Human T-cell lymphotropic/leukemia virus type 1 (HTLV-1) Tax-specific T-cell exhaustion in HTLV-1-infected individuals

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1INTRODUCTION

Adult T-cell leukemia/lymphoma (ATL) is caused by Human T-cell lymphotropic/leukemia virus type 1 (HTLV-1), and a higher HTLV-1 provirus load in PBMC is a risk factor for ATL development. Here, we document a significant inverse correlation between the function of HTLV-1 Tax-specific CTL (Tax-CTL), as assessed by ex vivo cytokine production in response to cognate peptide, and the HTLV-1 provirus load in PBMC in both HTLV-1 asymptomatic carriers (AC) (Spearman rank correlation coefficient $R_s = -0.494$, $P = .037$, $n = 18$) and ATL patients ($R_s = -0.774$, $P = .001$, $n = 15$). There was also a significant correlation between the HTLV-1 provirus load and the percentage of PD-1-positive Tax-CTL in both HTLV-1 AC ($R_s = 0.574$, $P = .013$) and ATL patients ($R_s = 0.676$, $P = .006$). Furthermore, the percentage of PD-1-positive Tax-CTL was inversely correlated with their function in HTLV-1 AC ($R_s = -0.542$, $P = .020$), and ATL patients ($R_s = -0.639$, $P = .010$). These findings indicate that the function of Tax-CTL decreased as their programmed cell death protein 1 (PD-1) levels increased, parallel to the increased HTLV-1 provirus load in PBMC. We propose that functional Tax-CTL are crucial for determining the HTLV-1 provirus load in PBMC, not only in HTLV-1 AC, but also in ATL, and that PD-1 expression levels are reliable markers of Tax-CTL function. Thus, modulating the immunological equilibrium between Tax-CTL and HTLV-1-infected cells to achieve dominance of functional effectors could represent an ideal strategy for controlling HTLV-1-associated disease.

KEYWORDS
adult T-cell leukemia/lymphoma, CTL, HTLV-1, programmed cell death protein 1, Tax
response for many years, and that eventually a small number escape immunosurveillance and develop into overt ATL. In this scenario, it is important to understand which antigens on the HTLV-1-infected cells are or could be targeted by the host immune response. HTLV-1-associated antigens such as Tax or HBZ,5-7 cancer testis antigens, 8 or neoantigens arising as a consequence of
tumor-specific mutations\textsuperscript{5,10} are all candidates. Of these candidates, immunogenicity of HBZ is not strong.\textsuperscript{2-3} We previously reported that HTLV-1 transmission from mothers to infants through breast milk in early life might induce tolerance to HBZ and result in insufficient HBZ-specific T-cell responses in HTLV-1 asymptomatic carriers or ATL patients.\textsuperscript{7} Cancer testis antigen expression profiles in ATL are variable, thus reducing their utility as therapeutic targets as well.\textsuperscript{8} Neoantigens are, by definition, most likely limited to individual cases.\textsuperscript{9,10} Therefore, here we focused on Tax, which is obligatory for transformation of infected cells by HTLV-1,\textsuperscript{11} and which is relatively strongly immunogenic.\textsuperscript{2,3,5,6} We explored the relationship between the function of HTLV-1 Tax-specific CTL (Tax-CTL) and the HTLV-1 provirus load in PBMC.

\section{Patients and Methods}

\subsection{Primary cells from HTLV-1 AC or ATL patients}

PBMC were isolated from 18 HTLV-1 AC and 15 ATL patients using Ficoll-Paque centrifugation (Pharmacia, Uppsala, Sweden). Of the 15 ATL patients, 1 with a chronic and 1 with a smoldering subtype were carefully observed using a watch-and-wait approach. Among the remaining 13 patients, 9 had been in remission for aggressive ATL after systemic chemotherapy and/or treatment with mogamulizumab\textsuperscript{12-14} for more than 6 months before blood draw for the present study. The remaining 4 were in remission after allogeneic hematopoietic stem cell transplantation (HSCT) from an unrelated HTLV-1-negative donor more than 2 years earlier. The transplanted patients had been free of any immunosuppressive treatment for more than 6 months prior to the study. All donors provided written informed consent before sampling, according to the Declaration of Helsinki, and the present study was approved by the institutional ethics committee of Nagoya City University Graduate School of Medical Sciences.

\section{Human leukocyte antigen typing}

Human leukocyte antigen (HLA)-A genotyping was carried out using WAKFlow\textsuperscript{9} HLA-typing kits (WAKUNAGA Pharmacy Co. Ltd, Hiroshima, Japan). In the present study, all enrolled individuals had at least 1 HLA-A*02:01, -A*02:06, or -A*24:02 allele.

\subsection{Antibodies, tetramers, and flow cytometry}

Phycoerythrin (PE)-conjugated HLA-A*02:01/Tax11-19 and HLA-A*24:02/Tax301-309 tetramers, peridinin chlorophyll protein-conjugated anti-CD8 monoclonal antibody (mAb) (SK1) (Medical & Biological Laboratories, Co., Ltd, Nagoya, Japan), allophycocyanin (APC) conjugated anti-PD-1 mAb (EH12.2H7; BioLegend, Inc., San Diego, CA, USA), FITC-conjugated anti-T-cell immunoglobulin and mucin domain-containing protein-3 (anti-TIM-3) mAb (344823), FITC-conjugated anti-lymphocyte-activation gene 3 (anti-LAG-3) Ab (FAB2319A) (both from R&D Systems Inc., Minneapolis, MN, USA), and APC-conjugated anti-cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) mAb (BN13) (BD Biosciences, San Jose, CA, USA) were used here. For intracellular staining, cells were cocultured with or without cognate peptide (final concentration 100 nmol/L) at 37°C in 5% CO\textsubscript{2} for 3 hours, after which brefeldin A (BD Biosciences) was added. The cells were then incubated for an additional 2 hours. Subsequently, they were stained with FITC-conjugated anti-interferon (IFN)-γ (45.15; Beckman Coulter, Fullerton, CA, USA) and APC-conjugated anti-tumor necrosis factor (TNF)-α (MAB11; eBioscience, San Diego, CA, USA) mAbs, using the Intracellular Fixation & Permeabilization Buffer Set (88-8824-00; eBioscience). An appropriate isotype control Ab for each Ab was used for drawing the quadrant lines in each case. Cells were analyzed on a FACSCalibur (BD Biosciences) with the aid of FlowJo software (Tree Star, Inc. Ashland, OR, USA). The HTLV-1 provirus load in PBMC was quantified by SRL, Inc. (Tokyo, Japan).
2.4 | Absolute number of Tax-CTL

Absolute numbers of PD-1-positive and -negative Tax-CTL were calculated from the total lymphocyte count (/µL) and the percentages of PD-1-positive and -negative Tax-CTL in this population.

2.5 | Statistical analysis

Correlations between 2 variables were assessed using the Spearman rank correlation coefficient (Rs). The analyses were carried out with SPSS Statistics 17.0 (IBM Corporation, Armonk, NY, USA). In this study, \( P < .05 \) (two-sided) was considered significant.

3 | RESULTS

There was a significant positive correlation between the HTLV-1 provirus load in PBMC and the percentage of PD-1-positive Tax-CTL in HTLV-1 AC (\( R_s = 0.574, \ P = .013 \); Figure 1A). In contrast, there were no significant correlations between provirus load in PBMC and TIM-3-, LAG-3-, or CTLA-4-positive Tax-CTL (\( R_s = -0.043, \ P = .864; \ R_s = 0.368, \ P = .133; \ R_s = -0.172, \ P = .494 \), respectively). Thus, PD-1 expression levels on Tax-CTL increased as the HTLV-1 provirus load in PBMC increased in HTLV-1 AC. Consistent with this, there was a significant inverse correlation between the percentage of PD-1-positive Tax-CTL and the percentage of Tax-CTL producing both IFN-\( \gamma \) and TNF-\( \alpha \) ex vivo in response to cognate peptide (\( R_s = -0.542, \ P = .020 \) (Figure 1B). Thus, cytokine production by Tax-CTL decreased as their PD-1 expression increased. In contrast, there were no significant correlations between the percentages of TIM-3-, LAG-3-, or CTLA-4-positive Tax-CTL and the frequency of cells producing both IFN-\( \gamma \) and TNF-\( \alpha \) ex vivo in response to cognate peptide (\( R_s = -0.038, \ P = .880; \ R_s = 0.044, \ P = .861; \ R_s = 0.003, \ P = .991 \), respectively). Finally, we did find a significant inverse correlation between the HTLV-1 provirus load in PBMC and the percentages of IFN-\( \gamma \) and TNF-\( \alpha \)-producing Tax-CTL in HTLV-1 AC (\( R_s = -0.494, \ P = .037 \) (Figure 1C). Thus, cytokine production by Tax-CTL decreases as the HTLV-1 provirus load in PBMC increases. Quantification of PD-1 and TIM-3, LAG-3 and CTLA-4 expression on Tax-CTL, and cytokine production in response to cognate peptide, is shown in Figure 1D (for HTLV-1 AC numbers 1-4).

Next, we undertook the same analysis with ATL patients. As observed in the HTLV-1 AC, there was a significant positive correlation between the HTLV-1 provirus load in PBMC and the percentage of PD-1-positive Tax-CTL (\( R_s = 0.676, \ P = .006 \); Figure 2A), but no correlations with TIM-3-, LAG-3-, or CTLA-4-positivity (\( R_s = 0.275, \ P = .321; \ R_s = -0.154, \ P = .584; \ R_s = -0.095, \ P = .737 \), respectively. Subsequently, we observed a significant inverse correlation between the percentage of PD-1-positive Tax-CTL, and the percentage of IFN-\( \gamma \)- and TNF-\( \alpha \)-producing Tax-CTL ex vivo, after stimulation with cognate peptide (\( R_s = -0.639, \ P = .010 \) (Figure 2B). However, in this instance, we also observed a significant inverse correlation between the percentage of TIM-3-positive Tax-CTL and the percentage of cells producing IFN-\( \gamma \)- and TNF-\( \alpha \)-ex vivo after stimulation with cognate peptide (\( R_s = -0.532, \ P = .041 \). Nonetheless, again there were no significant correlations for LAG-3 (\( R_s = 0.045, \ P = .874 \), or CTLA-4 (\( R_s = -0.004, \ P = .988 \) expression. Finally, there was a significant inverse correlation between the HTLV-1 provirus load and the percentage of IFN-\( \gamma \)- and TNF-\( \alpha \)-producing Tax-CTL (\( R_s = -0.774, \ P = .001 \) (Figure 2C). Note that the four gray circles in Figure 2A-C denote ATL patients who had received allogeneic HSCT. The levels of PD-1 and TIM-3 or LAG-3 and CTLA-4 expression by Tax-CTL, and their cytokine production in response to cognate peptide, are shown in Figure 2D for ATL patients numbers 1-4.

Because the present observations indicated that the function of Tax-CTL decreases as their PD-1 levels increase, parallel to the increased HTLV-1 provirus load in PBMC, we determined the absolute number of Tax-CTL in the blood. In HTLV-1 AC, there were no significant correlations between the HTLV-1 provirus load in PBMC and the absolute number of PD-1-negative Tax-CTL (\( R_s = -0.152 \).
**Figure 1:**

(A) Scatter plot showing the correlation between PD-1 positive cells on Tax-CTL (%) and HTLV-1 provirus load (copies/1000 PBMC). The correlation coefficient Rs = 0.676, P = 0.006.

(B) Scatter plot showing the correlation between IFNγ & TNFα producing cells of Tax-CTL (%) and HTLV-1 provirus load (copies/1000 PBMC). The correlation coefficient Rs = -0.639, P = .010.

(C) Scatter plot showing the correlation between IFNγ & TNFα producing cells of Tax-CTL (%) and HTLV-1 provirus load (copies/1000 PBMC). The correlation coefficient Rs = -0.774, P = .001.

(D) Flow cytometry analysis showing the expression of various markers on CD8+ T cells:
- **1.** CD8+ T cells with PD-1 and CTLA-4 expression.
- **2.** Increased expression of PD-1 and TIM-3.
- **3.** Increased expression of PD-1 and LAG-3.
- **4.** Increased expression of PD-1 and IFNγ.
FIGURE 3 Relationships between the absolute number of Tax-specific cytotoxic T cells (Tax-CTL) and the Human T-cell lymphotropic/leukemia virus type 1 (HTLV-1) provirus load in PBMC from HTLV-1 asymptomatic carriers (AC) and in adult T-cell leukemia/lymphoma (ATL) patients. A, No significant correlation between HTLV-1 provirus load in PBMC and the absolute number of PD-1-negative Tax-CTL in HTLV-1 AC ($Rs = -0.152$, $P = .548$). B, No significant correlation between HTLV-1 provirus load in PBMC and the absolute number of PD-1-positive Tax-CTL in HTLV-1 AC ($Rs = 0.126$, $P = .618$). C, Significant inverse correlation between HTLV-1 provirus load in PBMC and the absolute number of PD-1-negative Tax-CTL in ATL patients ($Rs = -0.561$, $P = .029$). D, No significant correlation between HTLV-1 provirus load in PBMC and the absolute number of PD-1-positive Tax-CTL in ATL patients ($Rs = -0.061$, $P = .830$). Plots labeled 1, 2, 3, and 4 in (A, B) correspond to 1, 2, 3, and 4 in Figure 1, respectively. Plots labeled 1, 2, 3, and 4 in (C, D) correspond to 1, 2, 3, and 4 in Figure 2, respectively. The 4 symbols in gray in (C, D) indicate ATL patients after allogeneic hematopoietic stem cell transplantation.
Tax, such as Tax peptide-pulsed dendritic cell vaccination, would development of overt ATL. In this context, a treatment strategy targeting CTL to HTLV-1-infected cells attenuates their function, which would present study indicates that persistent continuous exposure of Tax-immunodeficiency virus (HIV) infections, and also in cancer. The present study indicates that persistent continuous exposure of Tax-CTL to HTLV-1-infected cells attenuates their function, which would allow the latter to proliferate more and potentially lead to the development of overt ATL. In this context, a treatment strategy targeting Tax, such as Tax peptide-pulsed dendritic cell vaccination, would seem to be promising, especially for preventing ATL development in HTLV-1 AC by means of suppressing the proliferation of HTLV-1-infected cells. In addition, the present study indicates that PD-1 is a reliable marker of Tax-CTL exhaustion. Thus, a treatment strategy targeting PD-1 should also be effective for ATL patients. Accordingly, we are now conducting an investigator-initiated phase II study of nivolumab for relapsed/refractory ATL (UMIN0000020601). However, in this respect, it must be noted that rapid progression of ATL after nivolumab treatment has been reported by other investigators. The mechanisms responsible for ATL progression on nivolumab have not yet been determined, but, clearly, great caution is required in our own phase II study of nivolumab for ATL. Although TIM-3, LAG-3 and CTLA-4 have also been reported to be markers of exhausted T-cells, the present study showed that these were not reliable markers of Tax-CTL exhaustion. The reason for this is not clear, but it is conceivable that appropriate markers of exhaustion might vary according to the type of CTL.

Allogeneic HSCT is considered to be the only curative treatment for aggressive ATL. In this context, it is notable that the functions of the Tax-CTL originating from HTLV-1-negative donors in the present transplanted patients were generally well retained, and the fraction of PD-1-positive cells was low. This is likely to be related to the control of HTLV-1-infected cells in these patients after allogeneic HSCT. Although an earlier study analyzed PD-1 expression in general on lymphocytes in HTLV-1 AC or in ATL patients comprehensively, the present study quantified PD-1 expression levels on Tax-CTL. Relating these to the HTLV-1 provirus load in PBMC and to the functional integrity of Tax-CTL has not been previously reported. Herein lies the novelty and significance of this work.

In conclusion, the functional integrity of Tax-CTL is crucial for determining HTLV-1 provirus load in PBMC not only in HTLV-1 AC, but also in ATL patients. Exploiting the immunological equilibrium between Tax-CTL vs HTLV-1-infected cells to achieve long-term dominance of functional Tax-CTL would represent an ideal strategy for controlling HTLV-1-associated disease.

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CONFLICTS OF INTEREST

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