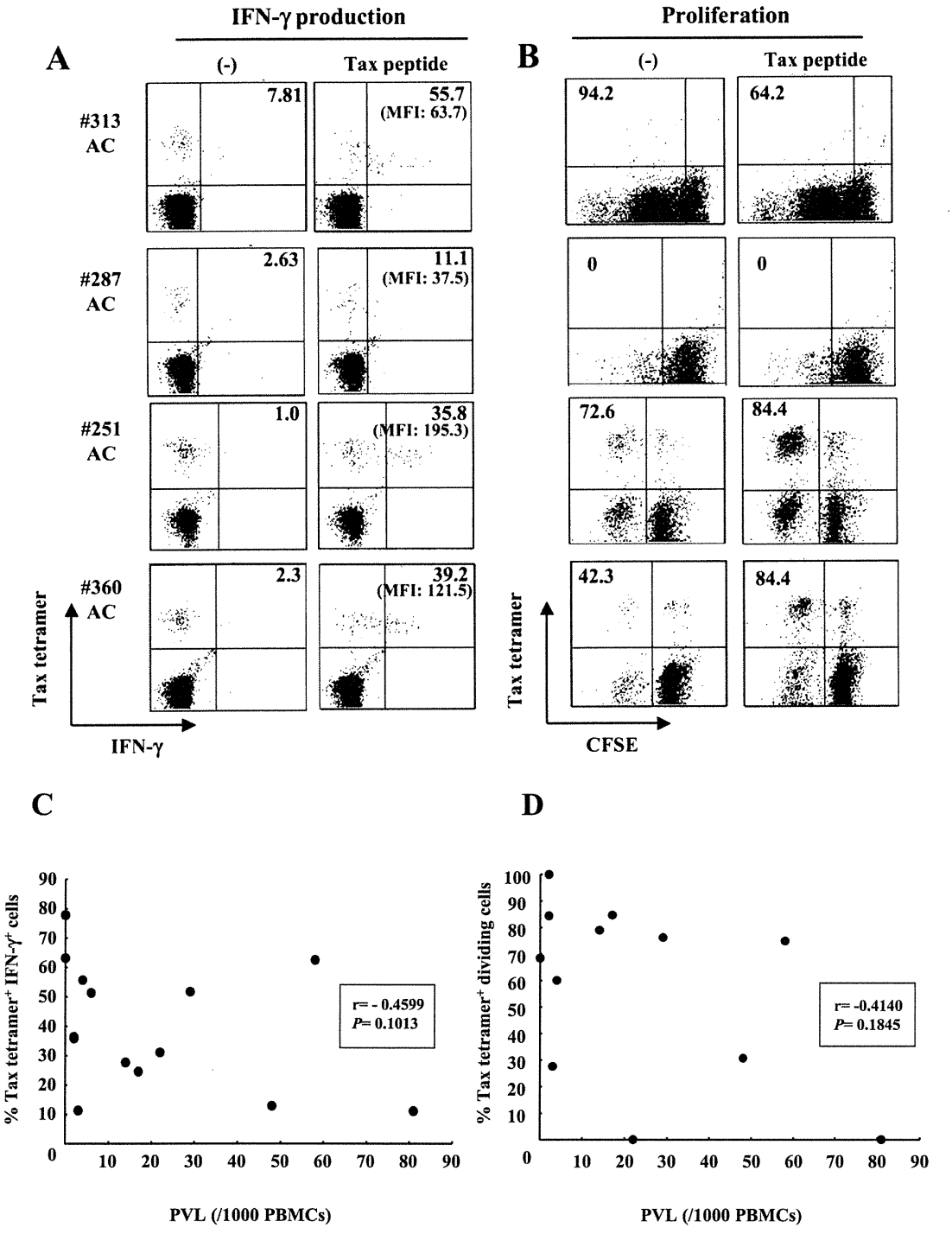

Figure 3 IFN-γ production and cell proliferation of Tax-specific CD8$^+$ T-cells in ACs. (A, B) IFN-γ production (A) and cell proliferation (B) of Tax-specific CD8$^+$ T-cells in PBMCs from 4 ACs were assessed as in Figure 2. The number given in parenthesis shows mean fluorescence intensity (MFI) of IFN-γ expression in the IFN-γ$^+$ tetramer$^+$ cells. (C, D) Relation between the percentage of IFN-γ$^+$ (C) or dividing (D) Tax-specific CD8$^+$ T-cells and proviral loads (PVL) in ACs. Dots represent individual ACs. The Spearman rank correlation test was used to determine correlations and *P* values.

to 0.2% after stimulation with Tax peptide, and was not recovered by LPS stimulation (Figure 4A). In addition, HTLV-1-infected cells have been reported to express C-C chemokine receptor type 4 (CCR4) and have FoxP3$^+$ Treg-like function[18,40]. However, the proliferative ability of Tax-specific CD8$^+$ T-cells in #287 was not restored even in the absence of CCR4$^+$ infected cells (data not shown).

To further examine the function of Tax-specific CD8$^+$ T-cells in #313 and #287, we observed the expression of CD69, an early activation marker transiently expressed on T lymphocytes that precedes cytokine secretion after antigenic stimulation, and CD107a, a marker of degranulation associated with cytotoxic activity in an antigen-specific manner[41]. CD69 was up-regulated on Tax-specific CD8$^+$ T-cells in #313 when stimulated with Tax peptide, but not in #287, which was in agreement with their abilities to produce IFN-γ (Figure 4B). In #313, 22.4% of Tax-specific CD8$^+$ T-cells mobilized CD107a to the surface during a 6-hr culture with Tax peptide stimulation, while CD107a surface expression was detected on 4% of Tax-specific CD8$^+$ T-cells in the culture without stimulation (Figure 4C). However, no CD107a mobilization was detected on the surface of Tax-specific CD8$^+$ T-cells in #287 with or without Tax peptide stimulation (Figure 4C). These results indicate that HTLV-1-specific CD8$^+$ T-cells in AC #287 did not properly activate upon antigen stimulation, and therefore failed to control HTLV-1-infected cells.

The Tax/HLA tetramers used in this study allow us to evaluate the functions of CD8$^+$ T-cells only against an immunodominant epitope, Tax. We therefore compared HTLV-1 Gag p19 in the culture between whole and CD8$^+$ cell-depleted PBMCs to examine the role of total HTLV-1-specific CD8$^+$ T-cells including the dominant Tax-specific CD8$^+$ T-cells, in suppression of HTLV-1 production from infected cells (Figure 4D). As expected, depletion of CD8$^+$ cells from PBMCs in #313 led to significantly higher HTLV-1 production compared to whole PBMCs (P = 0.0115). In contrast, HTLV-1 p19 production increased only a little in the culture of CD8$^+$ cell-depleted PBMCs in #287 (P = 0.1563), indicating that HTLV-1-specific CD8$^+$ T-cells other than the dominant Tax-specific CD8$^+$ T-cells might have a reduced ability to control the infected cells in this donor. It is of note that HTLV-1-infected cells from both two donors carried intact HTLV-1 proviral genomic DNA because HTLV-1 p19 could be detected after 7 day-culture.

Phenotypic analysis of functional and dysfunctional Tax-specific CD8$^+$ T-cells

We next characterized the differentiation status of memory T-cells in Tax-specific CD8$^+$ T-cells. Human CD8 T-cells may be classified as naïve T-cells (CD45RA$^+$CCR7$^+$CD27$^+$), T_{CM} (CD45RA$^-$CCR7$^+$CD27$^+$), T_{EM} (CD45RA$^-$CCR7$^-$CD27$^+$), and T_{Diff} (CD45RA$^+$CCR7$^-$CD27$^-$) cells[42-44]. As shown in Figure 5A, almost all Tax-specific CD8$^+$ T-cells in both #313 and #287 were skewed to CD45RA$^-$CCR7$^-$CD27$^+$ T_{EM} cells, and there was no essential difference between two donors.

A previous report has shown that PD-1 was highly up-regulated on Tax-specific CD8$^+$ T-cells in ATL patients and ACs[32]. We therefore examined PD-1 expression on Tax-specific CD8$^+$ T-cells in several AC samples, including #287. The frequency of PD-1$^+$ Tax-specific CD8$^+$ T-cells was very high in #309 (85.3%) and #313 (96%) (Figure 5B and Table 2) while those Tax-specific CD8$^+$ T-cells retained the proliferative and the cytokine-producing abilities (Figure 3A and Table 2). In #287, the frequency of PD-1-expressing Tax-specific CD8$^+$ T-cells (55.6%) was lower than #309 and #313, but higher than that of PD-1$^+$ CMVpp65-specific CD8$^+$ T-cells in the same donor (Figure 5B). The levels of PD-1 expression showed a similar tendency to the frequency of PD-1$^+$ T-cells. In addition, the blockade of PD-1/PD-ligand 1 (PD-L1) pathway did not restore the proliferative capacity of Tax-specific CD8$^+$ T-cells in #287 (data not shown).

Conserved functions of CMV-specific CD8$^+$ T-cells in #287

We next examined whether the impairment of proliferative capacity and effector functions observed in #287 CD8$^+$ T-cells were specific for HTLV-1 antigens or the result of general immune suppression. PBMC from #287 contained CMVpp65-specific CD8$^+$ T-cells (2.3% of CD8$^+$ T-cells), as detected by tetramer staining. The frequency of CMVpp65-specific CD8$^+$ T-cells increased from 2.3% to 66.0% following in vitro CMVpp65 peptide stimulation, but not without the peptide stimulation (Figure 6A). Antigen-specific IFN-γ and CD69 expression were clearly detected in CMVpp65-specific CD8$^+$ T-cells in #287 (Figures 6B and 6C). Furthermore, CMVpp65-specific CD8$^+$ T-cells mobilized CD107a to the surface in response to CMVpp65 peptide (Figure 6D). These results demonstrate that in #287, CMVpp65-specific CD8$^+$ T-cells, but not Tax-specific CD8$^+$ T-cells, have proliferative potential and effector functions, such as cytotoxic activity and IFN-γ release, suggesting that the impaired CD8$^+$ T-cell function in #287 was specific for HTLV-1.

Dysfunction of Tax-specific but not CMVpp65-specific CD8$^+$ T-cells also in sATL patients

Finally, we extended the study to see whether patients with early stage ATL might exhibit similar dysfunction selective for HTLV-1-specific CD8$^+$ T-cells. We found two smoldering ATL (sATL) patients (#110 and #353)

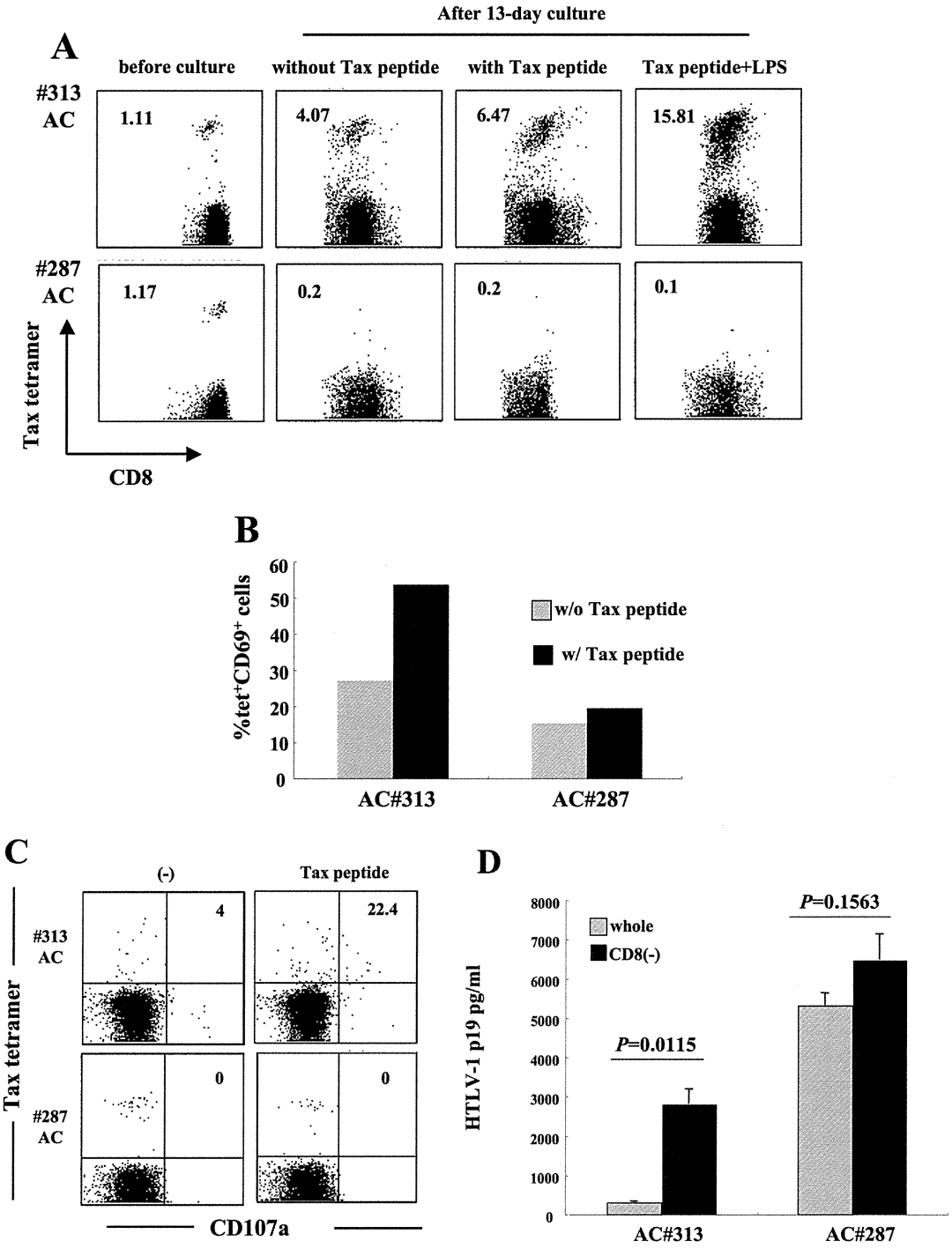

Figure 4 Dysfunction of Tax-specific CD8$^+$ T-cells and inefficient CD8$^+$ cell-mediated HTLV-1 control in AC#287. (A) For antigen-specific T-cell proliferation, PBMCs from #313 and #287 were cultured for 13 days with or without Tax peptide in the presence or absence of 0.1 μg/ml LPS. The number indicates the percentage of tetramer$^+$ cells in CD8$^+$ T-cells. (B, C) PBMCs were stimulated with or without 10 μM Tax peptide for 6 hrs. The expression of CD69 (B) and CD107a (C) in Tax-specific CD8$^+$ T-cells was analyzed by flow cytometry. (B) Bar indicates the percentage of CD69$^+$ cells in Tax-specific CD8$^+$ T-cells. (C) The number represents the percentage of CD107a$^+$ cells in Tax-specific CD8$^+$ T-cells. (D) Whole PBMCs and CD8-depleted fractions in ACs (#287 and #313) were cultured for 7 days and HTLV-1 p19 in the supernatants were measured by HTLV-1 p19 ELISA. *P* value was determined by the unpaired *t* test.

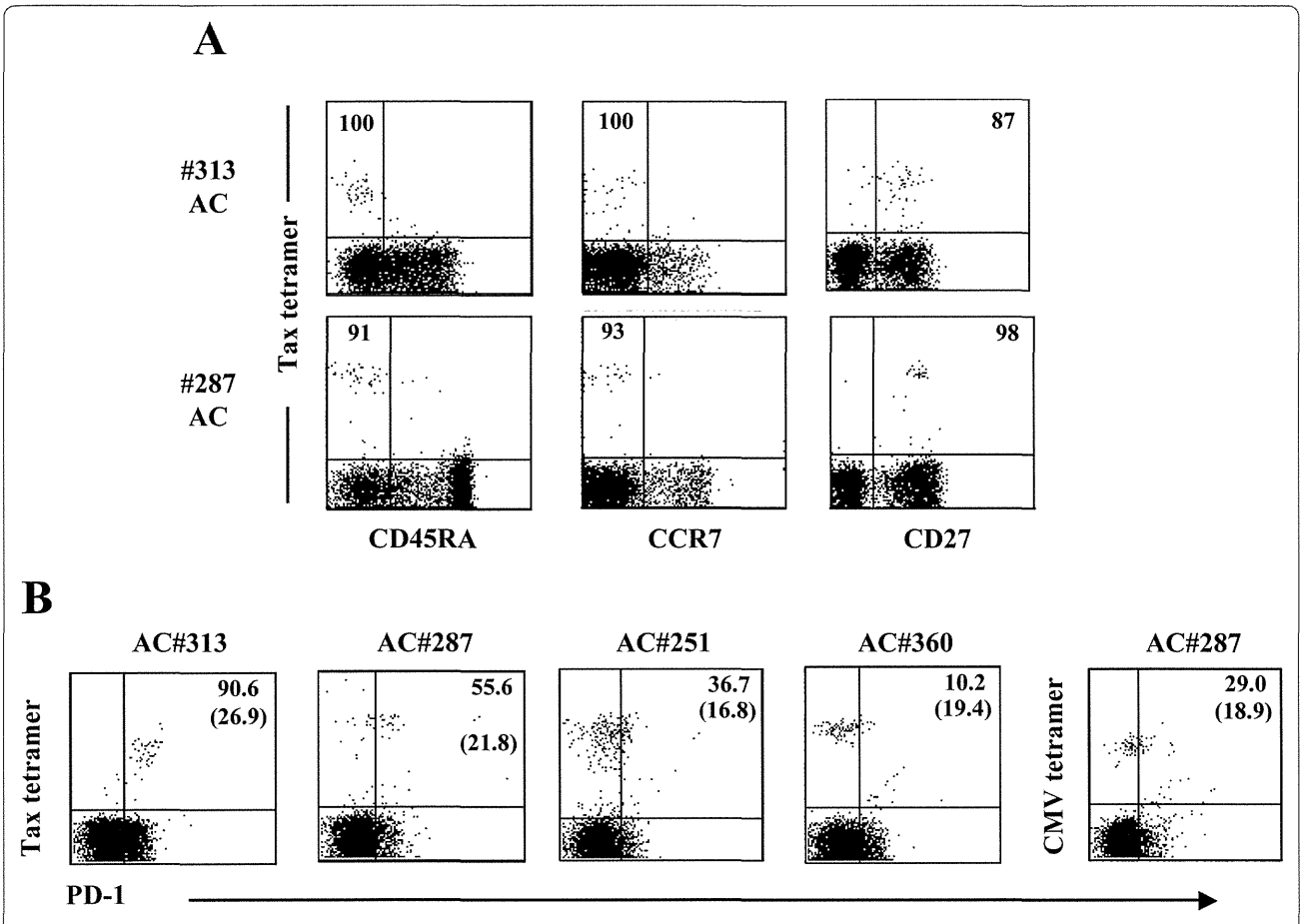

Figure 5 Phenotypic analysis of functional and dysfunctional Tax-specific CD8$^+$ T-cells. (A) Differentiation memory phenotype, based on the expression of CD45RA, CCR7, and CD27 and (B) PD-1 expression of Tax-specific CD8$^+$ T-cells from ACs were examined by flow cytometry. The number represents the percentage of indicated marker-positive or -negative cells in tetramer$^+$ CD8$^+$ T-cells. The number given in parenthesis shows MFI of PD-1 expression on the PD-1$^+$ tetramer$^+$ cells.

possessing 6.89% and 3.15% of tetramer-binding Tax-specific CD8$^+$ T-cells, respectively. The sATL patient #353 carried 5% of abnormal lymphocytes (ably) with a normal range of lymphocyte number, whose status is very close to the borderline with ACs. Patient #110 carried 4% of abnormal lymphocytes with mild lymphocytosis. Tax-specific CD8$^+$ T-cells of two sATL patients (#110 and #353) did not proliferate in response to Tax peptides as similarly observed in a cATL patient (#224) (Figure 7A) and most other cATL patients (Figure 2A and Additional file 1). In contrast, CMVpp65-specific CD8$^+$ T-cells in both sATL patients vigorously proliferated when stimulated with CMVpp65 peptides. CMVpp65-specific CD8$^+$ T-cells in a cATL (#224) also proliferated, but to a lesser degree, which might reflect general immune suppression in this patient (Figure 7).

Discussion

In this study, we detected Tax-specific CD8$^+$ T-cells in 87%, but not the rest of ACs tested, by using tetramers containing Tax major epitope-peptides presented by HLA-A*0201, A*1101, and A*2402. Tax-specific CD8$^+$ T-cells were also detected in 38% of cATL patients, but at reduced frequencies and with severely impaired functions. Further analysis of Tax-specific CD8$^+$ T-cells in 14 ACs indicated that they were functional in most of ACs tested except one (#287), whose Tax-specific CD8$^+$ T-cells poorly responded to specific peptides. However, CMVpp65-specific CD8$^+$ T-cells of this individual were fully functional. Similar T-cell dysfunction selective for HTLV-1, but not CMV, was also observed in sATL patients, one of which (#353) had no clinical symptoms but 5% abnormal lymphocytes. General immune suppression might partly account for the scarcity and/or the dysfunction of Tax-specific CD8$^+$ T-cells in ATL patients, but not those in the AC or the sATL patients as they were selective for HTLV-1. These findings suggest that HTLV-1-specific immune suppression is undergoing in a minor group of ACs and an early stage of ATL.

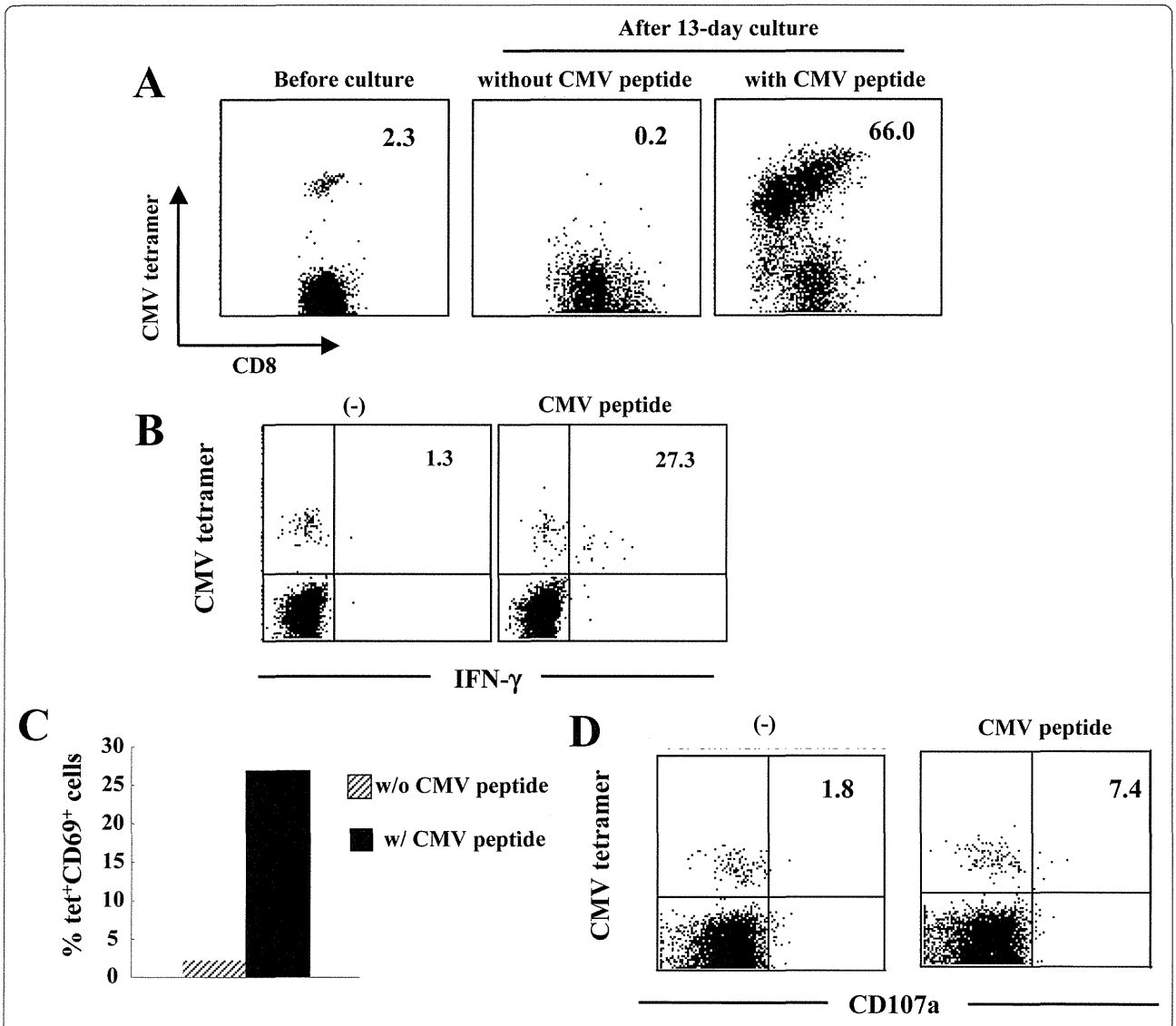

Figure 6 Conserved functions of CMV-specific CD8$^+$ T-cells in AC#287. (A) For antigen-specific T-cell proliferation, PBMCs from #287 were cultured for 13 days with or without 100 nM CMV peptide. The number indicates the percentage of CMV tetramer$^+$ cells in CD8$^+$ T-cells. (B-D) PBMCs were stimulated with or without 10 μM CMV peptide for 6 hrs. IFN-γ production (B), CD69 (C) and CD107a (D) expression of CMVpp65-specific CD8$^+$ T-cells in #287 was analyzed by flow cytometry. (B, D) The number represents the percentage of the indicated marker-positive cells in CMVpp65-specific CD8$^+$ T-cells. (C) Bar indicates the percentage of CD69$^+$ cells in CMV-specific CD8$^+$ T-cells.

The presence of tetramer-binding Tax-specific CD8$^+$ T-cells in cATL patients, although at low frequencies, implies that they have encountered antigen during the chronic phase of ATL disease, suggesting that Tax may be expressed *in vivo*. This may be supported by a previous report showing that virus-specific CD8$^+$ T-cells fails to acquire memory T-cell property of long-term antigen-independent persistence during chronic lymphocytic choriomeningitis virus (LCMV) infection[45]. However, there is no direct evidence that infected cells produce Tax in infected individuals. HTLV-1-specific T-cell responses in cATL patients are largely different from HAM/TSP patients. In HAM/TSP patients, Tax-specific CD8$^+$ T-cells proliferated vigorously and a large population of them produced IFN-γ. In contrast, the function of Tax-specific CD8$^+$ T-cells in cATL patients was profoundly suppressed, similarly to tumor infiltrating lymphocytes (TIL)[46]. In cATL patients, Tax-specific CD8$^+$ T-cells that were detected before culture decreased in number to undetectable or very low levels after 6 days, regardless of peptide stimulation (data not shown). This is not likely to be due to TCR down-regulation, because TCRs on Tax-specific CD8$^+$ T-cells in HAM/TSP patients are down-regulated on days 1 to 4

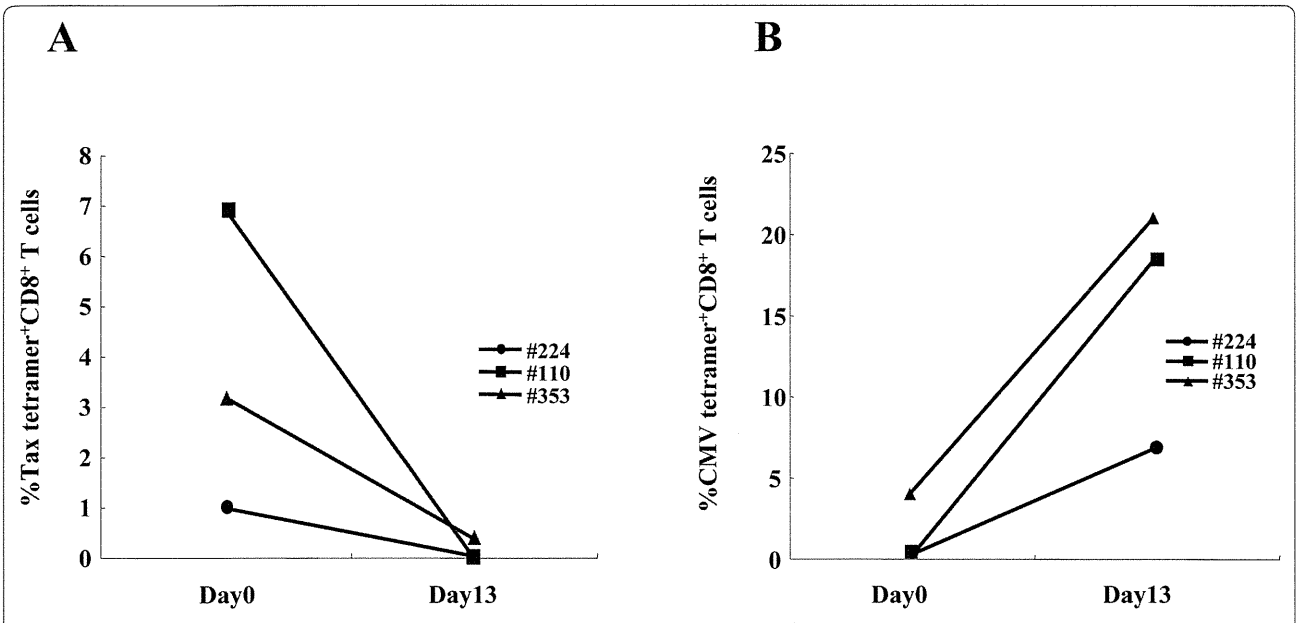

Figure 7 Impaired proliferation of Tax-specific but not CMVpp65-specific CD8$^+$ T-cells in sATL patients. For antigen-specific T-cell proliferation, PBMCs from sATL (#110; square, #353; triangle) and cATL (#224; circle) patients were cultured for 13 days with 100 nM Tax (A) or CMV (B) peptide. Each dot indicates the percentage of tetramer$^+$ cells in CD8$^+$ T-cells at day 0 and day 13 after culture. Clinical information on ATL patients used here is as follows; sATL#110: age; 40 s, gender; F, WBC#; 11,000/μL {lymphocyte (lym); 39%, abnormal lymphocytes (ably); 4%}, cATL#224: age; 50 s, gender; F, WBC#; 7900/μL (lym; 30%, ably; 33%), sATL#353: age; 60 s, gender; M, WBC#; 4620/μL (lym; 39%, ably; 5%).

and reappeared by day 6 in vitro[34]. Moreover, we could not observe any tetramer$^+$ CD8$^+$ T-cells even in the 13-day culture (data not shown), suggesting these cells might have died during the culture.

Severe dysfunction of Tax-specific CD8$^+$ T-cells was observed not only in cATL patients, but also in an AC #287. Fresh PBMCs of #287 contained 1.17% tetramer$^+$ cells in the CD8$^+$ T-cell fraction. However, none of these tetramer-positive T-cells proliferated in culture, with or without Tax peptide stimulation (Figure 3B). Although a few populations of them (11.1%) produced a small amount of IFN-γ, they lacked degranulation activity for cytotoxicity or expression of CD69, an early activation marker, upon specific stimulation (Figures 3 and 4). Importantly, CMVpp65-specific CD8$^+$ T-cells in the same donor were clearly activated, and exhibited these characteristics upon stimulation with pp65 peptides (Figure 6). These observations indicated that the impaired Tax-specific CD8$^+$ T-cells function in #287 was not attributable to general immune suppression, but to an HTLV-1-specific phenomenon. In addition, CD8-depletion study indicated that not only the dominant Tax-specific CD8$^+$ T-cell function but also other HTLV-1-specific CD8$^+$ T cell responses might be reduced in #287 (Figure 4D). Since CMV-specific CD8$^+$ T-cells responded well to the specific peptides, antigen-presenting cells in culture were not likely to be responsible for the selective suppression of Tax-specific CD8$^+$ T-cells. In addition, it has been shown that HTLV-1-infected cells generally express CCR4 and have Treg-like function[18,40]. However, depletion of CCR4$^+$ cells did not restore the proliferative ability of Tax-specific CD8$^+$ T-cells (data not shown), indicating that suppression of the infected cells were not likely to be the major reason for the impaired Tax-specific CD8$^+$ T-cell function in our culture system. These observations suggest that in #287, Tax-specific CD8$^+$ T-cells themselves might lose their functions.

Many chronic viral infections affect the phenotype, function, and maintenance of memory T-cells [24,42,47,48]. T_{EM} cells predominate in infections in which relatively high levels of antigen persist and continuous antigen stimulation are required for maintenance of T_{EM} cells. As described in HAM/TSP patients [34], Tax-specific CD8$^+$ T-cells in both ACs (#287 and #313) were primarily enriched in T_{EM} memory pool in spite of the functionality of Tax-specific CD8$^+$ T-cells (Figure 5A), which may support continuous or periodical expression of viral antigen in vivo during an asymptomatic stage.

PD-1 is known to play a major role in regulating T-cell exhaustion during chronic infection. In this study, we could not obtain any data supporting the involvement of PD-1 in the dysfunction of Tax-specific CD8$^+$ T-cells. However, we observed that Tax-specific CD8$^+$ T-cells in some ACs showed IFN-γ production, but not

proliferative capacity (Table 2). This partially lacked function of Tax-specific $CD8^+$ T-cells is similar to the features of T-cell exhaustion. Whether Tax-specific $CD8^+$ T-cells are exhausted in HTLV-1 infection, and whether other molecules associated with T-cell exhaustion are involved in the impairment of Tax-specific $CD8^+$ T-cell responses are necessary to be clarified because some inhibitory molecules such as T-cell immunoglobulin and mucin domain-containing protein-3 (TIM-3), lymphocyte activated gene-3 (LAG-3), and transcription factors including BLIMP-1 are also found to be associated with T-cell exhaustion [49].

The incidence of Tax-specific $CD8^+$ T-cell detection was high (87.0%) in ACs. Given the fact that the incidence of Tax-specific $CD8^+$ T-cells in HAM/TSP patients was 100%, a small fraction of ACs lacking detectable tetramer-binding cells might lack Tax-specific T-cell responses. Our previous study investigating GST-Tax protein-based T-cell responses supports this notion [20]. In the present study, even in ACs possessing Tax-specific $CD8^+$ T-cells, at least one individual exhibited T-cell dysfunction selectively for HTLV-1. The incidence of tetramer-positive cells was reduced in ATL patients (38.1%), and the function of these cells was impaired in all the ATL patients even with detectable tetramer-binding Tax-specific $CD8^+$ T-cells. Our findings suggest that HTLV-1-specific T-cell responses are selectively impaired in a small percentage of HTLV-1-infected individuals in the asymptomatic stages, and the proportion of individuals with such characteristics increase as the stages proceed towards ATL. Strategies to reactivate HTLV-1-specific T-cells at early stages might contribute to a reduction in the immunological risk of ATL.

Conclusions

Tax-specific $CD8^+$ T-cells were scarce and dysfunctional in a limited AC population and ATL patients, and the dysfunction of $CD8^+$ T-cells was selective for HTLV-1 in early stages. These results implied the presence of some HTLV-1-specific T-cell suppressive mechanisms even in asymptomatic stages, which are not a result of general immune suppression in ATL but could be underlying conditions toward disease progression.

Methods

Samples

Blood samples from 64 HTLV-1-seropositive individuals were used in this study: 23 asymptomatic carriers (ACs), 18 HAM/TSP patients, 2 smoldering type ATL (sATL) patients, and 21 chronic type ATL (cATL) patients. All blood samples were obtained following written informed consent, and this study was reviewed and approved by the Institutional Review Board of the Tokyo Medical and Dental University.

Peptides

Peptides used in this study were HLA-A2-restricted CTL epitopes (Tax11-19, LLFGYPVYV)[12] (Hokudo Co., Hokkaido, Japan) and (CMV495-503, NLVPMVATV)[50] (Sigma Aldrich St. Louis, MO), HLA-A11-restricted CTL epitope (Tax88-96, KVLTPPITH)[36] (Hokudo Co) and HLA-A24-restricted CTLs epitopes (Tax301-309, SFHSLHLF)[35] (Hokudo Co) and (CMV341-349, QYDPVAALF)[51] (Sigma Aldrich).

Cell Surface staining

To select samples carrying HLA-A2, -A11, or -A24, whole blood was screened with antibodies for HLA-A2, -A11, and -A24 subtypes (One Lambda, Inc., Los Angeles, CA). FITC-conjugated goat anti-mouse Ig (G+M) (Beckman Coulter Inc., Webster, TX) was used as a secondary antibody. For cell surface staining, whole blood samples were stained with the following fluorochrome-conjugated mouse anti-human mAbs; CD3-FITC, CD8-PE/Cy5, CD8-PerCP/Cy5.5 (RPA-T8, BioLegend), CD27-FITC (O323, BioLegend) CD45RA-FITC (HI 100, BD Biosciences), CD45RA-APC (HI 100, BioLegend), CD69-FITC (FN 50, BioLegend), PD-1-FITC (EH12.2H7, BioLegend), CCR7 (TG8/CCR7, Biolegend).

Tetramer staining

PE-conjugated HLA-A*0201/Tax11-19, HLA-A*1101/Tax88-96, HLA-A*2402/Tax301-309, HLA-A*0201/CMVpp65, HLA-A*2402/CMVpp65 tetramers were purchased from MBL (Nagoya, Japan). Whole blood samples or peripheral blood mononuclear cells (PBMCs) were stained with PE-conjugated Tax/HLA tetramer in conjunction with FITC-conjugated anti-CD3 (UCHT1, BioLegend San Diego, CA), and PE-Cy5-conjugated anti-CD8 monoclonal antibodies (mAbs) (HIT8a, BD Biosciences San Jose, CA). Whole blood samples were lysed and fixed in BD FACS lysing solution (BD Biosciences) before washing the cells. Samples were analyzed on a FACSCalibur (Becton Dickinson, San Jose, CA) and data analyses were performed using CellQuest software (Becton Dickinson).

Tetramer-based IFN-γ flow cytometry

Tetramer-based intracellular IFN-γ flow cytometry was performed as described previously[17], with slight modifications. In brief, PBMCs (2.0×10^5 cells) were incubated with HLA tetramer-PE and anti-CD8-PE/Cy5, washed, and stimulated with 10 μM antigenic peptide for 6 hrs at 37°C in the presence of brefeldin A (BFA, 10 μg/ml; Sigma Aldrich). The cells were stained with a

tetramer, permeabilized, and stained with anti-human IFN-γ-FITC (4S.B3, BD Biosciences).

T-cell proliferation

PBMCs (2.0-5.0 × 10^5 cells/well) labeled with carboxyfluorescein succinimidyl ester (CFSE; Sigma Aldrich) were cultured for 6 days with or without 100 nM antigenic peptide and then stained with Tax/HLA tetramer-PE and anti-CD8-PE/Cy5. In some experiments, PBMCs (2.0 × 10^5 cells) were cultured for 13 days with 100 nM antigenic peptide and 10 U/ml recombinant human IL-2 (IL-2; Shionogi, Osaka, Japan) in the presence or absence of 0.1 μg/ml Lipopolysaccharide (LPS; Sigma Aldrich). The cells were then stained with HLA tetramer-PE, anti-CD8-PE/Cy5 and anti-CD3-FITC, and analyzed by flow cytometry.

Quantification of HTLV-1 proviral load

The HTLV-1 proviral load was measured using LightCycler DNA Master SYBR Green 1 (Roche, Mannheim, Germany) with a LightCycler (Roche). Genomic DNA was extracted from PBMCs (2 × 10^6 cells) using DNeasy Blood & Tissue kits (QIAGEN, Courtaboeuf, France). The primer sets used in this study were as follows: pX2 (5'-CGGATACCCAGTCTACGTGTTTGGAGACTGT-3') and pX3 (5'-GAGCCGATAACGCGTCCATCGATGG GGTCC-3') for HTLV-1 pX, and B-globin (5'-ACA-CAACTGTGTTCACTAGC-3') and aB-globin (5'-CAACTTCATCCACGTTCACC-3') for β-globin. The proviral load was calculated as: [(copy number of pX)/(copy number of β-globin/2)] × 1000. HTLV-1 proviral loads in some of the PBMC samples were measured by the Group of Joint Study on Predisponsing Factors of ATL Development (JSPFAD, Japan) as described previously [20].

CD107a mobilization assay

PBMCs were stained with Tax/HLA tetramers-PE and anti-CD8-PE/Cy5, washed, and stimulated with 10 μM antigenic peptide for 6 hrs at 37°C in the presence of mouse anti-human CD107a-PerCP/Cy5.5 (H4A3, Biolegend) or mouse IgG_1-PerCP/Cy5.5 (MOPC-21, Biolegend). BFA (10 μg/ml) was added 1 hr after incubation was started. The cells were then collected and stained with an HLA tetramer.

Depletion of $CD8^+$ cells and Detection of HTLV-1 p19

$CD8^+$ cells were depleted from PBMCs by negative selection using 10-fold numbers of Dynabeads M-450 CD8 (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. The PBMCs were adjust to 1 × 10^6 cells/ml before depletion, and the resulting $CD8^+$ cell-depleted fractions were resuspended in medium with the same initial volume, irrespective of the remaining cell number. PBMCs (1 × 10^6 cells/ml) and $CD8^+$ cell-depleted PBMCs were cultured for 7 days. HTLV-1 p19 in the supernatants of those PBMCs were measured by HTLV p19 antigen ELISA (RETRO tek, Buffalo, NY).

Statistics

The Mann-Whitney U-test, the unpaired t test, and the Spearman rank correlation test were performed for statistical significance by using the Graphpad Prism software (Graphpad Software). In all cases, two-tailed *P* values less than 0.05 were considered significant.

Additional material

Additional file 1: Tax-specific $CD8^+$ T-cells in cATL patients could not proliferate against Tax-peptide stimulation. (A) CFSE-labeled PBMCs were cultured with or without 100 nM Tax-peptide for 6 days. The number indicates the percentage of $tetramer^+$ cells in $CD8^+$ T cells (Day 0) or the percentage of dividing ($CFSE^{low}$) cells in Tax-specific $CD8^+$ T-cells (Day 6). In a cATL sample #54, CFSE-labeled PBMCs were cultured in the presence of mouse IgG for other experiment. (B) PBMCs (#224) and CCR4-depleted PBMCs (#280) were cultured for 13 days in the presence of 100 nM Tax-peptide. The number indicates the percentage of $tetramer^+$ cells in $CD8^+$ T-cells.

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Authors' contributions

AT carried out immunological and virological analyses, and drafted the manuscript. AH conceived of the study, participated in its design and coordination, and drafted the manuscript. AU, YM, YY, MM, IC, NU, and JO provided clinical samples. YS, YT, AS, and NZ carried out a part of the experiments. TW provided the data on proviral load of some HTLV-1-infected individuals. TM helped to draft the manuscript. MK participated in study design and helped to draft the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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ORIGINAL ARTICLE

Unrelated cord blood transplantation for patients with adult T-cell leukemia/lymphoma: experience at a single institute

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Abstract We report the results of unrelated cord blood transplantation (UCBT) for patients with adult T-cell leukemia/lymphoma (ATLL) conducted in our single institute. Ten patients with ATLL (nine acute and one lymphoma-type) received UCBT during the period from August 2003 to July 2011. The median age at the time of diagnosis of ATLL was 51 years (range 37–64). The median period from diagnosis of ATLL to UCBT was 130 days (range 94–344). Conditioning regimens were myeloablative for six and reduced intensity for four. The median number of infused nucleated cells and CD34 positive cells were 2.52×10^7/kg and 1.04×10^5/kg, respectively. There was no engraftment failure. Three patients developed grade II acute graft versus host disease, and four developed grade III. The estimated 2-year overall survival was 40 % (95 % CI 12–67 %). Four of six chemosensitive patients prior to UCBT survived for 1035, 793, 712, and 531 days post-UCBT, respectively. There were no survivors among the four chemorefractory patients prior to UCBT. Our data indicates that UCBT is feasible and provides long-term survival in patients with chemosensitive ATLL.

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Introduction

Adult T-cell leukemia/lymphoma (ATLL) is a distinct peripheral T-lymphocytic malignancy associated with a retrovirus designated human T-cell leukemia virus type 1 or human T-cell lymphotropic virus type 1 (HTLV-1) [1–3]. The prognosis of ATLL has been dismal. However, improved results of combination chemotherapy are reported to some extent [4]. Allogeneic hematopoietic stem cell transplantation (HSCT) is now considered a promising treatment for patients with aggressive ATLL [5, 6]. A phase I trial of allogeneic HSCT with reduced-intensity conditioning (RIC) for ATLL also revealed promising results for relatively older patients [7, 8]. These data suggest a graft-versus-ATLL effect.

The number of unrelated cord blood transplantations (UCBTs) in adult patients with hematologic disorders has been increasing rapidly in Japan. Rapid availability of a CB unit is a great advantage for patients who need urgent HSCT.

In this study, we report the results of UCBT conducted in patients with ATLL in our single institute.

Patients and methods

Patients

Ten patients with ATLL (9 acute and 1 lymphoma type) received UCBT at our hospital during the period from August 2003 to July 2011. All the patients lacked

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HLA-matched sibling donors. The median age at the time of diagnosis of ATLL was 51 years (range 37–64). Clinical characteristics of ATLL patients are shown in Table 1.

Diagnosis and classification of clinical subtypes of ATLL

The diagnosis of ATLL was made according to the following criteria: (1) HTLV-I antibody positive in the serum; (2) presence in peripheral blood of abnormal lymphocytes with convoluted or lobulated nuclei, or histological findings compatible with ATLL in the biopsied lymph nodes; (3) tumor cells with mature CD4+ T-cell phenotype. Clinical subtypes of ATLL were classified according to the criteria of the Japanese Lymphoma Study Group [9]. Recently published simplified prognostic index (PI) for acute- and lymphoma-type ATLL (ATL-PI) [10] is shown in Table 1.

Definition of therapeutic response

Response to induction chemotherapy was divided into five categories: complete remission (CR), uncertified CR (CRu), partial remission (PR), stable disease (SD), and relapsed disease or progressive disease (PD), as defined by Tsukasaki et al. [11].

Statistical analysis

The overall survival (OS) was estimated by the Kaplan–Meier method. OS was calculated from the day of allo-HSCT until death or last follow-up.

Results

Several chemotherapy regimens including CHOPVMMV, CHOP, LSG15, and mLSG15 were used for initial treatments [4, 12, 13]. Chemotherapy before UCBT resulted in CR in 2 patients, PR in 4, SD in 1 and PD in 3. The median period from diagnosis of ATLL to UCBT was 130 days (range 94–344). Myeloablative conditioning was used for patients $\leq$55 years of age, and RIC was used for patients $\geq$55 years of age. Conditioning regimens were myeloablative cyclophosphamide (CY) (60 mg/kg/day $\times$ 2 days)/total body irradiation (TBI) (2 Gy $\times$ 6) for 6 patients and RIC with fludarabine (Flu) (25 mg/m^2/day $\times$ 5 days)/melphalan (MEL) (40 mg/m^2/day $\times$ 2 days)/TBI (2 Gy $\times$ 2) [14] for 4 patients. Most frequently used graft-versus-host disease (GVHD) prophylaxis was cyclosporin (CsA) + short term methotrexate (MTX) (5 mg/m^2, days 1, 3, and 6). CB units were obtained from the Japanese Cord Blood Bank Network. Anti-HLA antibodies (Abs) were screened before transplantation in 8 patients using a FlowPRA method (One Lambda), and LAB Screen PRA or Single Antigen (One Lambda) was used to identify HLA antibody specificities [15]. In 4 patients positive for anti-HLA Abs, CB units were selected for no reaction to patients' anti-HLA Abs. The median number of infused nucleated cells and CD34 positive cells was 2.52 (2.06–4.43) $\times$ 10^7/kg and 1.04 (0.80–3.51) $\times$ 10^5/kg, respectively. All the patients received CB units with HLA mismatches at 1 (n = 2) or 2 (n = 8) loci (Table 2). There was no engraftment failure. The median time of recovery to absolute neutrophil count >0.5 $\times$ 10^9/L was 20.5 days (range 16–26). The median time of recovery to platelet count >20 $\times$ 10^9/L was 31 days (range 24–56), except in one patient who did not achieve platelet recovery. Three patients developed grade II acute GVHD and 4

Table 1 Patient Characteristics at the diagnosis of ATLL

Case no.	Age/ sex	Subtype of ATLL	Performance status	WBC ($\times$ 10^9/L)	Abnormal lymphocyte (%)	Serum calcium (mg/dl)	LDH (IU/l)	sIL2R (U/ml)	Lymph node swelling	Organ involvement	ATL-PI
1	55/M	Acute	0	18	61	10.27	725	63060	(+)	Liver	3
2	58/F	Acute	0	5.9	10	9.86	852	42770	(+)	(−)	3
3	52/M	Lymphoma	0	11.4	0	10.22	499	1130505	(+)	(−)	3
4	48/M	Acute	0	61.9	85.5	9	401	12000	(+)	(−)	2
5	47/M	Acute	0	13.9	19	11.6	506	26800	(+)	CNS	3
6	55/M	Acute	0	7.7	3	9.66	780	7261	(+)	(−)	2
7	64/F	Acute	0	9.6	29.5	9.31	223	9163	(+)	(−)	2
8	37/F	Acute	1	10.8	38.5	8.93	235	3192	(+)	(−)	2
9	45/M	Acute	0	25.5	36.5	16.5	941	67100	(+)	Skin, CNS	3
10	50/F	Acute	0	6.6	11.5	11.85	223	10020	(+)	(−)	3

developed grade III acute GVHD. Two patients with stage II acute GVHD involving only skin were successfully treated with topical steroid. One patient with stage II acute GVHD involving skin and gut was successfully treated with 1 mg/kg dose of predonisolone. Three patients with stage III acute GVHD were treated with 1 mg/kg dose of predonisolone with success. Three patients developed limited type of chronic GVHD.

According to disease status at day 30 post-UCBT, 1 SD patient prior to UCBT attained CRu; among 3 PD patients prior to UCBT, 1 attained CR and 2 attained PR. Thus, UCBT induced 2 CR, 1 CRu, and 1 PR in 4 chemorefractory patients indicating anti-ATLL potential of UCBT.

Among 6 chemosensitive patients prior UCBT, one CR patient relapsed on day 100 and died of PD on day 141. All of the chemorefractory patients prior to CBT (SD and PD) attained PR or CR post-CBT, but eventually relapsed and died of PD.

One patient died of sepsis caused by *Pseudomonas aeruginosa* in CR status on day 163, and this was the only case of non-relapse mortality.

All but one of the patients became positive for cytomegalovirus antigenemia and were successfully treated with ganciclovir. Three patients developed adenovirus hemorrhagic cystitis which was treated conservatively. Two patients developed blood stream infection caused by *Enterococcus faecalis* in one, and *Staphylococcus hominis* in the other. These infectious complications were successfully treated (Table 3).

The estimated 2-year overall survival was 40 % (95 % CI 12–67 %) (Fig. 1a). Four of 6 chemosensitive patients prior to UCBT have survived for 1035, 793, 712, and 531 days, respectively. There was no long-term survival among the 4 chemorefractory recipients (Fig. 1b). Four of 4 patients with low simplified ATL-PI score [10] were disease-free, 5 of 6 patients with intermediate simplified ATL-PI score relapsed (Fig. 1c).

Discussion

According to a retrospective analysis of Japanese registry data comparing outcomes of patients with ATLL who underwent allogeneic HSCT using different graft sources between 1996 and 2005, multivariable analysis revealed that use of UCB compared with use of HLA-matched related grafts and status other than CR were significantly associated with lower survival rates [16]. Treatment-related mortality (TRM) rate was higher among patients given UCB. UCB recipients were older and were more likely to receive purine analog-containing regimens and 17 % (12/70) of UCBT recipients showed primary graft failure. As the authors mentioned, the study period was the developmental phase of UCBT for adult patients in Japan, and improvements in UCBT outcomes have been expected in more recent years.

The number of UCBTs in Japan is increasing rapidly, and more than 1000 UCBTs were performed in 2011,

Table 2 Unrelated cord blood transplantation for ATLL

Case no.	Induction chemotherapy	Interval from Dx to UCBT (days)	Disease Status at UCBT	HLA mismatch (GVH direction)	HLA mismatch (HVG direction)	Anti-HLA Abs	TNC ($\times 10^7$/kg)	$CD34^+$ cells ($\times 10^5$/kg)	Conditioning	GVHD prophylaxis
1	CHOPVMMV(3)	94	PR	1	1	NE	2.33	0.8	Flu + Mel + TBI	CsA
2	TCOP(8), LSG15(2)	296	SD	2	2	NE	2.96	0.8	Flu + Mel + TBI	CsA, MTX
3	CHOP(l), mLSG15(2), CHASE(1)	126	PD	2	2	(−)	2.38	0.89	Flu + Mel + TBI	CsA, MTX
4	mLSG15(3)	204	PR	2	2	(+)	2.74	3.51	CY + TBI	CsA, MTX
5	mLSG15(7)	344	PD	2	2	(+)	2.13	1.24	CY + TBI	CsA, MTX
6	mLSG15(4)	231	PR	1	2	(+)	2.17	1.03	CY + TBI	CsA, MTX
7	CHOP(4)	128	CR	2	2	(+)	2.65	1.68	Flu + Mel + TBI	CsA, MTX
8	mLSG15(2)	120	PR	2	2	(−)	4.02	1.04	CY + TBI	CsA, MTX
9	CHOP(2), mLSG15(2)	107	PD	2	2	(−)	2.06	0.86	CY + TBI	TAC, MTX
10	CHOP(2), mLSG15(2)	132	CR	2	1	(−)	4.43	1.64	CY + TBI	TAC, MTX

UCBT unrelated cord blood transplantation, *TNC* total nucleated cells, *GVHD* graft versus host disease, *NE* not examined, *Flu* fludarabine, *Mel* melphalan, *TBI* total body irradiation, *CY* cyclophosphamide, *CsA* cyclosporine, *MTX* methotrexate, *TAC* tacrolimus

Table 3 Outcome of unrelated cord blood transplantation for ATLL

Case no.	Days to ANC $>0.5 \times 10^9$/L	Days to PLT $>20 \times 10^9$/L	Disease status at day 30	Acute GVHD	cGVHD	Viral infections	Complications	Disease progression (days)	Survival	Survival from UCBT (days)	Cause of death
1	24	38	CR	Grade III (skin, liver, gut)	(+) (skin)	CMV, adenovirus HC	Liver abscess	(−)	Dead	163	MDRP sepsis
2	21	33	CRu	Grade II (skin, gut)	(−)	(−)	(−)	(+) 84	Dead	219	Recurrence
3	20	31	CR	Grade I (skin)	(−)	CMV, adenovirus HC	*Enterococcus faecium* bacteremia	(+) 105	Dead	164	Recurrence
4	20	32	PR	Grade III (skin, liver, gut)	(+) (skin)	CMV	Pyogenic spondylitis, Aspergillosis	(−)	Alive	1035	NA
5	26	Transfusion dependent	PR	Grade III (skin, liver)	(−)	CMV, BK HC	Leukoencephalopathy	(+) 110	Dead	131	Recurrence, GVHD
6	19	28	CR	Grade III (skin, liver, gut)	(−)	CMV, adenovirus, BK, JC HC	Aspergillosis	(−)	Alive	793	NA
7	20	29	CR	Grade II (skin, gut)	(+) (oral cavity)	CMV	(−)	(−)	Alive	712	NA
8	21	30	PR	Grade I (skin)	(−)	CMV	*Staphylococcus hominis* bacteremia	(−)	Alive	531	NA
9	24	56	CR	Grade I (skin)	(−)	CMV	(−)	(+) 122	Dead	213	Recurrence
10	16	24	CR	Grade II (skin, gut)	(−)	CMV, adenovirus, BK HC	(−)	(+) 100	Dead	141	Recurrence

ANC absolute neutrophil count, *PLT* platelet, *HC* hemorrhagic cystitis, *MDRP* multi-drug resistant *Pseudomonas aeruginosa*, *NA* not available

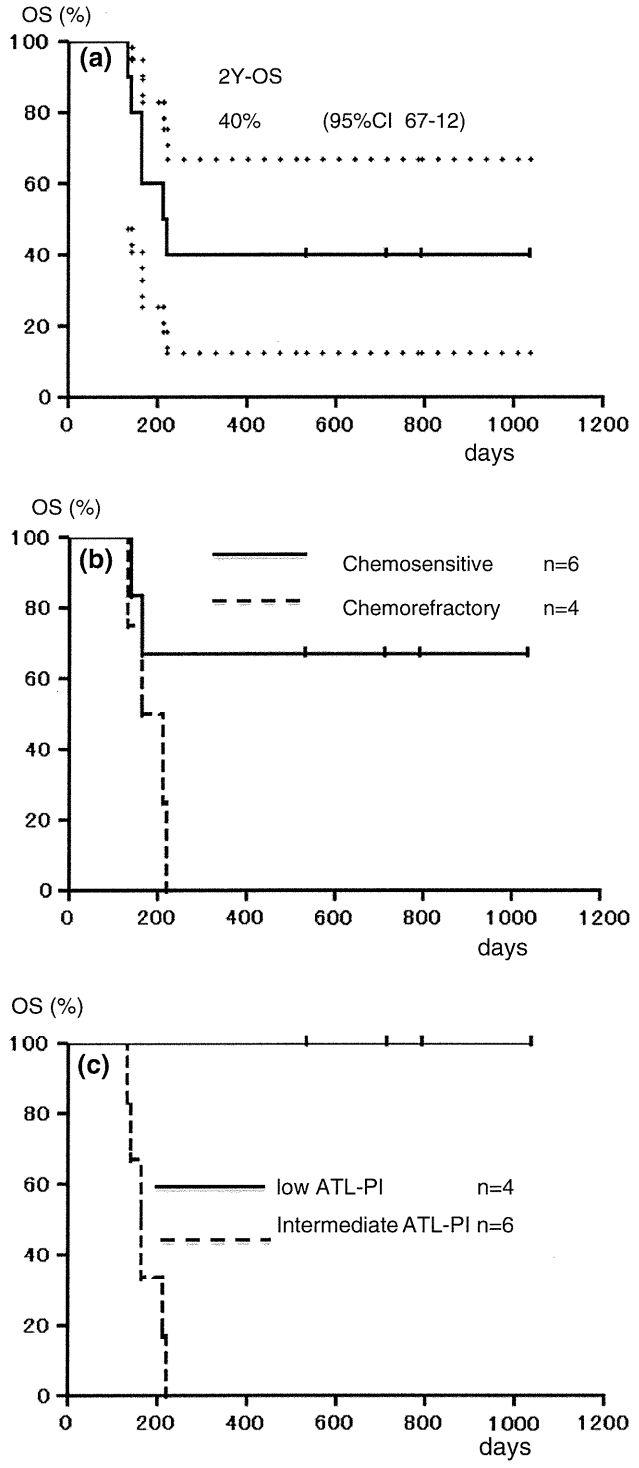

Fig. 1 **a** Overall survival of 10 patients with ATLL undergoing unrelated cord blood transplantation. **b** Overall survival according to the status of chemosensitivities prior to transplantation. **c** Overall survival according to simplified prognostic index (PI) for acute- and lymphoma-type ATLL (ATL-PI)

which is comparable to the number of unrelated bone marrow transplantations (UBMTs). The main drawback of UCBT is engraftment delay and failure [17]. Anti-HLA Abs have been reported to be a risk factor of engraftment failure; especially in cases where the HLA Abs were donor-specific [18]. With the routine screening of anti-HLA Abs, the rate of engraftment failure is supposed to be lowered. In our series of 10 UCBT recipients, we observed no engraftment failure.

There have been several reports of UCBT for ATLL [19–22]. Wake et al. [19] reported the outcome of RIC UCBT for 18 advanced ATLL patients with 80 % chemorefractory status. Estimated 1-year OS and progression-free survival (PFS) rates were 27.9 ± 9.0 % and 17.2 ± 12.8 %, respectively. These data suggest limited potential of UCBT for chemorefractory ATLL.

There have been several reports concerning graft-versus-ATLL effect. Yonekura et al. [23] reported that among 10 ATLL patients who relapsed after allogeneic HSCT, discontinued immunosuppressant therapy caused 8 cases of GVHD and 6 cases of CR. Tanosaki et al. [24] reported the results of two prospective clinical trials of RIC HSCT for ATLL disclosing that grade I–II acute GVHD was the only factor that favorably affected OS and PFS. According to a retrospective analysis of Japanese registry data comparing outcomes of ATLL patients who received allogeneic HSCT and survived at least 30 days post-transplant with sustained engraftment, multivariable analyses demonstrated that the development of grade I–II acute GVHD was significantly associated with higher OS compared with the absence of acute GVHD; the development of extensive chronic GVHD was associated with higher TRM compared with the absence of chronic GVHD. Collectively, these data suggest positive impact on survival of mild-to-moderate acute GVHD and not of chronic GVHD.

In spite of HLA incompatibility of UCBT, UCBT is reported to be associated with comparable acute GVHD and less chronic GVHD relative to UBMT [25, 26]. Thus, the GVHD characteristics of UCBT might be suitable for ATLL.

Disease status prior to HSCT is one of the most important prognostic factors in HSCT for ATLL. In this analysis, the OS of chemorefractory ATLL patients was zero. Chemotherapy for ATLL has been improved, and mLSG15 protocol is reported to have median PFS time and PFS rate at 1 year of 7 months and 28 %, respectively [4]. This means that even with the treatment with mLSG15, 7 months after initial presentation, half of the patients were PD, and HSCT outcome of ATLL patients with PD status has always been dismal irrespective of stem cell sources or conditioning intensities.

These data suggest that time from diagnosis to HSCT should be minimized to perform allograft while patients remain chemosensitive. On the contrary, it is not clear whether early HSCT really rescues high-risk patients with impending PD while remaining chemosensitive. There is a

possibility that patients with short-lasting chemosensitivity might not benefit from HSCT. Recently, simplified ATL-PI is proposed as a promising new tool for identifying patients with acute- and lymphoma-type ATLL at different risks [10], and the authors mentioned that simplified ATL-PI in allografted ATLL patients did not have prognostic power for OS. In our series, 4 of 4 patients with low simplified ATL-PI score were disease-free, 5 of 6 patients with intermediate simplified ATL-PI score relapsed, and there were no patients with high simplified ATL-PI score (Fig. 1c). The number of patients in this analysis was 10 and too small for definitive findings, while the meaning of ATL-PI should be evaluated further in UCBT recipients with ATLL.

In our series, chemorefractory patients with ATLL benefited from UCBT as shown by the fact that these patients achieved CR or PR at day 30, while these responses were not durable. Compared with other stem cell sources, UCBT has the disadvantage of not being eligible for donor lymphocyte infusion [27]. To achieve durable response, post-UCBT immunotherapy such as ex vivo expanded cord blood CD4 T lymphocytes [28] might be a candidate to be explored.

Another possibility would be a combination with novel agents, for which would expect the improvement of therapeutic outcome. Recently, promising result for mogamulizumab, a humanized anti-CCR4 monoclonal antibody with relapsed ATLL has been reported [29]. Mogamulizumab binds with high affinity to CCR4-positive cells and deplete normal regulatory T cells as well as CCR4-positive ATLL cells [30]. In the future, mogamulizumab could be used to lessen tumor burden prior to UCBT or to prevent relapse post-UCBT while pay attention to the immune-related adverse events, such as an increased risk of rejection caused by host regulatory T-cell depletion, and an increased risk of acute GVHD caused by recipient regulatory T-cell depletion.

In conclusion, our data indicate that UCBT is feasible and provides long-term survivals in patients with chemosensitive ATLL. Prospective clinical study can clarify the role of UCBT in the treatment strategy of ATLL.

Conflict of interest None.

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