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# Programmed death-1 (PD-1)/PD-1 ligand pathway–mediated immune responses against human T-lymphotropic virus type 1 (HTLV-1) in HTLV-1-associated myelopathy/tropical spastic paraparesis and carriers with autoimmune disorders

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ABSTRACT

Human T-lymphotropic virus-1 (HTLV-1) causes HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) and adult T-cell leukemia–lymphoma in individuals with dysfunctional immune responses. In this study, to characterize the HTLV-1-specific cytotoxic T lymphocyte (CTL) populations in asymptomatic HTLV-1 carriers (ACs), HAM/TSP patients, and carriers with autoimmune disorders (CAIDs), we examined the role of programmed death-1 and its ligand (PD-1/PD-L1) in HTLV-1-specific CTL functions using an HTLV-1 Tax/HLA-A*0201 tetramer and an HTLV-1 Tax/HLA-A*2402 tetramer. Interestingly, the percentage of HTLV-1 Tax301–309/HLA-A*2402 tetramer$^+$CD8$^+$ cells expressing PD-1 in ACs was significantly higher than the percentage of HTLV-1 Tax11–19/HLA-A*0201 tetramer$^+$CD8$^+$ cells expressing PD-1. PD-1 expression was significantly downregulated on HTLV-1-specific CTLs in HAM/TSP compared with ACs. PD-L1 expression was observed in a small proportion of unstimulated lymphocytes from ACs and was greater in ACs than in HAM/TSP and CAIDs after short-term culture. Furthermore, CTL degranulation was impaired in HAM/TSP, whereas anti-PD-L1 blockade significantly increased CTL function in ACs. Downregulation of PD-1 on HTLV-1-specific CTLs and loss of PD-L1 expression in HAM/TSP and CAIDs, along with impaired function of HTLV-1-specific CTLs in HAM/TSP, may underlie the apparently dysfunctional immune response against HTLV-1.



## 1. Introduction

Human T-lymphotropic virus-1 (HTLV-1) causes HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) and adult T-cell leukemia–lymphoma (ATL) after long-term infection [1–4]. The immune response to HTLV-1 is typically enhanced in HAM/TSP, whereas impaired cell-mediated immunity has been implicated in ATL [5–7]. A variety of autoimmune-like disorders are seen in HTLV-1-infected individuals [8], although the precise relationship between these disorders and HTLV-1 infection remains unclear [9–11].

Natural infection of goats with caprine arthritis encephalitis virus results in an arthropathy that mimics human rheumatoid arthritis (RA) [12]. HTLV-1 infection is a risk factor for the development of RA [13]. Endogenous murine retroviruses are involved in the etiology of a systemic lupus erythematosus (SLE)-like disease in specific inbred strains of mice [14]. Indeed, patients with SLE and concomitant HTLV-1 infection seem to have a more indolent clinical course compared with that of patients with SLE who are not infected with HTLV-1 [15]. Transgenic mice carrying retrovirus-specific genes, including HTLV-1 Tax, show autoimmune-like pathology, suggesting that these viruses have the potential to induce autoimmune disorders [16,17]. However, HTLV-1 is also associated with polyarthritis and proliferative synovitis, known as HTLV-1-associated chronic inflammatory arthropathy, in some HTLV-1 seropositive individuals [18,19]. In addition, there is serologic evidence linking HTLV-1 to Sjögren's syndrome (SS). In the Nagasaki region of Japan, where HTLV-1 is endemic, there is a high prevalence of anti-HTLV-1 antibodies, and 36% of SS patients are positive for these antibodies [20]. In another study from the same region, SS patients exhibited high serum reactivity to HTLV-1 (23%) compared with only 3% of the general population [10] and a high prevalence of HAM/TSP [21,22]. HTLV-1 infection may also play a role in the etiology of animal autoimmune disorders, and the pathogenesis of these diverse autoimmune conditions may be similar, but still different from HAM/TSP. At present, it is not known whether the HTLV-1 Tax-specific cytotoxic T lymphocytes (CTLs) involved in

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HAM/TSP are similar to, or different from, those observed in HTLV-1-associated autoimmune disorders.

Negative immunoregulatory programmed death-1 (PD-1) signaling is involved in autoimmunity, allergy, sites of immune privilege, and antitumor immunity [23–26]. Sharpe et al. demonstrated that PD-1/PD-1 ligand (PD-L1) interactions control the induction and maintenance of peripheral T-cell tolerance and indicated a previously unknown function of PD-L1 on nonhematopoietic cells in protecting tissues from autoimmune attack. The PD-1/PD-L1 pathway may also be exploited by a variety of microorganisms to attenuate antimicrobial immunity and facilitate chronic infection [25].

We previously reported on the repertoire, function, and upregulation of PD-1 expression on HTLV-1-specific CTLs in asymptomatic HTLV-1 carriers (ACs) and ATL patients [27,28]. This increased PD-1 expression suggests that PD-1 signaling plays a role in fostering persistent HTLV-1 infection, facilitating immune evasion by ATL cells, and thereby furthering ATL development [29]. However, despite recognition of the PD-1/PD-L1 pathway in immune dysregulation, there are no reports regarding the PD-1 expression of HTLV-1-specific CTL in diseases related to HTLV-1-host immune responses, such as HAM/TSP or other autoimmune disorders.

Both HAM/TSP and autoimmune disorders may be associated with host immune responses to the virus in HTLV-1-infected individuals [8]. The aim of this study was to characterize HTLV-1-specific CTLs in HTLV-1-infected subjects with dysfunctional immune responses, such as HAM/TSP patients and carriers with autoimmune disorders (CAIDs), and compare them with those from ACs. Therefore, we examined PD-1 expression by HLA-A*0201- or HLA-A*2402-restricted HTLV-1 Tax tetramer-specific CTLs in these groups and the effects of PD-L1 blockade on CTL function.

## 2. Subjects and methods

### 2.1. Subjects

The subjects enrolled in this study comprised 20 ACs (24–81 years old, mean = 58.3), 30 HAM/TSP patients (34–73 years old, mean = 54.6), and 15 HTLV-1-infected patients with autoimmune disorders (32–80 years old, mean = 59.3), all of whom were recruited through the Kagoshima University Hospital. Patients seropositive for HTLV-1, but without clinical symptoms of HTLV-1-related diseases, were designated ACs. Autoimmune disorders included 7 patients with SS, 5 with SLE, and 3 with RA. The study protocol was reviewed and approved by the Medical Ethical Committee of Kagoshima University.

### 2.2. Phenotypic analysis

Phenotypic analysis of peripheral blood mononuclear cells (PBMCs) using an HTLV-1 Tax/HLA-A*0201 tetramer, an HTLV-1 Tax/HLA-A*2402 tetramer, a CMV pp65 (QYDPVAALF)/HLA-A*2402 tetramer, an EBV BRLF1 (TYPVLEEMF)/HLA-A*2402 tetramer (Medical and Biological Laboratories, Nagoya, Japan), anti-PD-1 mAbs (eBioscience, San Diego, CA), and anti-PD-L1 monoclonal antibodies (mAbs; MIH1; eBioscience) was performed as previously described [30–32]. PBMCs were screened by serologic staining with mAbs against HLA-A*02 supertype (clone BB7.2) and HLA-A*24 supertype (clone 17A10) (Medical and Biological Laboratories), followed by secondary staining with goat antimouse IgG-FITC (Immunotech, Miami, FL). The cells were then analyzed by flow cytometry using a FACScan cytometer (BD Biosciences, Mountain View, CA). Subjects expressing neither HLA-A*02 nor HLA-A*24 were excluded from the study.

### 2.3. Intracellular Tax staining assay

PBMCs used for HTLV-1 Tax and PD-L1 expression analysis were cultured for 12 hours and stained as previously described [28]. Briefly, PBMCs ($1 \times 10^6$) were cultured for 12 hours in complete medium (CM; RPMI 1640 supplemented with 100 U/mL penicillin, 0.1 mg/mL streptomycin, 0.05 mM 2-mercaptoethanol, 50 U/mL recombinant human interleukin-2, and 10% heat-inactivated fetal calf serum). For cell-surface antigen analysis, PBMCs were labeled with Cy7-conjugated murine anti-PD-L1 mAbs, anti-CD4-PE, and anti-CD25-allophycocyanin antibody (Becton Dickinson, Mountain View, CA). These cells were further treated with permeabilizing solution (Becton Dickinson). After being washed, the cells were incubated with anti-Tax-FITC (clone Lt4; kindly provided by Y. Tanaka, Ryukyu University, Okinawa, Japan). As a negative control, staining was also performed with an isotype control IgG1-FITC for Tax (Becton Dickinson). Lymphocyte analysis was performed using a FACSCalibur (Becton Dickinson) and data were analyzed using FlowJo software (Tree Star, San Carlos, CA).

### 2.4. CD107a mobilization assay

Assessment of cytolytic ability in the presence of a blocking antibody specific to PD-L1 was performed using flow cytometric quantification of the surface mobilization of CD107a as a marker of degranulation after stimulation, as previously described [27,28]. Briefly, PBMCs ($1 \times 10^6$) were cultured for 6 hours in CM with or without 0.02 $\mu$M HTLV-1 Tax peptide (Sigma–Aldrich, Tokyo, Japan) in combination with anti-CD107a mAb-FITC (clone H4A3; Southern Biotech, Birmingham, AL) and the secretion inhibitor monensin (Becton Dickinson). A blocking antibody specific to PD-L1 (clone MIH1; eBioscience) was added to the cell cultures at a concentration of 10 $\mu$g/mL. The cells were further stained with HLA-tetramer-PE and anti-CD8 mAb-PE/Cy5 (Beckman Coulter) as previously described [28]. Aliquots of $1 \times 10^4$ $CD8^+$ T lymphocytes were examined using a FACSCalibur and data were analyzed using FlowJo software.

### 2.5. Statistical analysis

The Mann–Whitney *U* test and the Wilcoxon matched pairs test were performed using StatView software version 5.0 (SAS Institute, Inc., Cary, NC). $p$ values $< 0.05$ were considered significant.

## 3. Results and discussion

### 3.1. Frequency and PD-1 expression on the HTLV-1-specific tetramer$^+$ cells within the CD8$^+$ lymphocyte population

The frequency of $CD8^+$ T cells binding the Tax301–309/HLA-A*2402 tetramer ranged from 0.09 to 8.64% (2.78 ± 3.74) in AC and from 0 to 26.6% (7.56 ± 8.08) in HAM/TSP. Binding was significantly less in CAIDs compared with that in ACs and HAM/TSP, ranging from 0 to 2.73% (0.76 ± 0.82; $p < 0.05$ and $p < 0.01$, respectively; % ± SD; Table 1). A similar trend was observed for the HLA-A*0201 tetramer in ACs, HAM/TSP, and CAIDs [32], whereas the frequency of HTLV-1 Tax301–309/HLA-A*2402 tetramer$^+$$CD8^+$ cells tended to be lower than that of HTLV-1 Tax11–19/HLA-A*0201 tetramer$^+$$CD8^+$ cells. The frequency of $CD8^+$ T cells binding the Tax11–19/HLA-A*0201 tetramer was 1.65 ± 1.43% in AC, 4.99 ± 5.38% in HAM/TSP, and 0.40 ± 0.47% in CAIDs (data not shown). Furthermore, the percentage of HTLV-1 Tax301–309/HLA-A*2402 tetramer$^+$$CD8^+$ cells expressing PD-1 in ACs was significantly higher than the frequency of HTLV-1 Tax11–19/HLA-A*0201

**Table 1**
The percentage of tetramer$^+$ cells within the $CD8^+$ lymphocyte populations from ACs, HAM/TSP patients, and CAIDs

| | AC | HAM/TSP | CAIDs |
|---|---|---|---|
| HTLV-1 Tax/HLA-A*2402 Tetramer$^+$ cells in $CD8^+$ cells | 2.78 ± 3.74% ($N = 20$) | 7.56 ± 8.08%* ($N = 29$) | 0.64 ± 0.81%* ($N = 15$) |

Results represent the mean ± SD.
*$P < 0.05$ versus AC.