (A) CSF levels of total protein, cell count, IgG, neopterin, sIL-2R, and nine chemokines (CCL3, CCL4, CCL5, CXCL9, CXCL10, CXCL11, CCL17, CCL20, and CCL22) were measured and compared between HAM/TSP patients (HAM; $n = 30$) and HTLV-1-infected control subjects (control; $n = 8$): seven ACs and one ATL patient). Data is shown for the top six CSF markers ranked according to the significance of the difference between the HAM/TSP patients and the control subjects. Horizontal bars indicate the median values. The Mann-Whitney U -test was used for statistical analysis. (B) ROC analysis was employed to assess the sensitivities and specificities of the six markers exhibited in part (A) for discriminating HAM/TSP patients from controls. AUC = area under the ROC curve; 95% CI = 95% confidence interval. doi:10.1371/journal.pntd.0002479.g002

TSP patients that had not undergone HAM/TSP-targeting treatment). As shown in Figure 4A, the results indicated that the levels of five CSF markers, proviral load in PBMCs, and serum sIL-2R were significantly higher in deteriorating cases than in stable cases. Among them, CSF levels of CXCL10, CXCL9, neopterin, and CSF cell count exhibited particularly high sensitivities and specificities for detecting the deteriorating HAM/TSP cases in the Test set as well as Training set (AUC > 0.8, Figures 4B and S1).

Demographic and clinical characteristics of the subjects

The demographics of the HAM/TSP patients versus the control subjects for both the blood tests and CSF analyses were compared and evaluated for statistical significance (Table S1). There were no significant differences in age or gender distribution between the HAM/TSP patients and either control subject group.

Similarly, the demographic and clinical characteristics of stable versus deteriorating HAM/TSP subjects in both the Training and Test sets are shown in Tables 2 and 3, respectively. There were no significant differences in age or gender distribution among either set, but deteriorating patients in both sets were significantly older at disease onset and had been living with the disease for shorter periods of time. Deteriorating patients in the Training set scored higher OMDS values than their stable counterparts ($p < 0.01$), but there was no such significant difference in the Test set.

To investigate the potential influence of disease duration as a secondary variable, a new test group was created containing only those patients for whom the disease onset date was 7–13 years prior to the sample collection day. Patients fitting this criterion were selected from the 53 total available from both the Training and Test sets: eight stable patients and ten deteriorating patients; we confirmed that there was no significant difference in disease duration between these two groups. The results remained consistent with our previous findings: CSF CXCL10, CXCL9, and neopterin were all elevated in deteriorating patients with respect to stable patients ($p < 0.01$, Figure 5).

Follow-up mini-study on biomarker levels over time

Four stable HAM/TSP patients were left completely untreated and followed for a period of three to five years. Within this time, one patient rose one grade on the OMDS scale, and the other three experienced no change in OMDS grade at all. The levels of CSF CXCL10 and neopterin remained consistently low over time (Figure S6).

Discussion

To date, there have been few well-designed studies that have evaluated the relationship between biomarkers and HAM/TSP disease progression. In a previous retrospective study with 100 untreated HAM/TSP patients, a significant association was

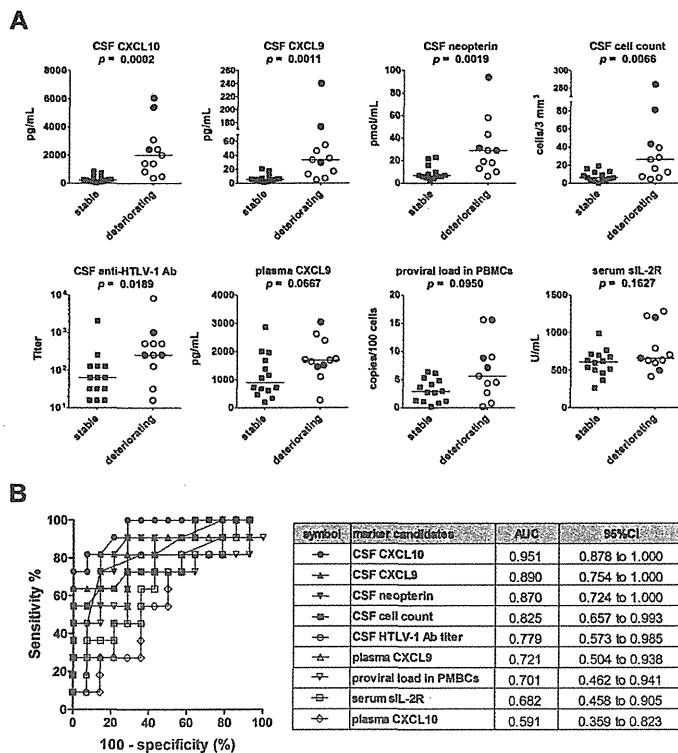


Figure 3. Identification of biomarkers associated with clinical progression of HAM/TSP. (A) Five CSF marker candidates (CXCL10, CXCL9, neopterin, cell count, and anti-HTLV-1 antibody titer) and four blood marker candidates (proviral load in PBMCs, serum sIL-2R, plasma CXCL9, and plasma CXCL10) were compared among a cohort of patients called the Training Set (deteriorating HAM/TSP, $n = 11$; stable HAM/TSP, $n = 14$). Data is shown for the top eight CSF markers ranked according to the significance of the difference between the deteriorating and stable subjects. Black circles indicate patients with particularly rapidly progressive HAM/TSP. Horizontal bars indicate the median values. The Mann-Whitney U -test was used for statistical analysis. (B) ROC analysis was employed to assess the sensitivities and specificities of the nine markers listed above for discriminating deteriorating HAM/TSP patients from stable patients. AUC = area under the ROC curve; 95% CI = 95% confidence interval. doi:10.1371/journal.pntd.0002479.g003

demonstrated to exist between higher HTLV-1 proviral load in PBMCs and poor long-term prognosis; however, the predictive value of high proviral load appeared to be too low to qualify it as a marker for disease progression in clinical practice [32]. Here we conducted a retrospective study to compare for the first time the relationships of PBMC proviral load and several inflammatory biomarker candidates to disease progression in untreated HAM/TSP patients.

In this study, elevated CSF cell count, neopterin concentration, and CSF levels of CXCL9 and CXCL10 were well-correlated with disease progression over the four year period under study, better even than HTLV-1 proviral load in PBMCs (Figures 3 and 4). As CSF pleocytosis, CSF CXCL10, CSF CXCL9, and CSF neopterin are known indicators of inflammation in the central nervous system [33,34], our findings indicate that the rate of HAM/TSP progression is more closely reflected by the amount of inflammatory activity in the spinal cord than by the PBMC proviral load. However, we also found a significant correlation between PBMC proviral load and the levels of the CSF markers identified in this study (Figure S7), indicating that a higher PBMC proviral load does indeed suggest more inflammation in the spinal cord and therefore a poorer long-term prognosis. These findings are consistent with the theory that HAM/TSP is the result of an excess of inflammatory mediators caused by the presence of HTLV-1-infected T-cells [35–37].

The HTLV-1 proviral load in the CSF as well as the ratio of the proviral load in the CSF to that in PBMCs have been reported to be effective for discriminating HAM/TSP patients from ACs or multiple sclerosis patients infected with HTLV-1 [38,39]. Some researchers have suggested that these values might be associated with the rate of disease progression, but there has been only one small cohort study and one case report investigating this point, and so the significance of this experimental evidence is still questionable [40,41]. In addition to statistical validation with multiple, larger cohorts, it would also be beneficial to use precise definitions for progressive versus stable patients, as we have done in this study. Although the volume of CSF available per sample was too limited to measure CSF proviral load in the present study, we plan to incorporate CSF proviral load in a future prospective study and compare its usefulness to that of other biomarker candidates.

From our results, we concluded that of the potential biomarkers under study, CXCL10, CXCL9, and neopterin are the most fit for determining the level of spinal cord inflammation, and thus the most fit for predicting disease progression in HAM/TSP patients. Although the CSF cell count is an easily measurable inflammatory marker, it is not sensitive enough to reliably detect the level of spinal cord inflammation. Numerous patients with CSF cell counts within the normal range exhibited high levels of other inflammatory markers, such as neopterin and CXCL10 (Figure S5). In fact, it has been reported that CSF pleocytosis is present in only approximately 30% of HAM/TSP patients [42]. Furthermore, in

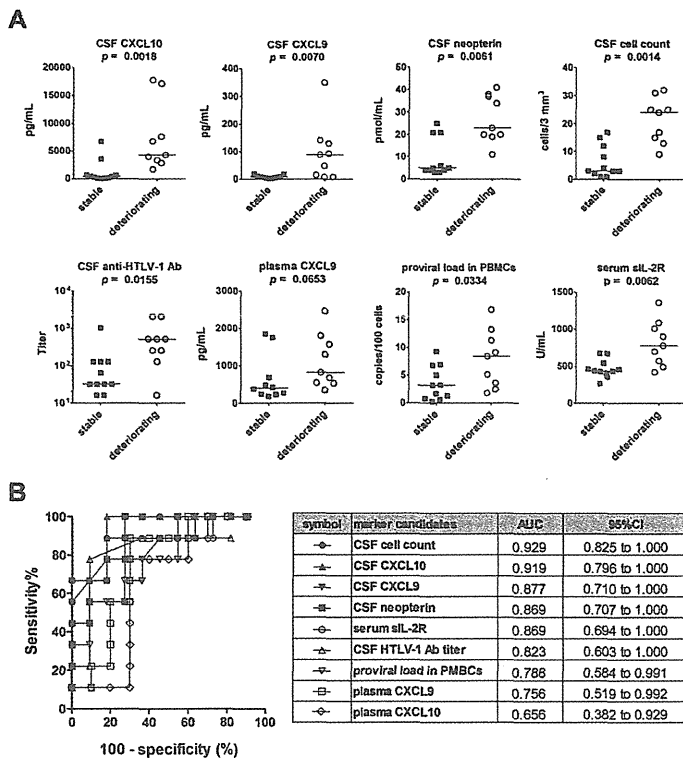


Figure 4. Validation of potential markers using the Test Set. (A) Five CSF marker candidates (CXCL10, CXCL9, neopterin, cell count, and anti-HTLV-1 antibody titer) and four blood marker candidates (proviral load in PBMCs, serum sIL-2R, plasma CXCL9, and plasma CXCL10) were compared among a second cohort of patients called the Test Set (deteriorating HAM/TSP, $n = 9$; stable HAM/TSP, $n = 11$). Data is shown for the top eight CSF markers ranked according to the significance of the difference between the deteriorating and stable subjects. Horizontal bars indicate the median values. The Mann-Whitney U -test was used for statistical analysis. (B) ROC analysis was employed to assess the sensitivities and specificities of the nine markers listed above for discriminating deteriorating HAM/TSP patients from stable patients. AUC = area under the ROC curve; 95% CI = 95% confidence interval.

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our study, there was no significant difference in CSF cell count between the control subjects and the stable HAM/TSP patients (Figure S8).

We also explored the possibility of combining multiple biomarkers via multiple logistic regression to form a combination more sensitive and specific than individual markers, but the results indicated that there is not much to be gained from combinations (data not shown).

While there were no significant demographic differences between subject groups, the clinical characteristics of stable versus deteriorating HAM/TSP patients of course differed widely (Tables 2, 3, and S2). We confirmed the already well-reported statistic that deteriorating patients experience HAM/TSP onset relatively late in life [12,14,20]; our data also reflected the short disease duration expected of deteriorating patients, who by definition progress through the disease more rapidly than their stable counterparts. As patients in all groups were of similar age at sample collection, the significant difference in age of onset should not have any impact on our findings. However, it was necessary to consider the possibility that those patients in a later stage of the disease (i.e. those listed with longer disease durations) might possess elevated or diminished biomarker levels regardless of rate of disease progression. We confirmed that this difference in disease duration was not a confounding factor in our selection of candidate biomarkers by comparing stable and deteriorating HAM/TSP patients with similar disease durations (7–13 years),

and we were able to obtain results consistent with our earlier findings (Figure 5). Finally, the OMDS values for the stable and deteriorating patient groups in the Test set were perfectly identical, eliminating the need to consider the possibility that the biomarkers could have been elevated according to disease severity regardless of rate of progression.

The main limitation of our retrospective study is that our samples were collected from patients at the end of the four year period during which the extent of progression was analyzed as opposed to the beginning of the four year period, which would have been optimal for directly measuring their prognostic powers. Of course, the patients with severe HAM/TSP symptoms began undergoing treatment soon after sample collection, rendering any observations on disease course after sample collection un-useable for analysis in this study. While this situation is non-ideal, we hypothesize that biomarker levels in a given patient do not substantially change over a few years' time. We were actually able to monitor the biomarker levels of four untreated HAM/TSP patients over 3–5 years, and the levels remained relatively stable in all four subjects over time (Figure S6), supporting our hypothesis. However, these were all stable HAM/TSP patients (hence the lack of treatment), and so we cannot rule out the possibility that biomarker levels in untreated deteriorating patients may dramatically rise, fall, or fluctuate. The results of the analysis of patients with similar disease durations (Figure 5) also support our hypothesis that disease duration is not an important determinant

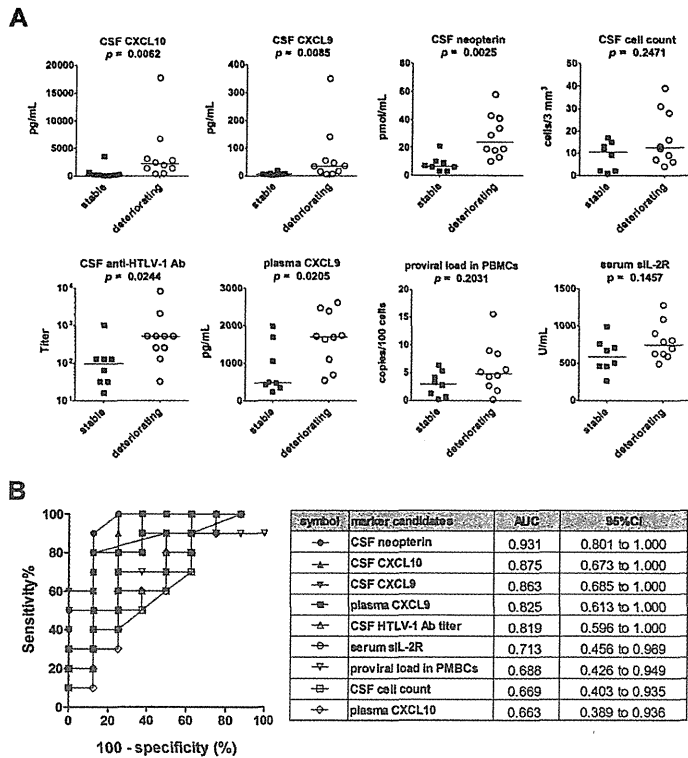


Figure 5. Comparison of potential markers in stable and deteriorating HAM/TSP patients with similar disease durations. (A) Five CSF marker candidates (CXCL10, CXCL9, neopterin, cell count, and anti-HTLV-1 antibody titer) and four blood marker candidates (proviral load in PMBCs, serum sIL-2R, plasma CXCL9, and plasma CXCL10) were compared among all patients from both the Training and Test Sets pooled together with similar disease durations (range: 7–13 years; no significant difference in duration between stable ($n=8$) and deteriorating ($n=10$) groups). Data is shown for the top eight CSF markers ranked according to the significance of the difference between the deteriorating and stable subjects. Horizontal bars indicate the median values. The Mann-Whitney U -test was used for statistical analysis. (B) ROC analysis was employed to assess the sensitivities and specificities of the nine markers listed above for discriminating deteriorating HAM/TSP patients from stable patients while controlling for disease duration. AUC = area under the ROC curve; 95% CI = 95% confidence interval. doi:10.1371/journal.pntd.0002479.g005

of biomarker levels, but it is of course not conclusive. We expect that a prospective study in the future will reveal the answer to this question.

The results of this study indicate that CXCL9 and/or CXCL10 may play a key role in the pathogenesis of HAM/TSP by recruiting more inflammatory cells to the spinal cord lesions. In this study, we measured the levels of the chemokines in the CSF that might play a part in inducing the migration of T-helper (Th) cells. $CD4^+$ Th cells differentiate from naive T-cells to members of the Th subset (e.g., Th1, Th2, Th17, or Treg cells), and each one expresses its own characteristic chemokine receptors [43]. Usually, Th1 cell express CCR5/CXCR3 receptors, Th2 and Treg cells express CCR4, and Th17 express CCR6. Interestingly, CCR4 ligands (CCL17 and CCL22) and the CCR6 ligand (CCL20) were not detected in the CSF of HAM/TSP patients. Moreover, of the CCR5 ligands, only CCL5 was elevated, but only slightly, and there was no association with rate of disease progression. Of the CXCR3 ligands, only CXCL9 and CXCL10 were correlated with the rate of disease progression. These results show that the pathology of HAM/TSP is unique among immune disorders in that, unlike other inflammatory disorders such as multiple sclerosis or rheumatoid arthritis that exhibit Th17 as well as Th1 involvement, the chemokine involvement in HAM/TSP is Th1-dominant. In a previous study, cytokines produced by HTLV-1-

infected T-cells in HAM/TSP patients were analyzed, and the results showed that $IFN-\gamma$ was elevated and IL-17 reduced [43,44]. Taken together, the results of these studies indicate that the characteristics of HTLV-1-infected T-cells themselves may be responsible for the Th1-dominant chemokine production observed in HAM/TSP. Also, these results suggest that the CXCR3-ligand (CXCL9 and CXCL10) interactions play an important role in the pathophysiology of HAM/TSP. Recently it was established that these CXCR3-ligand interactions are extremely important for the pathogenesis of several neurological disorders [33]. Therefore, future research on the significance of these interactions in the pathogenic process of HAM/TSP will be important for clarifying the suitability of CXCL9 and CXCL10 as biomarkers or therapeutic targets.

In conclusion, in this retrospective study, we have demonstrated that CSF levels of CXCL10, CXCL9, and neopterin are promising candidate prognostic biomarkers for HAM/TSP. These biomarkers may provide a means for the early identification of patients at increased risk of debilitating disease progression, those that may need anti-inflammatory therapies to limit or prevent this, and for evaluating the efficacy of such therapies. This initial identification of prognostic biomarkers for HAM/TSP should be followed by a future multicenter prospective clinical study.

Supporting Information

Figure S1 Diagram illustrating the biomarker selection process. A total of 26 biomarker candidates including 9 in the blood and 17 in the CSF underwent the following selection processes: 1) pre-screening of the cytokines for presence in HAM/TSP patients, 2) selection for markers elevated in HAM/TSP patients with respect to controls (AUC>0.8), 3) selection for markers elevated in deteriorating HAM/TSP patients with respect to stable patients (AUC>0.8) in a cohort termed the Training Set, 4) validation of the selected markers by evaluating again (AUC>0.8) in a second cohort termed the Test Set. The darkening of an arrow's color represents that marker's failure to meet the selection criteria, and the termination of an arrow indicates that no further testing was conducted for that marker. CYT = cytokine, HTLV-1 PVL = HTLV-1 proviral load, Ab Titer = anti-HTLV-1 antibody titer, AUC = area under the ROC curve. (TIF)

Figure S2 Questionnaire on the development of motor disability over time as measured using Osame's Motor Disability Score (OMDS). The first and second columns indicate the OMDS numerical value and description, respectively. Doctors interviewed the patients and filled in the table according to the following instructions: in the bottom row, write the ages at which symptoms listed to the left first appeared, and above the age check the box in the row corresponding to the symptom. (TIF)

Figure S3 Rate of disease progression in HAM/TSP patients without any history of HAM/TSP-targeting treatment. Each line illustrates the change in OMDS over time for an individual patient after disease onset for (A) all patients in the Training Set (n = 30) and (B, left) only deteriorating patients (n = 11) including three particularly rapidly progressive patients (shown as solid black circles) and (B, right) only stable patients (n = 14). (TIF)

Figure S4 Comparison of CSF levels of nine chemokines in control subjects and HAM/TSP patients. The CSF levels of nine chemokines (CCR5 ligands: CCL3, CCL4, and CCL5; CXCR3 ligands: CXCL9, CXCL10, and CXCL11; CCR4 ligands: CCL17 and CCL22; CCR6 ligand: CCL20) were compared between control subjects (control; n = 8) and HAM/TSP patients (HAM; n = 30). Horizontal bars indicate median values. The Mann-Whitney *U*-test was used for statistical analysis. (TIF)

Figure S5 Low sensitivity of CSF cell count for detection of HAM/TSP. (A) Sensitivities of four potential CSF markers for detection of HAM/TSP. For CSF CXCL10, CXCL9, and neopterin, dotted lines indicate reference values, defined as mean for control subjects +3 standard deviations. For CSF cell count, the dotted line represents the pre-established reference value of $15/3 \text{ mm}^3$. The sensitivity of CSF cell count was much lower than those of the other CSF markers. (B) Direct comparison of the sensitivities of CSF cell count and the other three CSF markers. The horizontal dotted lines all represent the reference value for CSF cell count ($\leq 15/3 \text{ mm}^3$), and each vertical dotted line

indicates the reference value for each of the other CSF markers. With these lines drawn, one can see in the shaded area the numerous patients with CSF cell counts within the normal range but abnormally high levels of each of the other inflammatory markers, thus directly illustrating the comparatively low sensitivity of CSF cell count. (TIF)

Figure S6 Changes in levels of CSF markers and OMDS over time in four untreated HAM/TSP patients. The three graphs illustrate the changes over time in CSF CXCL10 (top), neopterin (middle), and OMDS (bottom) for four untreated stable HAM/TSP patients. The patients were observed for 60 months (No. 1), 56 months (No. 2), 49 months (No. 3), and 39 months (No. 4). (TIF)

Figure S7 Significant positive correlation between the proviral load in PBMCs and four CSF markers. HTLV-1 proviral load in PBMCs was compared with the levels of each of four CSF markers (CXCL10, CXCL9, neopterin, and cell count) in HAM/TSP patients (n = 53). Data analysis was performed using the Spearman's rank correlation test. (TIF)

Figure S8 Significant higher CSF levels of CXCL10, CXCL9, and neopterin even in stable HAM/TSP compared to controls. The levels of four CSF markers (CXCL10, CXCL9, neopterin, and cell count) were compared among three groups (HTLV-1-infected controls, n = 8; stable HAM/TSP patients, n = 25; and deteriorating HAM/TSP patients, n = 20) assembling patients from both Training and Test Sets combined. The horizontal bar indicates the median value for each group. Statistical analysis was performed using the Kruskal-Wallis test followed by Dunn's post-hoc tests. ns: not significant, * $P < 0.05$, *** $P < 0.001$. (TIF)

Table S1 Demographics of HAM/TSP patients and control subjects. There were no significant differences in the demographics of HAM/TSP patients versus control subjects. (DOCX)

Table S2 Demographics and clinical characteristics of HAM/TSP patients (Training set + Test Set). Among the HAM/TSP patients from the Training and Test Sets pooled together, deteriorating patients experienced disease onset significantly later in life and had lived with the disease for shorter periods. (DOCX)

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Author Contributions

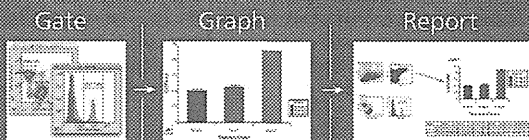
Conceived and designed the experiments: YY TS SJ SI. Performed the experiments: TS HA NA JY. Analyzed the data: TS AU NA NY HA JY EI TU YH KN TN. Contributed reagents/materials/analysis tools: YY AU YH. Wrote the paper: YY TS ACR.

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

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Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is an effective treatment for adult T cell leukemia/lymphoma (ATL) caused by human T cell leukemia virus type 1 (HTLV-1). We previously reported that Tax-specific CD8⁺ cytotoxic T lymphocyte (CTL) contributed to graft-versus-ATL effects in ATL patients after allo-HSCT. However, the role of HTLV-1–specific CD4⁺ T cells in the effects remains unclear. In this study, we showed that Tax-specific CD4⁺ as well as CD8⁺ T cell responses were induced in some ATL patients following allo-HSCT. To further analyze HTLV-1–specific CD4⁺ T cell responses, we identified a novel HLA-DRB1*0101–restricted epitope, Tax155–167, recognized by HTLV-1–specific CD4⁺ Th1-like cells, a major population of HTLV-1–specific CD4⁺ T cell line, which was established from an ATL patient at 180 d after allo-HSCT from an unrelated seronegative donor by in vitro stimulation with HTLV-1–infected cells from the same patient. Costimulation of PBMCs with both the identified epitope (Tax155–167) and known CTL epitope peptides markedly enhanced the expansion of Tax-specific CD8⁺ T cells in PBMCs compared with stimulation with CTL epitope peptide alone in all three HLA-DRB1*0101⁺ patients post-allo-HSCT tested. In addition, direct detection using newly generated HLA-DRB1*0101/Tax155–167 tetramers revealed that Tax155–167-specific CD4⁺ T cells were present in all HTLV-1–infected individuals tested, regardless of HSCT. These results suggest that Tax155–167 may be the dominant epitope recognized by HTLV-1–specific CD4⁺ T cells in HLA-DRB1*0101⁺–infected individuals and that Tax-specific CD4⁺ T cells may augment the graft-versus-Tax effects via efficient induction of Tax-specific CD8⁺ T cell responses. *The Journal of Immunology*, 2013, 190: 4382–4392.

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

ATL is characterized by extremely poor prognosis, mainly because of intrinsic drug resistance to cytotoxic agents. It has been reported that allogeneic hematopoietic stem cell transplantation

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

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Abbreviations used in this article: AC, asymptomatic carrier; allo-HSCT, allogeneic stem cell transplantation; ATL, adult T cell leukemia/lymphoma; HAM/TSP, HTLV-1–associated myelopathy/tropical spastic paraparesis; HTLV-1, human T cell leukemia virus type 1; ILT, IL-2–dependent T cell line; LCL, lymphoblastoid B cell line; rhIL-2, recombinant human IL-2; RIC, reduced intensity conditioning; Treg, regulatory T.

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

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

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

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

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

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

Materials and Methods

Subjects

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

Generation of cell lines derived from patients and donors

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

Synthetic peptides

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

GST-Tax fusion protein-based immunoassay

HTLV-1 Tax-specific T cell responses were evaluated using GST-fusion proteins of the N-terminal (residues 1–127), central (residues 113–237), and C-terminal (residues 224–353) regions of HTLV-1 Tax (GST-Tax-A, -B, and -C, respectively) as described previously (13, 31). PBMCs (1 \times 10⁶ cells/ml) were incubated with or without a mixture of GST-Tax-A, -B, and -C proteins (GST-TaxABC) in 200 μ l RPMI 1640 medium supplemented with 10% FCS. After 4 d, the supernatant was collected, and the concentration of IFN- γ in the supernatant was determined using an OptiEIA Human IFN- γ ELISA Kit (BD Biosciences, San Jose, CA). The minimum detectable dose for this assay was determined to be 23.5 pg/ml IFN- γ . CD8⁺ cells were depleted from PBMCs by negative selection using Dynabeads M-450 CD8 (Invitrogen, Carlsbad, CA), according to the

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

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

PBMCs (1×10^6 cells/ml) from patient #350, in complete remission at 180 d after allo-HSCT, were cultured for 2 wk with 100 nM Tax301-309 peptide in 96-well round-bottom tissue culture plate (BD Biosciences) in a final volume of 200 μ l RPMI 1640 medium with 20% FCS and 10 U/ml rIL-2. CD4⁺ cells were then isolated by negative selection using a Human CD4 T lymphocyte Enrichment Set-DM (BD Biosciences) and maintained in RPMI 1640 medium with 20% FCS and 100 U/ml rIL-2. Cells (1×10^6 cells/ml) were stimulated with formaldehyde-fixed ILT-#350 (2.5×10^5 cells/ml) every 2-3 wk. After multiple rounds of stimulation, the resulting CD4⁺ T cell line was assessed for HTLV-1 specificity by comparing IFN- γ production against ILT-#350 to that against an HTLV-1-negative cell line, LCL-#350.

RT-PCR

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

Flow cytometry

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

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

Epitope mapping

T4 cells (3×10^5 cells/ml) were stimulated with LCL-#350, pulsed with various concentrations of synthetic peptides for 1 h at 37°C, at a responder/stimulator (R/S) ratio of 3. The culture supernatant was collected at 6 h poststimulation, and peptide-specific IFN- γ production from T4 cells was determined by ELISA.

HLA class II restriction assay

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

To identify a HLA class II molecule responsible for Ag presentation to T4 cells, Tax155-167 peptide-specific IFN- γ responses were evaluated using various HLA-typed LCLs (LCL-#350, LCL-#341, LCL-#307, and LCL-Kan). These LCLs (1×10^5 cells/ml) were pulsed with 100 ng/ml Tax155-167 peptide for 1 h, fixed with 2% formaldehyde, and then cultured with T4 cells (3×10^5 cells/ml) for 6 h. The culture supernatant was collected, and IFN- γ in the supernatant was measured by ELISA.

Tetramer-based proliferation assay

PBMCs (1.0×10^6 cells/ml) were cultured for 13 or 14 d with or without 100 nM antigenic peptides in the presence of 10 U/ml rIL-2. Cells were stained with HLA/Tax tetramer-PE, CD3-FITC, and CD8-PE-Cy5 or CD4-PE-Cy5 and then analyzed by flow cytometry.

Statistic analysis

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

Results

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

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

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

ID (Age, Sex)	ATL Subtype	Type of Donor	Donor-HLA	Donor HTLV-1 Sero Status	Chimerism (%) ^a	Tetramer (%) ^b	Proviral Load ^c
239 (55, M)	Lymphoma	r-PB	A 26/33, DR 4/13	(-)	<5	NT	0.1
241 (61, F)	Acute	r-PB	A 2/26, DR 10/18	(-)	<5	0.00	0.1
247 (52, F)	Lymphoma	r-PB	A 24/-, DR 9/15	(-)	<5	0.07	0.1
270 (57, M)	Lymphoma	r-PB	A 24/33, DR 13/15	(-)	<5	0.00	0.0
300 (53, F)	Lymphoma	r-PB	A 24/26, DR 4/15	(+)	<5	1.34	4.8
301 (57, F)	Acute	ur-BM	A 24/33, DR 13/15	(-)	<5	0.72	0.0
307 (68, F)	Acute	r-PB	A 2/11, DR 14/15	(+)	<5	0.10	5.4
317 (60, M)	Acute	ur-BM	A 2/24, DR 14/15	(-)	<5	0.92	0.0
328 (62, M)	Acute	ur-BM	A 11/24, DR 8/9	(-)	<5	0.75	NT
340 (50, M)	Acute	r-PB	A 2/24, DR 4/8	(-)	<5	1.40	0.7
341 (61, F)	Acute	ur-BM	A 24/33, DR 1/15	(-)	<5	0.45	0.1
344 (58, M)	Lymphoma	ur-BM	A 2/24, DR 4/-	(-)	<5	0.44	0.0
349 (53, M)	Acute	r-PB	A 24/-, DR 8/15	(+)	<5	0.00	0.0
350 (60, F)	Acute	ur-BM	A 24/26, DR 1/14	(-)	<5	0.59	0.6
351 (57, F)	Acute	ur-BM	A 24/26, DR 9/12	(-)	<5	0.45	0.0
358 (63, F)	Lymphoma	r-PB	A 2/11, DR 4/14	(-)	<5	0.42	0.0
352 (61, M)	Acute	ur-BM	A 11/26, DR 8/15	(-)	<5	0.14	0.0
364 (52, M)	Acute	r-PB	A 24/26, DR 1/-	(-)	<5	0.11	0.0

^aIndicates percentage of recipient-derived T cell chimerism.

^bIndicates percentage of tetramer⁺ cells among CD8⁺ T cells in PBMCs.

^cIndicates copy number per 1000 PBMCs.

F, Female; M, male; NT, not tested; r-PB, related donor-derived peripheral blood stem cell; ur-BM, unrelated donor-derived bone marrow cell.

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

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

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

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

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

We next attempted to induce HTLV-1-specific CD4⁺ T cells from the PBMCs of patient #350 at 180 d after allo-HSCT, using an HTLV-1-infected T cell line (ILT-#350) as APCs. Freshly isolated PBMCs were stimulated for 2 wk with Tax301–309, a dominant CTL epitope presented by HLA-A*2402, to eliminate HTLV-1-infected cells, which potentially existed in PBMCs. The CD4⁺ cells were then isolated from the cultured cells and stimulated with formaldehyde-fixed ILT-#350 every 2–3 wk. The established cell line was found to be a CD4⁺ T cell line (designated as T4 cells thereafter) because cells expressed CD3 and CD4 but not CD8

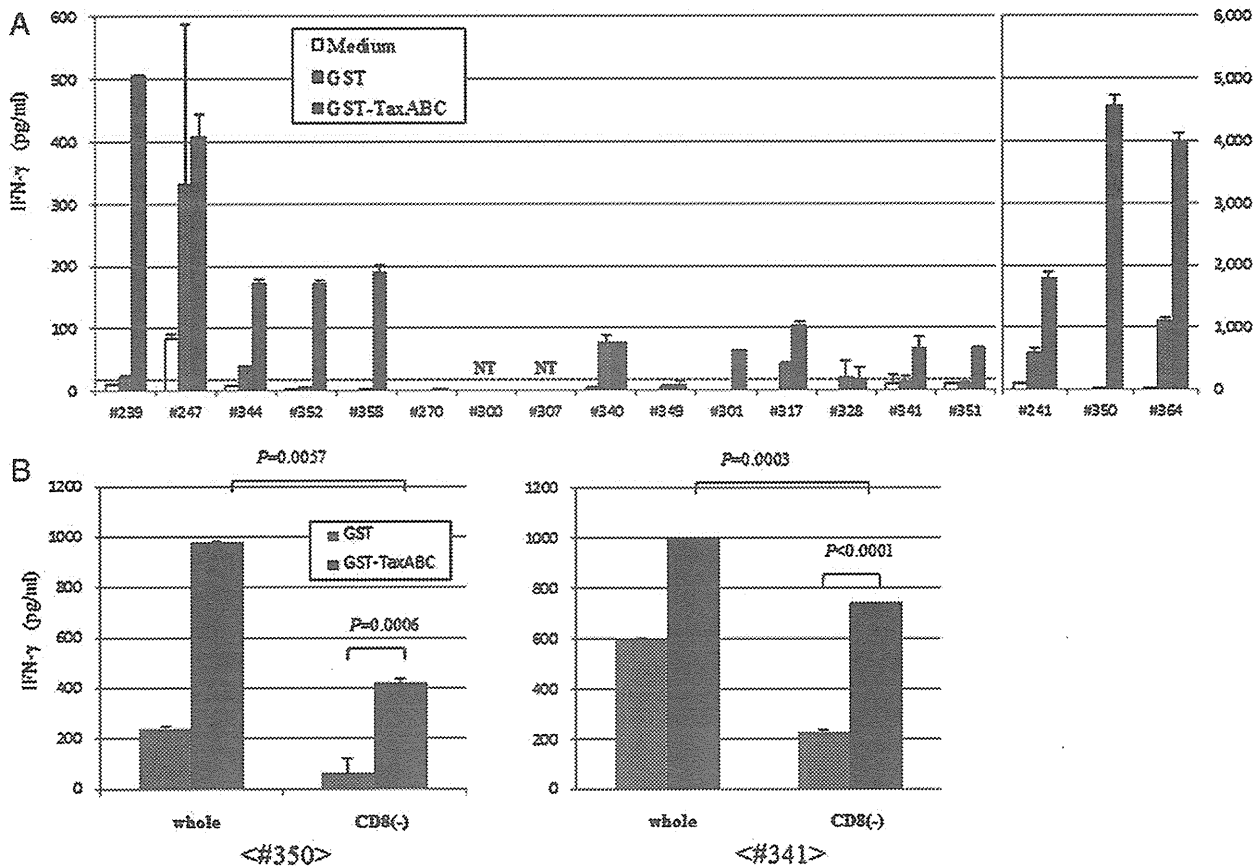


FIGURE 1. Diversity of Tax-specific T cell responses in ATL patients who received allo-HSCT with RIC. (A and B) PBMCs from 18 ATL patients at 180 d after allo-HSCT (A) or whole and CD8⁺ cell-depleted PBMCs from two patients at 540 d after allo-HSCT (#350 and #341) (B) were cultured for 4 d in the absence (open square) or presence of GST (gray square), or GST–Tax (black square) proteins. The concentration of IFN- γ in the supernatant was determined by ELISA. The y-axis on the right side indicates the results from three patients (#241, #350, and #364). The dotted horizontal line indicates the detection limit (23.5 pg/ml). The error bars represent SD of duplicated wells. The representative result of two independent experiments is shown in (B).

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

Determination of the minimum epitope recognized by T4 cells

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

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

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

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

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

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

As T4 cells were established from PBMCs of an HTLV-1-infected patient #350, it is suggested that Tax155–167-specific CD4⁺ T cells may be maintained in the HLA-DRB1*0101⁺ patient #350.

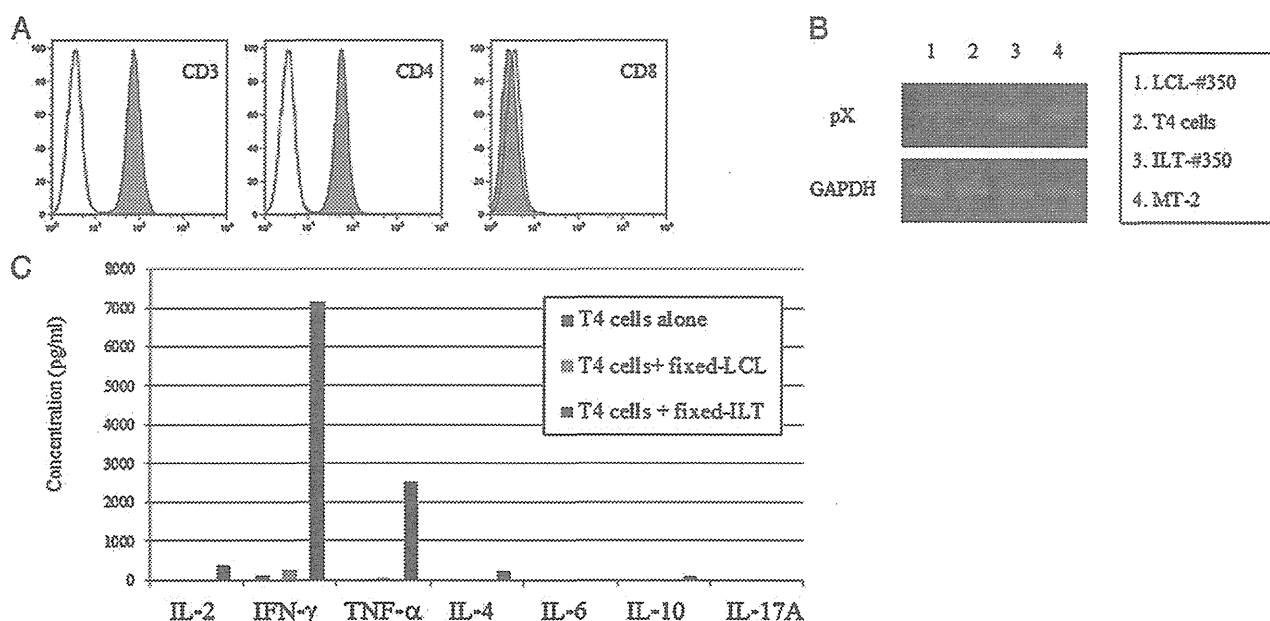


FIGURE 2. Phenotype and function of CD4⁺ T cell line (T4) generated from patient #350. (A) Cell surface phenotype of T4 cells was analyzed by flow cytometry. (B) Total RNA was extracted from LCL-#350 (lane 1), T4 cells (lane 2), ILT-#350 (lane 3), and MT-2 (lane 4). Tax mRNA expression for each cell type was analyzed by RT-PCR. GAPDH was used as an internal control. (C) T4 cells were stimulated for 24 h with or without formaldehyde-fixed ILT-#350 or LCL-#350 cells. The concentration of indicated cytokines in the supernatants was measured using a cytometric bead array system.

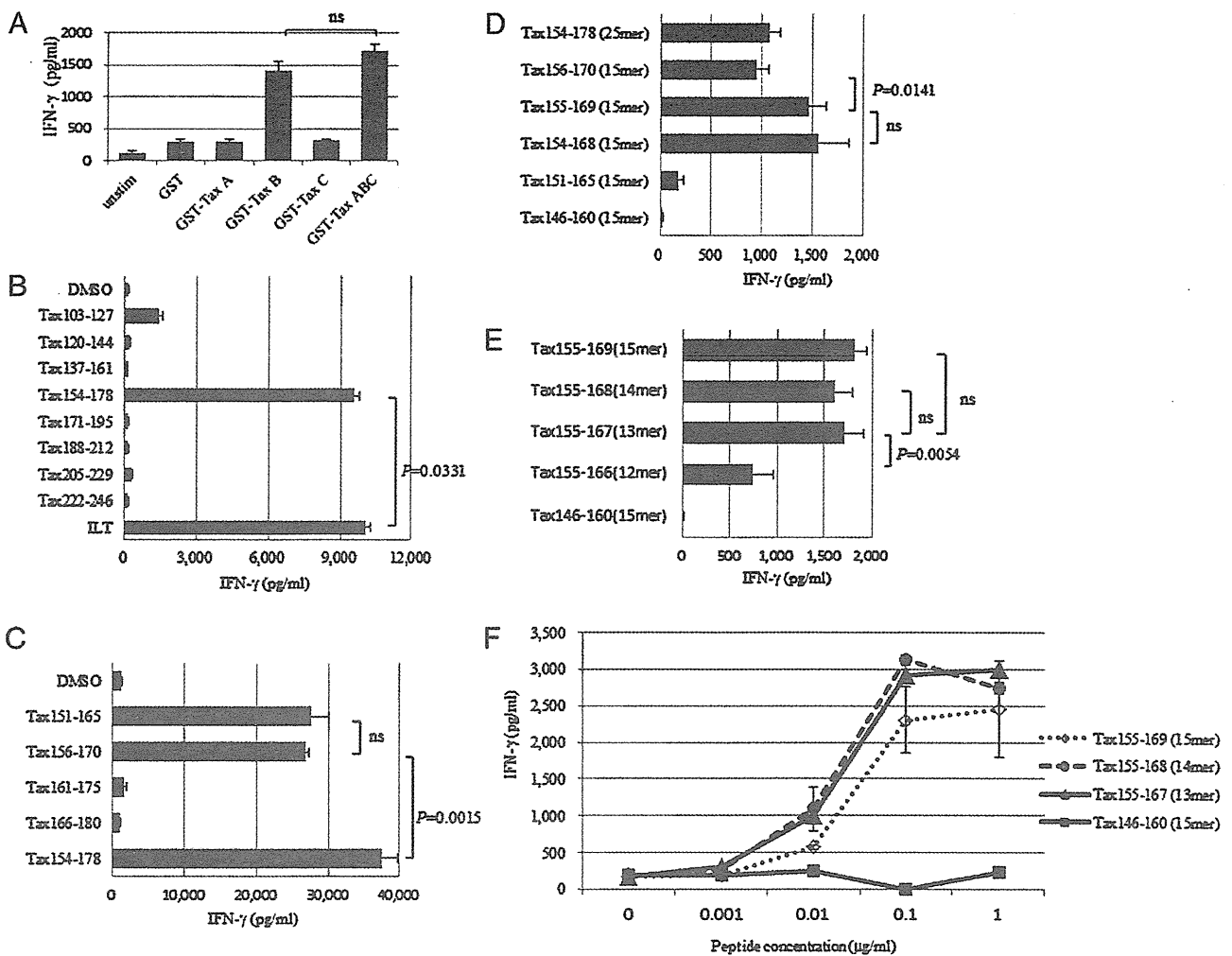


FIGURE 3. Identification of the dominant Tax-derived epitope recognized by established T4 cells. (A) Donor-derived LCL-#350 was pulsed with GST, GST-Tax-A, GST-Tax-B, GST-Tax-C, or a mixture of GST-Tax-A, -B, and -C (GST-TaxABC) for 24 h and then cocultured for 24 h with the T4 cells at a responder/stimulator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

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

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

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

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

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