Leukemia (2009) 23, 375–382

www.nature.com/leu

ORIGINAL ARTICLE

PD-1/PD-L1 expression in human T-cell leukemia virus type 1 carriers and adult T-cell leukemia/lymphoma patients

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Adult T-cell leukemia/lymphoma (ATLL) develops after infection with human T-cell leukemia virus-1 (HTLV-1) after a long latency period. The negative regulatory programmed death-1/programmed death-1 ligand 1 (PD-1/PD-L1) pathway has been implicated in the induction of cytotoxic T-lymphocyte (CTL) exhaustion during chronic viral infection along with tumor escape from host immunity. To determine whether the PD-1/PD-L1 pathway could be involved in the establishment of persistent HTLV-1 infections and immune evasion of ATLL cells in patients, we examined PD-1/PD-L1 expression on cells from 27 asymptomatic HTLV-1 carriers (ACs) and 27 ATLL patients in comparison with cells from 18 healthy donors. PD-1 expression on HTLV-1-specific CTLs from ACs and ATLL patients was dramatically elevated. In addition, PD-1 expression was significantly higher on CD8+ T cells along with cytomegalovirus (CMV)- and Epstein–Barr virus (EBV)-specific CTLs in ATLL patients compared with ACs and control individuals. Primary ATLL cells in 21.7% of ATLL patients expressed PD-L1, whereas elevated expression was not observed in cells from ACs. Finally, in functional studies, we observed that an anti-PD-L1 antagonistic antibody upregulated HTLV-1-specific CD8+ T-cell response. These observations suggest that the PD-1/PD-L1 pathway plays a role in fostering persistent HTLV-1 infections, which may further ATLL development and facilitate immune evasion by ATLL cells.
Leukemia (2009) **23,** 375–382; doi:10.1038/leu.2008.272; published online 2 October 2008
Keywords: human T-cell leukemia virus-1; adult T-cell leukemia/lymphoma; cytotoxic T lymphocytes; programmed death-1; programmed death-ligand 1

Introduction

Adult T-cell leukemia/lymphoma (ATLL) is a highly aggressive peripheral T-cell neoplasm that develops only after long-term chronic infections with human T-cell leukemia virus-1 (HTLV-1).[1–4] Despite recent progress in both chemotherapy and supportive care for hematological malignancies, the prognosis of ATLL is still poor; overall survival at 3 years is only 24%.[5–8]

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Received 12 February 2008; revised 23 June 2008; accepted 25 August 2008; published online 2 October 2008

Human T-cell leukemia virus-1 is a human retrovirus that has infected approximately 10–20 million people worldwide, particularly in southern Japan, the Caribbean Basin, South America, Melanesia and Equatorial Africa.[9] Despite this high frequency of human infection, only 2–5% of HTLV-1-infected individuals develop ATLL.[7,10] Multiple factors (for example, virus, host cell and immune factors) have been implicated in the development of ATLL, although the underlying mechanisms of leukemogenesis have not been fully elucidated.[5,8,10] Recently, lines of evidence suggest that HTLV-1-specific cytotoxic T lymphocytes (CTLs) play an important role in suppressing the proliferation of HTLV-1-infected or transformed T cells, and thus may prevent the development of ATLL.[11,12] We have also reported on the reduced frequency and function of HTLV-1 Tax-specific CD8+ T cells in ATLL patients.[13] Taken together, it is thus of great interest to further explore the role of the host T-cell immune system in understanding the persistence of HTLV-1 infections and ATLL leukemogenesis.

The T-cell receptor costimulatory pathways assist in regulating T-cell activation and tolerance.[14–16] The B7-CD28 superfamily membership has been expanded to include costimulatory and inhibitory T-cell receptors, including CD28 and programmed death-1 (PD-1).[15,17] Indeed, studies in PD-1-deficient mice have indicated that PD-1 serves as a negative regulator of immune responses.[18] PD-1 signaling is involved in autoimmunity, allergy, sites of immune privilege and antitumor immunity.[14,16] The interaction of PD-1 with the ligand, programmed death-1 ligand 1 (PD-L1), has been reported to negatively regulate the cytokine production and proliferation of T cells.[14,19,20] Interestingly, PD-L1 expression is also associated with poor prognosis in many cancers, including those of the larynx, lung, stomach, colon, breast, cervix, ovary, renal cell, bladder and liver, as well as in glioma and melanoma.[14] Further, it has been suggested that such tumors may actually evade the host immune system by attenuation of tumor-specific T-cell responses through the PD-1/PD-L1 pathway.[21–23] In addition, PD-1 has recently been shown to be involved in chronic viral infections. For example, Barber *et al.*[24] reported that PD-1 is upregulated in T cells that become exhausted during the chronic phase of viral infection in lymphocytic choriomeningitis virus infected mice. Moreover, blocking the interaction between PD-1 and PD-L1 reactivates the CTL function in those mice.[24] Studies have also suggested that the PD-1/PD-L1 pathway in virus-specific CD8+ T cells may be operating in chronic HIV infection.[25–27] However, there is no report on PD-1 expression in tumor-associated antigen-specific CTLs in humans. Therefore, it is important to explore the association between the PD-1/

PD-L1 pathway and HTLV-1 infection, in terms of both maintenance of the chronic infection and possible effects on the host immune system in this virus-induced malignancy in humans.

In this study, we examined the expression and interaction of PD-1/PD-L1 in asymptomatic HTLV-1 carriers (ACs), ATLL patients and non-HTLV-1-infected individuals to investigate the role of this negative regulatory axis in chronic HTLV-1 infection and associated leukemia.

Materials and methods

Patients

The patients in this study included 27 ACs (24–81 years of age, mean = 59.3), 27 ATLL patients (31–83 years of age, mean = 62.2; two with chronic type, three with lymphoma type and 22 with acute type) and 18 non-HTLV-1-infected healthy donors (HDs; 23–57 years of age, mean = 34.9), all of whom were recruited from the Kagoshima University Hospital. Patients were examined by a standard serological testing for the presence of HTLV-1 and by hematological/Southern blotting analysis for the diagnosis of ATLL. Those patients seropositive for HTLV-1 without clinical symptoms of HTLV-1-related diseases[28] were designated as ACs. Classification of ATLL was made according to Shimoyama's criteria.[29] All patients were inhabitants of Kagoshima prefecture, southern Kyushu and Japan, where ATLL is endemic. All patients gave their written informed consent to participate in this study and to allow review of their medical records, and they also provided a sample of peripheral blood for human leukocyte antigen (HLA) typing and for the HLA tetramer assay. The study protocol was reviewed and approved by the Medical Ethical Committee of Kagoshima University.

Phenotypic analysis

Cells from patients positive for HLA-A*24 were screened by serological staining with monoclonal antibodies (mAbs) for HLA-A*24 subtypes (clone: 17A10; Medical and Biological Laboratories, Nagoya, Japan), followed by secondary staining with goat anti-mouse IgG-fluorescein isothiocyanate (FITC) (Immunotech, Miami, FL, USA) according to the manufacturer's instructions, and subjected to flow cytometry analyses on FACScan (Becton Dickinson, Mountain View, CA, USA). Phenotypic analysis using HLA tetramers was performed as described earlier.[13] Briefly, aliquots of 1×10^6 freshly isolated peripheral blood mononuclear cells (PBMCs) were incubated with the HLA tetramers, FITC-conjugated murine anti-PD-1 mAbs (clone: MIH4; eBioscience, San Diego, CA, USA) and peridinin chlorophyll-a protein-conjugated murine anti-CD8 mAbs (Becton Dickinson, San Jose, CA, USA). Lymphocytes, CD8+ lymphocytes and tetramer-positive CD8+ lymphocytes were analyzed using the FACScan instrument (Becton Dickinson)[30] and FlowJo software (Tree Star, San Carlos, CA, USA).[31] HTLV-1 Tax/HLA tetramers; Tax 301–309 (SFHFLHLLF) with HLA-A*2402 were used in this study (Beckman Coulter Co., Fullerton, CA, USA). Phycoerythrin (PE)-labeled HLA tetramers were produced as described earlier.[32,33] Staining was also performed with an HIV (RYLRDQQLL)/HLA-A*2402 tetramer (Medical and Biological Laboratories) as a negative control, a cytomegalovirus (CMV) (QYDPVAALF)/HLA-A*2402 tetramer and an Epstein–Barr virus (EBV) BRLF1(TYPVLEEMF)/HLA-A*2402 tetramer (Beckman Coulter).[13] We estimated the surface PD-1 expression by subtracting the percentage of isotype control-positive cells from the percentage of anti-PD-1-positive cells. Aliquots of 1×10^6 PBMCs were stained with an FITC-conjugated murine anti-CD25 mAb (Beckman Coulter), a PE-conjugated murine anti-CD4 mAb (Beckman Coulter), a Cy7-conjugated murine anti-PD-L1 mAbs (clone: MIH1; eBioscience) or an isotype control mouse IgG1 antibody (eBioscience). We estimated the surface PD-L1 expression by subtracting the percentage of isotype control-positive cells from the percentage of anti-PD-1-positive cells.

Intracellular Tax, IFN-γ and TNF-α staining assay

Peripheral blood mononuclear cells (1×10^6) for HTLV-1 Tax expression analysis were cultured for 12 h in complete medium (CM; RPMI-1640 supplemented with the following reagents: 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.05 mM 2-mercaptoethanol, 50 U/ml of recombinant human interleukin-2 and 10% heat-inactivated FCS). PBMCs were labeled with Cy7-conjugated murine anti-PD-L1 mAbs, anti-CD4-PE and anti-CD25-allophycocyanin antibody (Becton Dickinson) for cell-surface antigen analyses.[34] PBMCs (1×10^6) for interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) analyses were cultured for 16 h with or without 0.02 μM HTLV-1 Tax peptide (Sigma Aldrich, Tokyo, Japan) in combination with brefeldin A (Becton Dickinson) in CM. Blocking antibody specific to PD-L1 (clone: MIH1; eBioscience) was added to cell cultures at a concentration of 10 μg/ml.[21,35,36] Cells were collected and labeled with HTLV-1/HLA-tetramer-PE and anti-CD8-allophycocyanin antibodies (Becton Dickinson).[34] These cells were further treated with permeabilizing solution (Becton Dickinson). After washing, the cells were incubated with anti-Tax-FITC (clone: Lt4; kindly provided by Tanaka Y, Ryukyu University), IFN-γ-FITC or TNF-α-FITC antibody (Becton Dickinson). As a negative control, staining was also performed with isotype control IgG1-FITC (Becton Dickinson) for Tax and FastImmune Control γ2aFITC/γ1PE for IFN-γ and TNF-α (Becton Dickinson). Lymphocyte analyses were performed using FACSCalibur and analyzed with FlowJo software.[13]

CD107a mobilization assay

Assessment of cytolytic ability was performed using flow cytometric quantification of the surface mobilization of CD107a—an integral membrane protein in cytolytic granules—as a marker of degranulation after stimulation, as described earlier.[13,37] Briefly, PBMCs (1×10^6) were cultured for 6 h with or without 0.02 μM HTLV-1 Tax peptide (Sigma Aldrich) in combination with anti-CD107a mAbs-FITC (clone: H4A3; Southern Biotech, Birmingham, AL, USA) and the secretion inhibitor monensin (Becton Dickinson) in CM. Blocking antibody specific to PD-L1 (clone: MIH1; eBioscience) was added to cell cultures at a concentration of 10 μg/ml. The cells were further stained with HLA-tetramer-PE and anti-CD8 mAb-PE/Cy5 (Beckman Coulter) as described earlier.[13] Aliquots of 1×10^4 CD8+ T lymphocytes were examined using FACSCalibur and analyzed with FlowJo software.

Statistical analysis

Mann–Whitney- and Wilcoxon-matched pair tests were performed using a StatView software version 5.0 (SAS Institute Inc., Cary, NC, USA). *P*-values of less than 0.05 were considered significant.

Results

PD-1 expression in ACs and ATLL patients

Freshly isolated or cryopreserved PBMCs from 27 ACs and 25 ATLL patients were stained for PD-1 in combination with CD8 and HTLV-1 Tax, CMV or EBV tetramers, allowing for the direct visualization of PD-1 expression on tetramer-positive CD8+ lymphocytes. In this study, the cell-surface phenotype of ATLL cells determined by flow cytometric analyses showed the $CD4^+/CD25^+/CD8^-$ (data not shown). We were able to exclude the contribution of PD-1 expression from ATLL cells by analyzing PD-1 expression on CD8+ cells. PD-1 expression was readily apparent on lymphocytes and tetramer-positive CD8+ T cells (Figure 1). Percentages of total and CD8+ lymphocytes from ACs and ATLL patients expressing PD-1 were significantly higher than those from HDs (Figure 1b, $P<0.01$ and 0.001, respectively). Importantly, percentages of total and CD8+ lymphocytes in ATLL patients who expressed PD-1 were significantly even higher than those from ACs ($P<0.01$). Overall, there was no correlation between age and the rates of PD-1 expression, although ATLL patients with high PD-1 expression were significantly younger than those with low PD-1 expression (58.1 years of age versus 67.3 years of age, $P<0.05$, data not shown).

We next assessed PD-1 expression on viral antigen-specific CD8+ lymphocytes (Figure 1c). The rates of PD-1 expression on CMV- and EBV-tetramer-positive CD8+ lymphocytes from ATLL patients were significantly higher than those in HDs (Figure 1c, $P<0.01$ and 0.005, respectively) and ACs ($P<0.01$ and 0.05, respectively). In contrast, we found that 72.7 ± 27.1% of HTLV-1-tetramer-positive CD8+ lymphocytes from ACs expressed PD-1 and that a similar number of HTLV-1-tetramer-positive lymphocytes from ATLL patients expressed PD-1 ($P=0.32$). Furthermore, PD-1 was expressed on significantly higher percentages of HTLV-1-specific cells as compared with CMV- and EBV-specific CD8+ T cells in ACs ($P<0.01$ and 0.05, respectively), but not in ATLL patients. These observations regarding PD-1 cell-surface expression were unique as we did not observe simultaneous increased cell-surface expression of the inhibitory receptor CTL antigen-4 (data not shown).

PD-L1 expression in ACs and ATLL patients

To explore whether ATLL cells and HTLV-1-infected cells could possibly evade the host immune system by expression of the

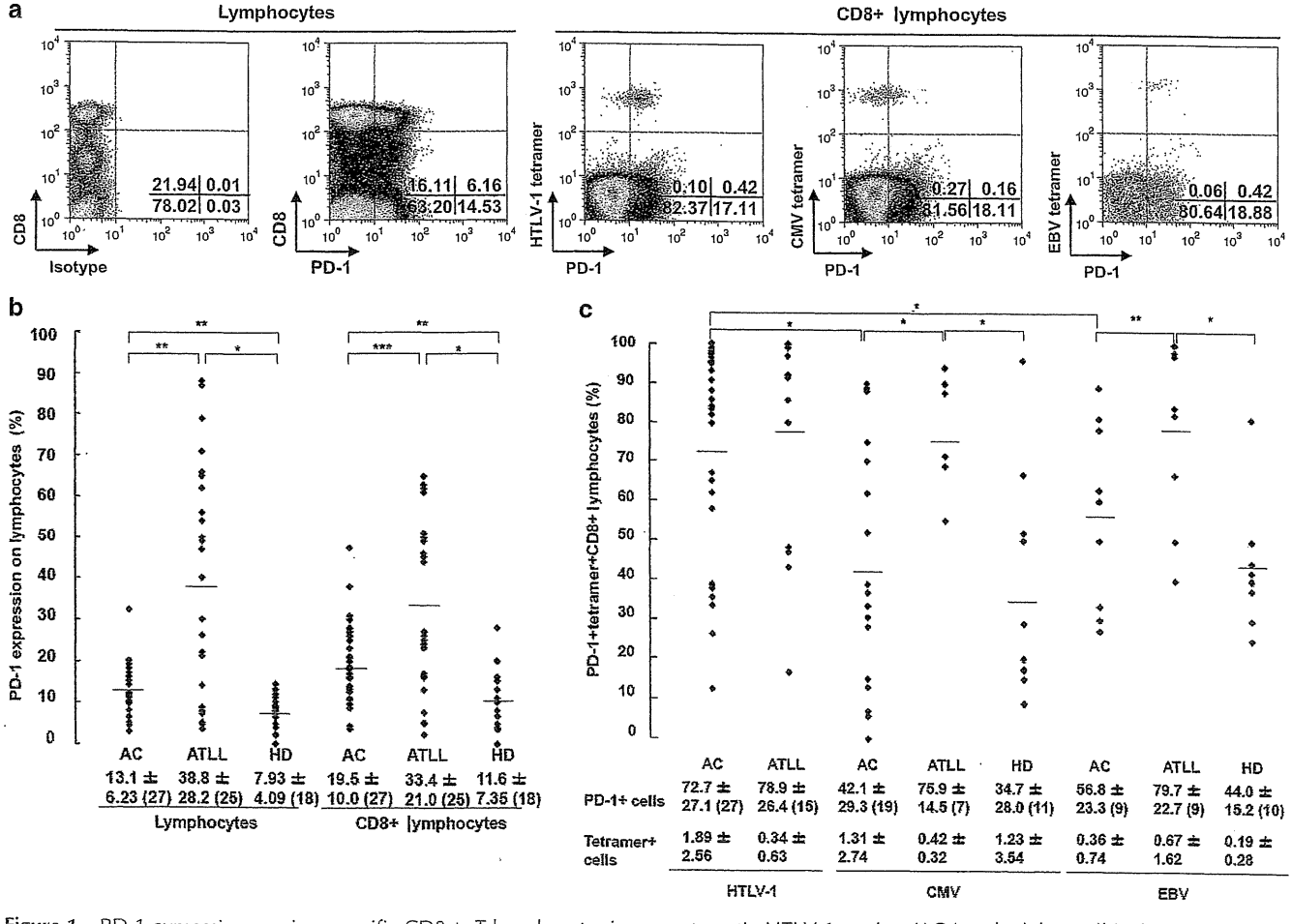

Figure 1 PD-1 expression on virus-specific CD8+ T lymphocytes in asymptomatic HTLV-1 carriers (ACs) and adult T-cell leukemia/lymphoma (ATLL) patients. (**a**) Representative flow cytometric plots of programmed death-1 (PD-1) expression on total lymphocytes and virus-specific CD8+ T lymphocytes in AC. Numbers indicate the percentages of lymphocytes or CD8+ T lymphocytes. (**b**) Percentage of PD-1 expression on lymphocytes (left column) and CD8+ lymphocytes (right column) in AC, ATLL and healthy donor (HD). Horizontal bars indicate the mean percentage of PD-1-positive cells. The numbers below each patient represent the means ± s.d. (**c**) Percentage of PD-1 expression on human T-cell leukemia virus-1 (HTLV-1)-, cytomegalovirus (CMV)- and Epstein–Barr virus (EBV)-specific CD8+ lymphocytes and the percentage of tetramer+ cells in CD8+ lymphocytes in AC, ATLL and HD. Horizontal bars indicate the mean percentage of PD-1-positive cells. The numbers below each patient represent the means ± s.d. $*P<0.001$; $**P<0.01$; $***P<0.05$ (Significant differences by Mann–Whitney *U*-test).

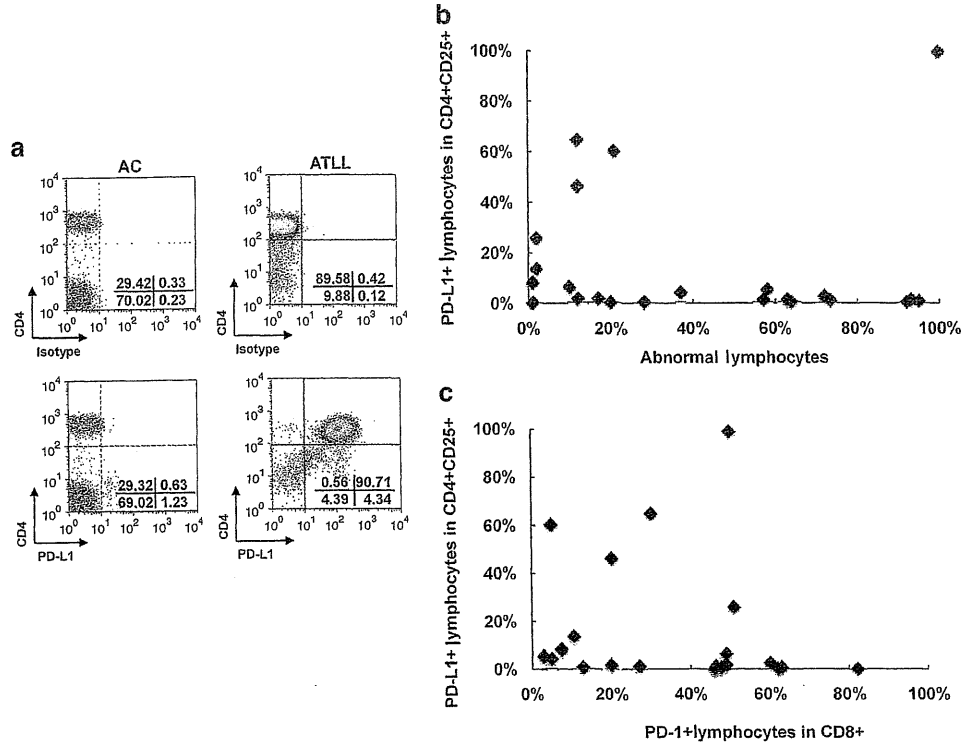

Figure 2 Programmed death-ligand 1 (PD-L1) expression on lymphocytes in asymptomatic HTLV-1 carriers (ACs) and adult T-cell leukemia/lymphoma (ATLL) patients. (**a**) Representative flow cytometric plots of PD-L1 expression in AC and ATLL patients. Numbers indicate the percentages in lymphocytes. (**b**) Percentages of PD-L1 expression on CD4+25+ lymphocytes in AC and ATLL along with the percentage of abnormal lymphocytes in peripheral blood. (**c**) Percentages of PD-L1 expression on CD4+25+ lymphocytes in AC and ATLL along with the percentage of programmed death-1 (PD-1)+ lymphocytes in CD8+ cells.

ligand of this axis, PD-L1 expression was measured. Freshly isolated or cryopreserved PBMCs from 16 ACs, 23 ATLL patients and 17 HDs were stained for PD-L1 expression in combination with CD4 and CD25 expression. Representative PD-L1+ staining patterns on CD4+-gated lymphocytes are shown in Figure 2a. The percentage of PD-L1 expression on CD4+CD25 cells was summarized in Figure 2b along with the percentage of abnormal lymphocytes in peripheral blood. There was no correlation between the percentage of PD-L1 expression on CD4+CD25 cells and the percentage of abnormal lymphocytes in peripheral blood. Lymphocytes from ACs were negative for PD-L1 expression (mean = 0.3 ± 0.57%, data not shown), whereas 5 of 23 ATLL patients (21.7%) showed higher rates of PD-L1 expression on lymphocytes (mean = 11.6 ± 27.8%, Figure 2b). Among HDs, the percentage of lymphocytes expressing PD-L1 was 0.43 ± 0.62 (data not shown). PD-L1 expression on the CD4+CD25 lymphocytes appeared to vary among individuals. Such variation is also seen *in vitro*; the cloned HTLV-1-infected Oh13T cell line and HTLV-1-negative Jurkat cell line expressed PD-L1, whereas HTLV-1-infected Su9TO1, K3T, F6T, SIT[38] and HTLV-1-negative MOLT-4 and KG-1 had no measurable PD-L1 expression (data not shown). The percentage of PD-L1 expression on CD4+CD25+ cells was summarized in Figure 2c along with the percentage of PD-1 expression in CD8+ lymphocytes. There was no correlation between the percentage of PD-L1 expression on CD4+CD25 cells and the percentage of PD-1 expression in CD8+ lymphocytes. We compared the clinical characteristics of two groups of ATLL cases, in which one group showed high expression and the other showed low expression of PD-L1 on ATLL cells. There are no differences between those groups in terms of chemosensitivity, survival, hypercalcemia, LDH, percentage of ATLL cells in peripheral blood and involvement of other organs. However, PD-L1 expression was not observed in the CD4+CD25+ cells at diagnosis in all ATLL patients. Samples from all the five patients who express high PD-L1 on CD4+CD25+ cells (>10%) were collected at least 12 months after diagnosis (21.0 ± 15.3 months). Three of the five patients were severely refractory to chemotherapy, and the other two patients were in chronic stage.

To explore whether PD-L1 expression occurs on HTLV-1-infected cells, we examined T cells expressing Tax, an HTLV-1 oncoprotein. PD-L1 expression was observed on PBMCs cultured for 12 h from ACs (6 of 7), but not from HDs (0 of 6, representative data shown in Figure 3). Overall, 84.7% of the HTLV-1 Tax+ cultured cells expressed PD-L1, although there was no HTLV-1 Tax expression on cells freshly isolated from ACs. Interestingly, all HTLV-1 Tax+PD-L1+lymphocytes were CD4+CD25+ (data not shown). Thus, HTLV-1-infected cells were conditionally capable of inducing PD-L1 expression.

Blockade of the PD-1/PD-L1 pathway enhances CD8+ T-cell function

To assess whether PD-1 expression on CD8+ lymphocytes from ACs and ATLL patients could affect function, cytolytic reactions were performed using flow cytometric quantification of intracellular IFN-γ and TNF-α produced in HTLV-1 Tax-specific CD8+ cells. These cytokines are primarily produced by activated lymphocytes and act as multifunctional immunomodulators with antitumor and antiviral activity.[31] Representative results regarding IFN-γ, TNF-α and CD107a production are

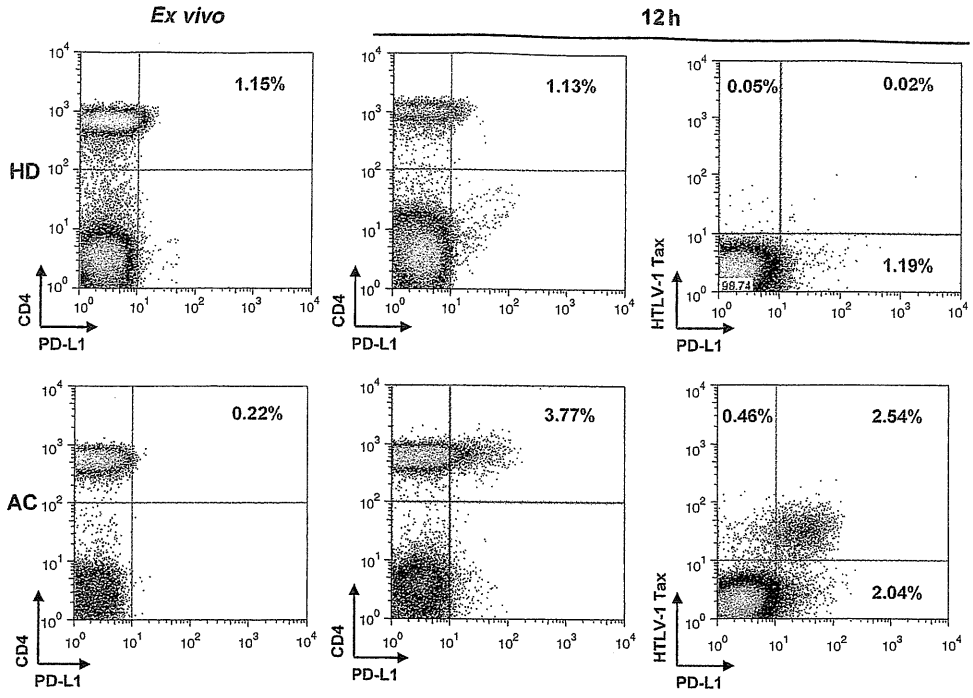

Figure 3 Human T-cell leukemia virus-1 (HTLV-1)-infected cells are capable of inducing programmed death-ligand 1 (PD-L1). Representative flow cytometric plots of PD-L1 expression gated by lymphocytes in healthy donor (HD) (upper) and asymptomatic HTLV-1 carrier (AC) (lower). The left panel shows the results obtained *ex vivo*, whereas the right panel shows those after 12 h in culture gated by lymphocytes. Increases in PD-L1 expression were observed after cultivation in AC but not in HD. The numbers indicate the percentage of lymphocytes. The figure shows one representative result of PD-L1 expression in six HDs and seven ACs. There was no HTLV-1 Tax expression *ex vivo*. All HTLV-1 Tax + PD-L1 + lymphocytes were CD4 + CD25 + cells.

shown in Figure 4a. We incubated freshly isolated PBMCs from ACs and ATLL patients in culture medium alone or medium containing anti-PD-L1 antibody in the presence or absence of HTLV-1 Tax peptide for 6 or 16 h. Intracellular cytokine + cells were estimated from HTLV-1-tetramer + CD8 + T lymphocytes. The HTLV-1 Tax peptide tetramer + CD8 + T cells produced only low levels of IFN-γ, TNF-α and CD107a after short-term cultures in the absence of HTLV-1 Tax peptide (<0.6, <1.0 and <0.8%, respectively). However, the percentage of cells producing IFN-γ when incubated in the presence of Tax peptide increased to 24.1 ± 20.2% in the tetramer + CD8 + T cells (Figure 4a), whereas the percentage of cells producing IFN-γ when incubated in the presence of anti-PD-L1 antibody increased to 29.0 ± 21.7% ($P<0.05$). The percentage of cells expressing TNF-α when incubated with HTLV-1 Tax peptide increased to 14.0 ± 7.4% in the tetramer + CD8 + T cells, whereas the percentage of cells producing TNF-α in the presence of anti-PD-L1 antibody increased to 20.0 ± 11.9% ($P<0.05$). CD107a expression with added HTLV-1 Tax peptide increased to 36.8 ± 18.5% of the tetramer + CD8 + T cells, whereas CD107a expression in the presence of the anti-PD-L1 antibody increased to 42.2 ± 15.8% ($P<0.05$). Thus, stimulation with HTLV-1 Tax peptide in the presence of PD-L1 antibody further enhanced IFN-γ, TNF-α production and CD107a expression, which were significantly increased in those in tetramer-positive cells, when compared with peptide-only stimulation in ACs and ATLL patients ($P<0.05$, Figure 4b). There was no different PD-L1 blockage response between the tetramer + CD8 + functional molecule + from the ACs and ATLL patients. Interfering with the PD-1/PD-L1 pathway using an anti-PD-L1 antibody enhanced the production of IFN-γ, TNF-α and expression of CD107a on HTLV-1 tetramer-positive CD8 + cells, indicating the restored function of HTLV-1-specific CD8 + cells.

Discussion

A higher percentage of PD-1 expression was observed on total and CD8 + T cells in ACs compared with HDs (Figure 1b). Although no earlier study of other oncogenic viruses has shown a correlation between PD-1 expression and the carrier state or eventual progression to cancer, in this study, we report that the percentage of CD8 + lymphocytes expressing PD-1 was higher for ATLL patients compared with ACs (Figure 1b). Even though we failed to measure HTLV-I provirus load with those samples, this may reflect the higher load of virus-associated antigens in ATLL patients than in ACs, as it has also been shown that the PD-1 expression is increased in HIV viremic compared with aviremic individuals.[27] Indeed, our Tax data here (Figure 3) indicate that there may be some correlation between these effects. The observation of fairly high rates of PD-1 expression on CD8 + lymphocytes in ATLL patients and ACs compared with HDs prompted us to investigate PD-1 expression on viral antigen-specific CTL from ATLL patients, ACs and HDs. PD-1 expression on HTLV-1-specific CD8 + lymphocytes from ACs was just as high as that observed on cells from ATLL patients, whereas PD-1 expression on CMV- and EBV-tetramer-positive CD8 + lymphocytes was also upregulated, but to a lesser extent than on cells from in ATLL patients (Figure 1c). These observations suggest relatively skewed exhaustion of CTLs against HTLV-1 more than against other viruses in the carrier state, which is consistent with the observation that ATLL patients show a broadly compromised immune system.[11,39] This may be

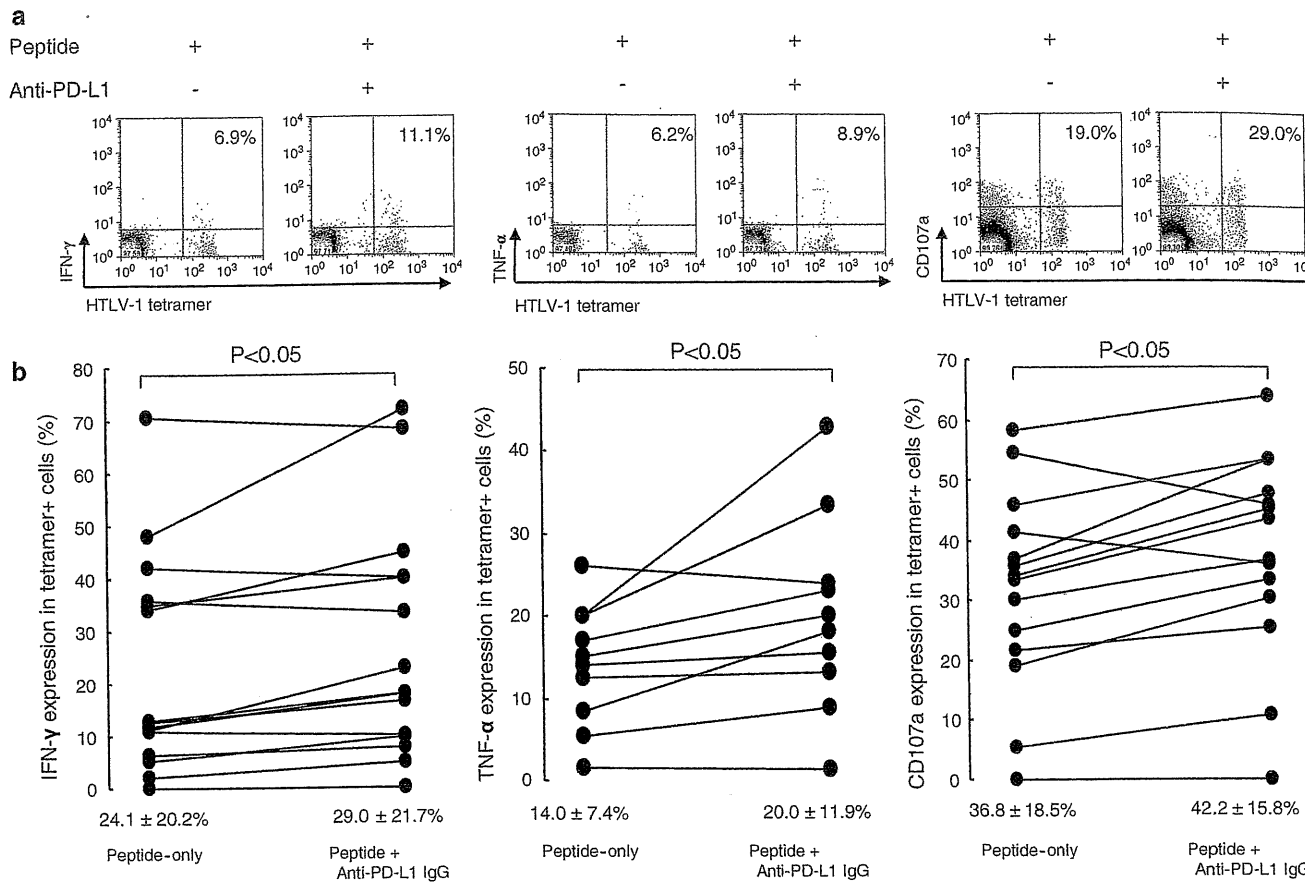

Figure 4 Blocking the programmed death-1/programmed death-ligand 1 (PD-1/PD-L1) pathway increases cytolytic reaction in human T-cell leukemia virus-1 (HTLV-1)-specific CD8+ cells. (**a**) Representative flow cytometric plots of interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and CD107a expression in HTLV-1-specific CD8+ T cells are shown. The numbers indicate the percentages in HTLV-1-specific CD8+ T lymphocytes. The left panel shows the results in the presence of 0.02 μM peptide, whereas the right panel shows peptide treatment in the presence of anti-PD-L1 blocking antibody. (**b**) Percentage of intracellular IFN-γ, TNF-α and CD107a expression in HTLV-1-specific CD8+ T lymphocytes treated with peptide and with anti-PD-L1 IgG. The numbers represent the means ± s.d. Statistical comparisons were made using the Wilcoxon-matched pair test.

one factor facilitating the persistence of HTLV-1-infected cells even in the presence of anti-HTLV-1 antigen-specific CTL. Furthermore, PD-1 expression on HTLV-1-specific CD8+ T cells was unique as we did not see upregulation of the inhibitory receptor CTL antigen-4 (data not shown) on CD8+ T cells, consistent with earlier reports related to HIV infection.[25,40]

The interaction of PD-1 with PD-L1 has been shown to negatively regulate the cytokine production and proliferation of T cells, allowing tumor evasion of the host immune system by negative attenuation of tumor-specific T-cell responses.[19,23] PD-L1 expression is found on many solid tumors, and increased expression of PD-L1 is associated with poor prognosis.[19,22,41] In this study, we showed that some primary ATLL cells express PD-L1 on the cell surface (Figure 2); however, the expression patterns varied considerably between individual patients. Notably, none of the ATLL patients expressed PD-L1 at diagnosis on ATLL cells in this cohort. Samples from all five patients who express high PD-L1 on CD4+CD25+ cells (>10%) were collected at least 12 months after diagnosis. Shimauchi *et al.*[42] also reported that CD4+CD25+ tumor cells expressed a low but discernible level of PD-L1 in only one of four cases. This and our data are in contrast with an earlier study that assessed PD-L1 expression in 30 heterogenous acute leukemia patients and found that 57% of patients expressed PD-L1 at diagnosis on leukemic cells.[43] The serial measurement of PD-L1 expression on ATLL cells clearly needs to be carried out to determine the significance of PD-L1 expression for the disease characteristics.

Nakanishi *et al.*[44] reported increased PD-1 expression on tumor-infiltrating lymphocytes; however, the specificity of tumor-infiltrating lymphocytes against tumor-associated antigens was not addressed because of technical limitations. In our study, we assessed CTL specific to HTLV-1, which is a tumor-causing virus, using a well-established and highly sensitive tetramer assay. This may provide direct evidence that the PD-1/PD-L1 interaction is one of the mechanisms allowing ATLL evasion from the host immune system. Although approximately 60% of ATLL cells do not express Tax protein *in vivo*,[7] it has been reported that HTLV-1 Tax-specific CD8+ lymphocytes play an important role in ATLL tumor immunity after hematopoietic stem cell transplantation.[12] This tumor-associated antigen-specific tetramer assay will be used further to assess whether therapeutic intervention against the PD-1/PD-L1 pathway can restore PD-1-expressing CD8+ lymphocytes in a malignancy setting, as has been reported in chronic viral infection models.[25] Although some ATLL cells from uncultured peripheral blood did not express PD-L1, we found that HTLV-1-infected cells are capable of producing PD-L1 in short-term

culture assays (Figure 3). PD-1 expression in HTLV-1-specific CD8+ T cells in ACs and ATLL patients is similarly increased. These results suggest that interaction of high PD-1 expression on HTLV-1-specific CD8+ T cells could occur with PD-L1 in ATLL patients. Indeed, an earlier study has shown that PD-L1 is upregulated in HIV-infected individuals;[26] yet, to date, it is unknown whether HIV-infected cells that express viral-derived protein are capable of producing PD-L1.

Our earlier study revealed that HTLV-1-specific CD8+ T cells, which produce intracellular molecules relevant to cytolytic effector function (IFN-γ, perforin and granzyme B), are lacking in effector functions in ATLL patients.[13] Here, we also examined whether interrupting the ligation of PD-1 by its ligand PD-L1 could increase the cytolytic potential of HTLV-1-specific CD8+ T cells, similar to reports in a mouse model *in vivo* using cells from glioma and HIV patients *in vitro*.[24,25,27,41] Our results showed that blockade of the PD-1/PD-L1 pathway augmented the production of IFN-γ, TNF-α and expression of the degranulation marker, CD107a, by effector cells. Blank *et al*.[21] also reported that blockade of endogenous PD-L1 expression on human cancer cells can still improve the immune functions of tumor-specific T cells *in vitro*. Furthermore, Maier *et al*.[45] reported that blockade of PD-1/PD-L1 pathways may reverse viral persistence and chronic infection in cases, in which the CTL secretion of IFN-γ, after antigen recognition in the liver is suppressed. Taken together, the results of these studies suggest that an effective blockade of PD-L1 interactions with immune effector cells *in vivo* may provide a promising strategy of immunotherapy for selected tumors expressing PD-L1.[14,21,23]

In summary, this study indicates that the immunoregulatory PD-1/PD-L1 pathway is operative during persistent viral infection, and suggests that this pathway may contribute to exhaustion of the CTL response in HTLV-1-infected individuals, which may facilitate immune evasion and ATLL development. Furthermore, interventions that lead to reactivation of exhausted T cells by blocking the PD-1/PD-L1 axis may be an effective immunological strategy for the clearance of persistent HTLV-1 infection and the treatment of ATLL.

Acknowledgements

Research support: This study was supported by a Grant-in-Aid (to NA) from the Japanese Ministry of Health, Labor, and Welfare, and by the Kagoshima University for Frontier Science Research Center Program (to NA). We thank Dr Jeffrey A Medin for a critical reading of the paper, Ms Hokkoku for preparation of clinical samples, and Ms Ariyoshi for HLA typing.

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A second NOTCH1 chromosome rearrangement: t(9;14)(q34.3;q11.2) in T-cell neoplasia

Leukemia (2009) **23,** 1003–1006; doi:10.1038/leu.2008.366; published online 8 January 2009

NOTCH1 encodes a transmembrane signaling protein that plays key roles in development and neoplasia. Its leukemogenic involvement was first revealed by analysis of t(7;9)(q34;q34.3) in a T-cell acute lymphocytic leukemia (T-ALL) cell line (SUP-T1), which juxtaposes truncated NOTCH1 with TCRB, directing overexpression of N-terminally truncated polypeptides (reviewed in Grabher *et al*[1]). Wild-type NOTCH1 is initially cleaved (S1 cleavage) into twin polypeptides: an extracellular N-terminal subunit (NEC) and a transmembrane C-terminal

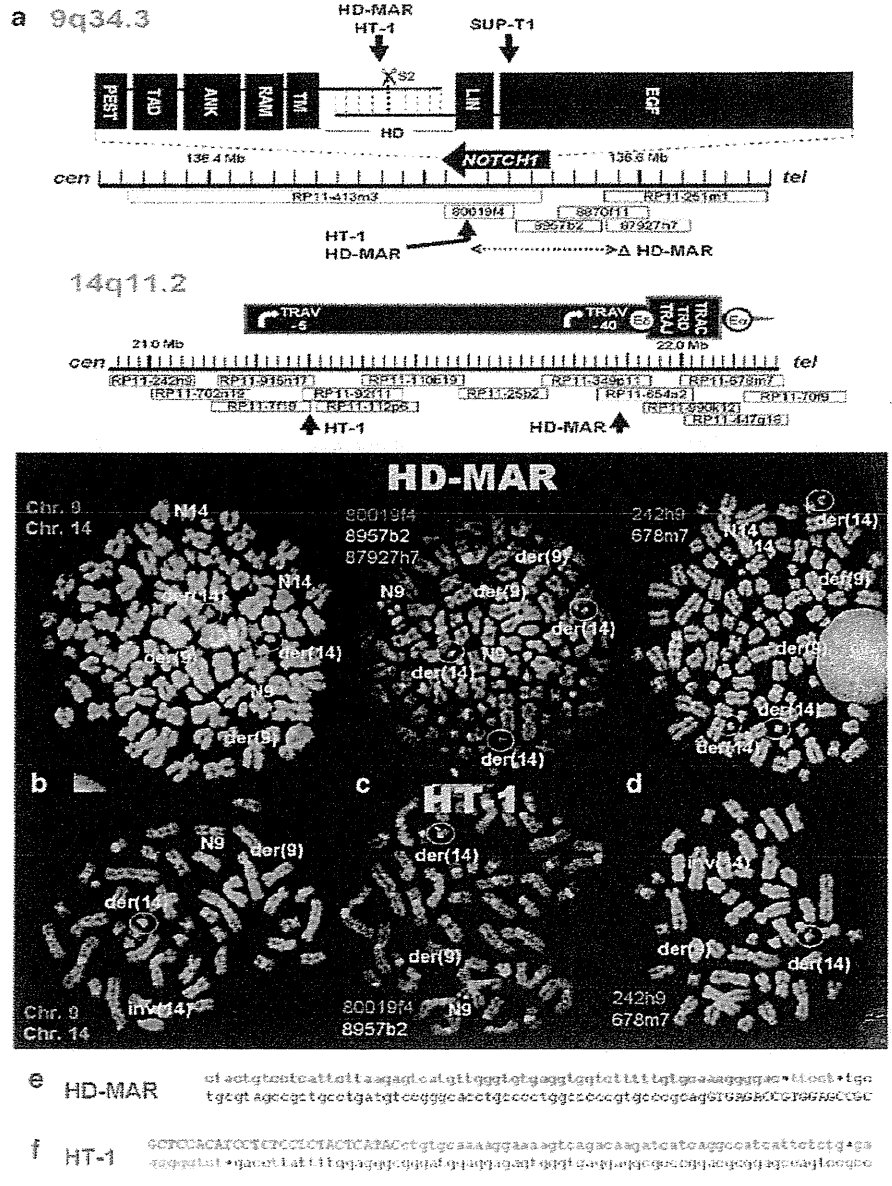

Figure 1 Cytogenetic and molecular breakpoint analysis of t(9;14)(q34;q11). (a) Breakpoint data, clone maps and the genomic structures of NOTCH1, including protein domain schema, and TRAD Note the intra-heterodimerization domain (HD) breakpoints in HD-MAR/HT-1 downstream of the S2 metalloprotease cleavage site, and the *cis*-HD breakpoint in SUP-T1. (b) Chromosome painting of t(9;14), and (c and d) FISH analyses of NOTCH1 and TRAD, respectively, in HD-MAR[4] and HT-1.[5] TRAD/NOTCH1 breakpoint junction sequences determined by long-distance inverse (LDI)-PCR are shown in (e) and (f), respectively. Nucleotides are shown in red, green and black to indicate TRAD, filler DNA and NOTCH1, respectively, with breakpoints shown as black dots. LDI-PCR was performed as described elsewhere.[6] Primer sequences are given in the Supplementary data.

subunit (NTM). Binding of NOTCH ligands to the epidermal growth factor-like repeats region of NEC promotes metalloprotease cleavage of NTM (S2 cleavage) to create membrane-bound NTM*I monomers (Figure 1a). These are cleaved in turn (S3 cleavage) at multiple sites within the heterodimerization domain (HD) binding extracellular and transmembrane subunits by the γ-secretase (GS) protease complex to release the intracellular domain (ICN1), which forms a ternary complex stimulating effector transcription.[1] ICN1 contains regulator of amino acid metabolism (RAM), ankyrin repeat and transcriptional activation domains, and a C-terminal polypeptide enriched with proline, glutamate, serine and threonine (PEST). Ankyrin and transcriptional activation domains are required for induction of T-ALL in mice.[2] Whereas γ-secretase inhibitors (GSi) may prove effective against T-ALL subsets, most T-cell lines are resistant, including SUP-T1.[3] We describe a second NOTCH1 rearrangement, t(9;14)(q34.3;q11.2), in T-ALL cell lines, HD-MAR[4] and HT-1,[5] both highly GSi-sensitive despite overexpressing truncated NOTCH1.

Cytogenetic analysis revealed t(9;14)(q34.3;q11.2) in both HD-MAR and HT-1 cells, with NOTCH1 breakpoints inside fosmid 80019F4 accompanied in HD-MAR cells by deletion of ~80 kbp corresponding to NEC (Figures 1a–d). Fluorescence *in situ* hybridization (FISH) also showed 14q11.2 breakpoints in both HD-MAR and HT-1 cells within the TRAV locus. These observations define an hitherto unlisted rearrangement (http://atlasgeneticsoncology.org//index.html), t(9;14)(q34.3;q11.2) that juxtaposes the intragenic regions of both NOTCH1 and TRAV. Long-distance inverse (LDI)-PCR confirmed and extended these findings, revealing tail-to-tail fusions of intron-27 of NOTCH1 at 138 516 818 and 138 516 905 bp, with 5′-TRAV40 (21 852 526 bp) and intron-1 of TRAV5 (21 287 494 bp) in HD-MAR and HT-1, respectively (Figures 1e and f, Supplementary Figure S1 A/B). t(9;14) in HD-MAR and HT-1 cells places NOTCH1, truncated inside HD (predicted 5 amino acids *trans-* of the S2 cleavage site), under transcriptional control of TRAV (Figure 1a). The TRAV-40 breakpoint in HD-MAR lies close to the proximal Eδ enhancer, whereas that in HT-1 at TRAV-5 lies ~600 kbp upstream near a cluster of DNase-I hypersensitive sites known to drive oncogene transcription in T-ALL. Thus, both t(9;14) cell lines carry breakpoints located inside HD, in contrast with that lying upstream (*cis-*) of HD in t(7;9) SUP-T1 cells.[1]

Protein (western blot) analyses of the minimal transforming regions, ankyrin and transcriptional activation domains, together with TM/RAM domains in T-ALL cells are shown in Figure 2a (top/middle). High protein expression of full-length NOTCH1 (~300 kDa) and NTM (~120 kDa) was detected in non-translocation cell lines excepting TALL-1 cells, which is monosomic for chromosome 9. Contrastingly, in NOTCH1 translocation cell lines the ~300-kDa band was weak (HD-MAR, HT-1) or absent (SUP-T1), indicating translocation-driven expression therein (Figure 2b). N-truncated NTMs in both t(9;14) cells undercut 116 kDa wild-type polypeptide (Figure 2a top), in contrast with t(7;9) SUP-T1 cells, which also translated longer species implying impaired cleavage therein. Correspondingly, ~110 TM/RAM+ forms in t(9;14) cells are taken to represent ICN1. Weak TM/RAM signals in *cis*-HD breakpoint SUP-T1 cells (Figure 2a bottom) also imply impaired GS-cleavage, as Ab8925 epitopes require prior GS exposure. These findings, linking peri-HD breakpoint location to aberrant protein expression, prompted further investigation into the effect of GSi treatments on NOTCH1 signaling and proliferation.

γ-Secretase inhibitor treatment effected dose-dependent reductions of cell growth and viability in HD-MAR and HT-1,

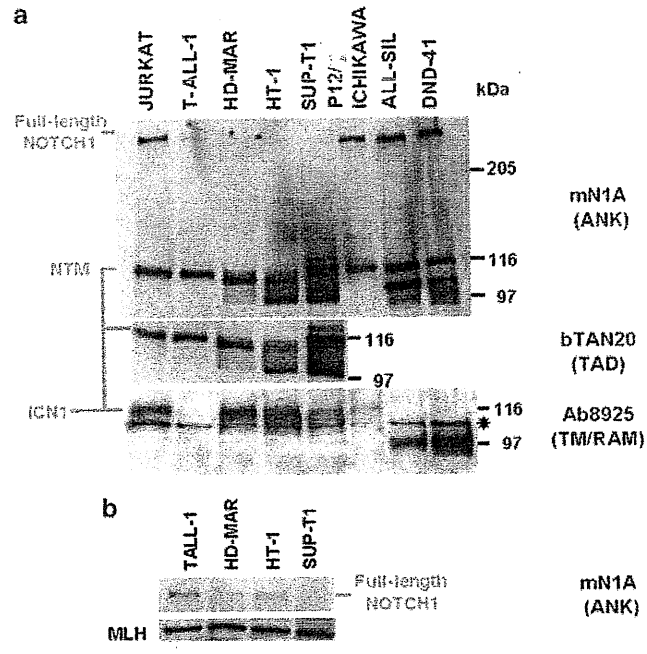

Figure 2 NOTCH1 protein expression in T-cell acute lymphocytic leukemia (T-ALL) cell lines with t(9;14). (**a**) Western analysis of T-ALL cell lines: NOTCH1-ANK domain antibody (Ab) to mN1A (top), bTAN20 Ab to transcriptional activation domains (TADs) (middle) and Ab8925 (activated NOTCH1) to TM/RAM (regulator of amino acid metabolism) (bottom). Ab8925 epitopes include a non-specific band (star) and require prior γ-secretase exposure (manufacturer's data). Note the weak TM/RAM epitopes in SUP-T1 NTM, which are attributable to impaired γ-secretase cleavage. (**b**) Western analysis of full-length NOTCH1 at longer exposure: note the faint (HD-MAR/HT-1) or absent (SUP-T1) full-length NOTCH1 bands, implying translocation-driven expression. Western blots were performed after lysis with 50 μl of RIPA buffer (1 μl aprotinin (1 mg/ml), 5 μl PMSF (20 ng/ml) and 50 μl 2 × SDS). Antibodies used: NOTCH1 (mN1A; BD Biosciences, San Diego, CA, USA, bTAN-20; Hybridoma Bank, Iowa City, IA, USA and Ab8925; Abcam, Cambridge, UK). Loading was checked using Ponceau dye and anti-human-MSH6 Ab (Santa Cruz Biotechnology, Heidelberg, Germany).

while sparing SUP-T1 cells (Figure 3a). The GSi sensitivities of both t(9;14) cell lines significantly exceeded that of T-ALL1, which was described earlier as sensitive.[3] Proliferation arrest in HD-MAR and HT-1 paralleled dose-dependent increases in G0/G1 arrest after GSi treatments, which evinced progressively weaker responses in TALL-1 and SUP-T1 cells (Figure 3b). To investigate these differential GSi sensitivities, downregulation of NOTCH1 transcriptional target genes, HES1 and MYC were respectively, measured in HD-MAR (92, 66%), HT-1 (94, 79%) and SUP-T1 (75, 39%) cells. Differential Gsi sensitivities of NOTCH1 effector gene expression thus paralleled both cell survival and cell cycle progression endpoints (Figures 3c and d), confirming the greater GS-dependence of NOTCH1 signaling in intra-HD breakpoint cells.

Inhibiting cleavage by GS-dependence (GSi) treatment effected the accumulation of four ANK+ species (105–116 kDa) taken as NTM/NTM*I (Figure 3e brackets), and dissipation of ~110 kDa TM/RAM+ (ICN1), that is, a picture consistent with interconversion. This result underlines the dependence of TM/RAM expression on GS activity in t(9;14) cells. A fifth truncated ~100 kDa NTM/NTM*I accumulated in HT-1 cells (arrow). Immunostaining revealed greater accumulation of

perimembraneous NOTCH1 at the expense of intranuclear forms in HD-MAR than in SUP-T1 (Figure 3f). Taken together, GSi treatments revealed increased dependence of intra-HD breakpoint cells on GS activity for NOTCH1 expression, signaling and proliferation. The enhanced TM/RAM epitope exposure of both t(9;14) cell lines (Figure 2a bottom) suggests a protein structural basis underlying the reduced dependence of GS cleavage on prior S2 cleavage owing to their adjacent breakpoints. The correlation of GS sensitivity with intra-HD breakpoint placement is consistent with data from a second t(7;9) cell line CUTLL1 with an intra-HD breakpoint, also sensitive to GSi.[7]

In T-ALL mutations occur in the F-box protein FBXW7 (alias FBW7, hCdc4), which abrogate its binding to NOTCH1, affecting NOTCH1 longevity and GSi responses. However, analysis of HD-MAR, HT-1 and SUP-T1 cells showed normal sequences around Arg465, Arg^{479} and Arg^{505} hotspots, discounting a major contribution in modulating GSi sensitivity thereby, refocusing the spotlight on GS cleavage.

All the three molecularly karyotyped t(9;14)(q34;q11) cases report TRAD/NOTCH1 involvement (Gesk *et al.*,[8] this report). All the five age-recorded t(9;14) cases were hitherto described in young adults (Table 1), whereas all four t(7;9) cases are pediatric (http://atlasgeneticsoncology.org/), raising the question whether different age groups might be targeted. However, only a few piecemeal cases have been described for either translocation and additional mapped examples are required to delineate breakpoints in both t(9;14) and t(7;9) T-ALL and help determine the clinical relationship of the cytogenetic entities.

Our findings highlight the role of intra-HD NOTCH1 breakpoint locations in promoting ligand-independent transcription and translation of GSi-sensitive polypeptides. Hightened GSi sensitivity bestowed by such intra-HD breakpoints may reflect increased molecular exposure near the HD region facilitating NOTCH1 cleavage. Independence from ligand-mediated cleavage may serve to promote the 'non-oncogene addiction' of intra-HD breakpoint cells on GS cleavage for NOTCH1 signaling.

In summary, we have characterized a new NOTCH1 alteration, t(9;14)(q34;q11), in T-ALL, which is only the second neoplastic NOTCH1 chromosome rearrangement described hitherto. Our data highlight breakpoints in the peri-HD region in determining GS activity and responses to inhibitor. Cell line models for the entity described here provide singular tools for

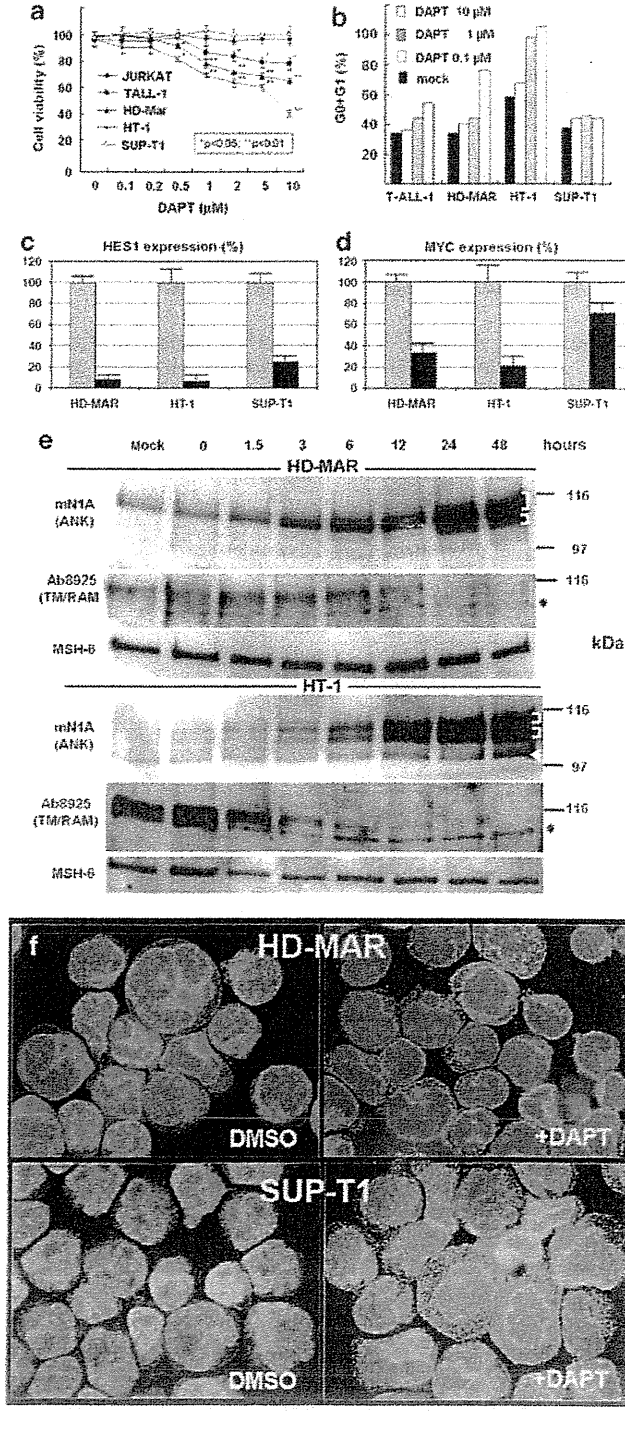

Figure 3 Responses of t(9;14) cells to γ-secretase inhibitors (GSi) treatment .T-ALL cell lines were cultured for 96 h with the indicated concentrations of DAPT or mock-treated with dimethylsulfoxide (DMSO). Cellular proliferation and DNA content were, respectively, measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (**a**) and by flow cytometry (**b**) after staining with propidium iodide. Note GSi hypersensitivities of HD-MAR and HT-1 t(9;14) cells at both endpoints. To analyze the effects of GSi on NOTCH1 signaling, t(9;14) and t(7;9) cells were treated for 16 h with 2 μM DAPT and subsequently analyzed by RQ-PCR to measure expression of HES1 (**c**) and MYC (**d**), both shown in black. The DMSO-control was set at 100% (gray). Bars show standard deviations. Again note the greatest HES1/MYC dependence on NOTCH1 signaling in t(9;14) cells. For reverse transcription quantitative (RQ)-PCR, cDNA was synthesized from 5 μg total RNA extracted from 2×10^6 cells with TRIzol (Invitrogen, Karlsruhe, Germany) by random priming in 20 μl using Superscript II (Invitrogen). RQ-PCR used the 7500 Real-Time System (Applied Biosystems, Darmstadt/Germany). Expression of HES1, MYC and the control gene TBP was analyzed using commercial primer sets (Applied Biosystems). To analyse the effects of GSi on NOTCH1 polypeptides, western analysis was performed on HD-MAR and HT-1 cells treated with 2 μM DAPT for the times indicated. (**e**) NOTCH1 polypeptides were immunoprecipitated from whole cell extracts with antibodies recognizing either the intracellular domain of NOTCH1 (mN1A) or the GS-cleaved form of NOTCH1 (ab8925). Brackets show four accumulating ankyrin-positive (ANK+) species (taken as NTM/NTM*I) and concomitant loss of TM/RAM+ (regulator of amino acid metabolism-positive) species (taken as ICN1) following GSi treatment. A fifth truncated ~100 kDa NTM/NTM* accumulated in HT-1 cells (arrow). The starred band is unspecific. (**f**) Effects of GSi on nuclear localization of NOTCH1 polypeptides in HD-MAR and SUP-T1. Cells treated for 24 h with DMSO (left) or 2 μM DAPT (right) were stained with the intracellular domain of NOTCH1 (mN1A) Ab. Reticular staining after GSi treatment reflects the redistribution of NOTCH1 polypeptide to the cell membrane.

Table 1 Summary of *t*(9;14) cases

Case	Diagnosis	Age (years)	Cell line	Reference
1	T-cell neoplasia[a]	20	HD-MAR	4
2	T-ALL	32	HT-1	5
3	T-ALL	24	—	8
4	T-ALL	23	—	9
5	T-ALL	29	—	9
6	T-CLL	Unknown	—	10

Abbreviations: ALL, acute lymphocytic leukemia; CLL, chronic lymphocytic leukemia; HD, heterodimerization domain.
[a]After Hodgkin's lymphoma.

investigating the leukemic role of NOTCH1, a topic of pressing clinical and scientific interest.

Acknowledgements

We thank H Ben-Bassat and M Abe for HD-MAR and HT-1 cells, respectively, A Ferrando for reading the paper, and the José Carreras Leukemia Research Fund for support.

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Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)

Nuclear entrapment of BCR–ABL by combining imatinib mesylate with leptomycin B does not eliminate CD34$^+$ chronic myeloid leukaemia cells

Leukemia (2009) **23**, 1006–1008; doi:10.1038/leu.2008.367; published online 8 January 2009

Chronic myeloid leukaemia (CML) arises from the formation of the Philadelphia (Ph$^+$) chromosome in haematopoietic stem cells. The translated fusion oncoprotein, BCR–ABL (p210$^{BCR-ABL}$), is a constitutively active tyrosine kinase (TK), which activates multiple proliferative and anti-apoptotic signalling pathways, causing deregulated cell growth.[1] Despite impressive rates of complete cytogenetic response (CCyR) in the majority of patients treated with the targeted TK inhibitor (TKI), imatinib mesylate (IM; Glivec, Novartis, Basle, Switzerland),[2] few patients achieve sustained molecular remission and a significant proportion develops resistance to IM.[3] The presence of pre-existing or acquired BCR–ABL kinase domain mutations, which decrease IM binding,[3] and the innate insensitivity of primitive quiescent CML stem cells to IM[4] are two contributing resistance mechanisms. Consequently, many strategies have been investigated to overcome IM resistance, including the development of second-generation TKIs, nilotinib (Tasigna, Novartis) and dasatinib (Sprycel, Bristol–Myers Squibb, Princeton, NJ, USA), although these remain ineffective against the T315I mutation.

In search of alternative strategies for the elimination of Ph$^+$ cells, Vigneri and Wang [5]showed an intriguing strategy of tricking BCR-ABL$^+$ cells into committing cell death. This mechanism was based on the observation that on treatment with IM, inactive BCR-ABL translocates into the cell nucleus. Entrapment of BCR–ABL there, coupled with re-activation of its TK activity, reversed its role to that of an activator of apoptosis. On the basis of the mounting evidence that BCR–ABL$^+$ stem cells are not eliminated by novel targeted therapies, such as IM and the second-generation TKIs, we investigated whether combination treatment of IM, with the nuclear export inhibitor leptomycin B (LMB Calbiochem), could drive CML CD34$^+$ cells into apoptosis.

Our initial experiments, over a period of 16 days, on the effects of IM, LMB and the combination of these drugs on the Ph$^+$ cell line K562, showed that treatment with either drug alone for the first 72 h was at least cytostatic. However, 5–8 days after drug washout, cells were able to recover, an effect not seen with the drug combination,in which irreversible growth

Molecular Immunology 47 (2009) 606–613

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Short communication

Efficient induction of human T-cell leukemia virus-1-specific CTL by chimeric particle without adjuvant as a prophylactic for adult T-cell leukemia

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ARTICLE INFO

Article history:
Received 25 June 2009
Received in revised form 1 September 2009
Accepted 3 September 2009
Available online 3 November 2009

Keywords:
Human T-cell leukemia virus-1
Adult T-cell leukemia/lymphoma
Cytotoxic T lymphocytes
Chimeric virus-like particle
Vaccine

ABSTRACT

Adult T-cell leukemia-lymphoma (ATL) is an aggressive peripheral T-cell neoplasm that develops after long-term infection with the human T-cell leukemia virus-1 (HTLV-1). HTLV-1-specific cytotoxic T lymphocytes (CTLs) play an important role in suppressing proliferation of HTLV-1-infected or transformed T-cells *in vitro*. Efficient induction of antigen-specific CTLs is important for immunologic suppression of oncogenesis, but has evaded strategies utilizing poorly immunogenic free synthetic peptides. In the present study, we examined the efficient induction of HTLV-1-specific CD8+ T-cell response by an HTLV-1/hepatitis B virus core (HBc) chimeric particle incorporating the HLA-A*0201-restricted HTLV-1 Tax-epitope. The immunization of HLA-A*0201-transgenic mice with the chimeric particle induced antigen-specific gamma-interferon reaction, whereas immunization with epitope peptide only induced no reaction as assessed by enzyme-linked immunospot assay. Immunization with the chimeric particle also induced HTLV-1-specific CD8+ T-cells in spleen and inguinal lymph nodes. Furthermore, upon exposure of dendritic cells from HLA-A*0201-transgenic mice to the chimeric particle, the expression of CD86, HLA-A02, TLR4 and MHC class II was increased. Additionally, our results show that HTLV-1-specific CD8+ T-cells can be induced by peptide with HTLV-1/HBc particle from ATL patient, but not by peptide only and these HTLV-1-specific CD8+ T-cells were able to lyse cells presenting the peptide. These results suggest that HTLV-1/HBc chimeric particle is capable of inducing strong cellular immune responses without adjuvants via effective maturation of dendritic cells and is potentially useful as an effective carrier for therapeutic vaccines in tumors, or in infectious diseases by substituting the epitope peptide.

1. Introduction

Adult T-cell leukemia-lymphoma (ATL) is an aggressive peripheral T-cell neoplasm, developing after long-term infection with HTLV-1 (Uchiyama, 1997). HTLV-1-specific CTLs play an important role in suppressing proliferation of HTLV-1-infected or transformed T-cells *in vitro* (Bangham, 2008; Jacobson et al., 1990). Additionally, we have previously reported the decreased frequency and function of HTLV-1 Tax-specific CD8+ T-cells in ATL patients, as characterized by insufficient cytolytic effector molecules, and have described the upregulation of the negative immuno-regulatory programmed death 1 (PD-1) marker on HTLV-1-specific CTLs from asymptomatic HTLV-1 carriers (ACs) and ATL patients (Kozako et al., 2006, 2009). Impaired host CTL function abrogates protection against accumulation of HTLV-1-transformed cells, and circumventing this hurdle may yield an effective immune strategy against leukemogenesis (Harashima et al., 2004; Yasunaga et al., 2001).

Antigen-specific CTL induction is an attractive immunotherapeutic strategy against hematologic malignancies and other cancers (Albert et al., 1998; Kawakami et al., 2008). The difficulty in inducing antigen-specific CTLs in individual patients vitiates a more widespread use of adoptive T-cell therapy. Whereas free synthetic peptides have proven to be relatively poor immunogens, viral-like particles (VLPs) have been consistently shown to induce strong antibody responses and CTLs, even without adjuvant (Grgacic and

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doi:10.1016/j.molimm.2009.09.006

Anderson, 2006; Zhang et al., 2009). The hepatitis B core antigen (HBcAg), a potent immunogen eliciting strong humoral, T-helper and CTL responses and amenable to a variety of heterologous epitopes without adjuvant (Milich et al., 1987), is a potentially effective carrier protein for T-cell mediated vaccine development (Storni et al., 2002).

In the present study, we fused HTLV-1 Tax11–19 peptide, recognized by HLA-A*0201-restricted HTLV-1-specific CD8+ T-cells with high frequency (Kozako et al., 2006), to the HBcAg to synthesize an HTLV-1/HBc chimeric particle. We further examined the efficient induction of the HTLV-1-specific CD8+ T-cell response in HLA-A*0201-transgenic mice by the chimeric particle without adjuvant. This is the first study demonstrating the successful induction of HTLV-1-specific CD8+ T-cells in HLA-transgenic mice *in vivo*.

2. Materials and methods

2.1. Expression of recombinant chimeric particle in Pichia pastris

A synthetic DNA fragment, the HTLV-1 Tax sequence from amino acid positions 8–22 including the Tax11–19 epitope recognized by HLA-A*0201, was inserted into the HBc gene. The chimeric genes were PCR amplified by using pUC18 DNA vector (Takara, Japan) as a template. The sense primer 5′CGGGATCCACCATGGACATGGACCCGTATAAA3′ included a BamHI site (underlined) in the context of Kozac sequence. The antisense primer 5′GGAATTCTAACATTGAGATCCCCGAGA3′ included an EcoRI site (underlined). The purified PCR product was digested with the appropriate enzymes, and ligated to the BamHI and EcoRI treated *P. pastris* expression vector, pPIC3.5 (Fig. 1A, Invitrogen BV, Groningen, The Netherlands). The chimeric protein was expressed in *P. pastris* KM71.

P. pastris His+ transformants were grown in an L-tube in 5 ml of a rich standard medium [1% (w/v) yeast extract, 2% (w/v) peptone, 1.34% (w/v) yeast nitrogen base, 0.4 mg/l biotin and 100 mM potassium phosphate (pH 6.0)] containing 1% (w/v) glycerol (BMG), until OD600 reached 2–6. To begin the induction phase, cells were harvested by centrifugation and resuspended to an OD600 of 1 in rich standard medium containing 0.5% of methanol (BMM). Each subsequent day, 100% methanol was added to a final concentration of 0.5%. On the second day the yeast cells were harvested. Methanol-induced cultures were analyzed for HBcAg expression by using the ELISA. One transformant, isolated from the parental strain KM71, was selected for its high level of expression and was retained for large scale fermentation. The following large scale fermentation conditions were used: the dissolved oxygen concentration was maintained above 20% saturation; the pH was maintained at 5.5 throughout the fermentation process [with 28% (v/v) ammonium hydroxide], and the temperature was set at 30 °C using BMS-P I (Biot, Osaka).

The transformant was grown at 30 °C until OD600 reached 2–6 in a 5 L culture BMG medium. After exhausting the glycerol from the culture medium, 50% (w/v) glycerol, YPD and 100% methanol were supplied. Methanol supply was added to a final concentration of 0.5% in the culture medium over a 2-day period. The culture was harvested by centrifugation at 3000 × *g* for 10 min at 4 °C, and the cell pellet was recovered and stored at −80 °C until purification.

2.2. Preparation of the recombinant HTLV-1/HBc chimeric particle

Cell extracts were prepared by sonication at 0.02 g/ml in PBS containing 1 mM PMSF. After destruction, the cell suspension was centrifuged for 20 min at 10,000 rpm at 4 °C. The supernatant was filtered through a 0.45 μm membrane and then loaded onto anion-exchange DEAE-Toyopearl 650 mol/L chromatography column. HBcAg positive fractions detected by ELISA were examined by sedimentation through a sucrose step-wise gradient centrifugation. An aliquot (20 ml) of each sample was layered on top of a 40-ml step-wise sucrose gradient (30–60% [w/w]) in PBS and centrifuged at 30,000 rpm for 14 h at 4 °C with an RPST40T rotor (Hitachi, Japan). HBcAg positive fractions were dialyzed against a Tris–saline buffer on a 30,000 Da cutoff membrane and concentrated in an AMICON cell (Millipore S.A., St-Quentin-Yvelines, France). The products of HTLV-1/HBc chimeric particle were detected using methods previously described (Shiosaki et al., 1991). The monoclonal antibody used for the antigen analysis by ELISA and Western immunoblot was anti-HBc antibody (Miyanohara et al., 1986).

2.3. Animals

HLA-A2.1 (HHD) Tgm; H-2Db−/−β2m−/− double knockout mice introduced with human β2m-HLA-A2.1 (1 2)-H-2Db (three transmembrane cytoplasmic) (HHD) monochain construct gene were generated in the Department SIDA-Retrovirus, Unite d'Immunite Cellulaire Antivirale, Institut Pasteur, France (Pascolo et al., 1997).

2.4. Induction of HTLV-1-specific mouse CTLs

HLA-A*0201 (HHD) transgenic mice were immunized intradermally through the tail at days 0 and 14 with HTLV-1/HBc chimeric particle (20 μg), or Tax11–19 peptide (1 μg: LLFGYPVYV), assuming Tax11–19 epitope comprises one-twentieth of the HTLV-1/HBc protein. Simultaneous but uncombined immunization with HBc particle (20 μg) and peptide (1 μg) was also done. Cells (2×10^6 cells/well) from spleens and inguinal lymph nodes, harvested 7 days after the last immunization, were stimulated with Tax11–19 peptide *in vitro*. Then 6 days later, the frequency of cells producing gamma-interferon (IFN-γ) per 5, 10, 20×10^4 inguinal lymph node cells upon stimulation with syngenic bone marrow-derived dendritic cells (BM-DC) (1×10^4 cells/well) (Senju et al., 2003) (pulsed with or without each peptide) was assayed by enzyme-linked immunospot (ELISPOT) using the ELISPOT Set (Becton Dickinson, San Jose, CA) as previously described (Komori et al., 2006).

2.5. Maturation of dendritic cells

Murine immature dendritic cells (iDCs) were obtained from bone marrow (BM) precursors using a previously described method (Senju et al., 2003).

2.6. Flow cytometry

Phenotypic analysis using HTLV-1 Tax11–19 (LLFGYPVYV)/HLA-A*0201 tetramers was performed as described previously (Kozako et al., 2006, 2009). Matured DCs were immunostained with anti-mouse CD86 mAbs (clone: GL1; BD PharMingen, San Diego, CA), anti-mouse MHC class II mAbs (clone: NIMR-4; eBioscience, San Diego, CA), anti-mouse toll-like receptor (TLR4)/CD284 mAbs (clone: UT49; Medical and Biological Laboratories), and anti-HLA-A02 (clone: BB7.2; Santa Cruz Biotechnology, Santa Cruz, CA) mAbs as maturation markers by flow cytometry (FCM) on a FACScan (BD Biosciences). The data were expressed as mean fluorescence intensity (MFI) compared to unpulsed iDC controls.

2.7. Clinical samples

The subjects in this study included 6 ACs and 5 ATL patients, all of whom were recruited from Kagoshima University Hospital. Subjects were examined by standard serological testing for the

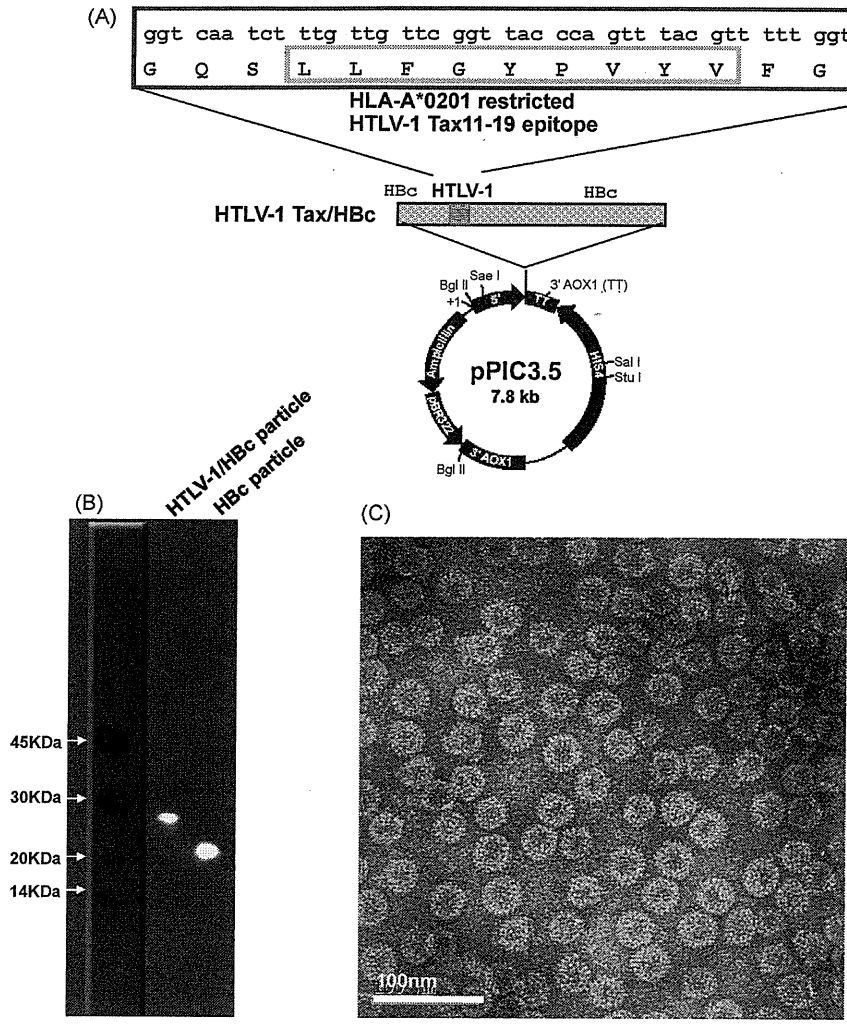

Fig. 1. Expression of recombinant HTLV-1/HBc chimeric particle. (A) The construction of HTLV-1/HBc chimeric protein. (B) Western blot profiles of the HTLV-1/HBc chimeric particle (left) and the HBc particle (right). The gene products were detected with anti-HBc antibody. (C) Electron micrograph of the HTLV-1/HBc chimeric particle. Original magnification × 100,000.

presence of HTLV-1 and by hematological/Southern blotting analysis for diagnosis of ATL. All subjects gave their written informed consent for participation in this study and to allow review of their medical records, and provided a sample of peripheral blood mononuclear cells (PBMCs) for human leukocyte antigen (HLA) typing and for the HLA tetramer assay (Kozako et al., 2006). The study protocol was reviewed and approved by the Medical Ethics Committee of Kagoshima University.

2.8. Preparation of PBMCs

PBMCs were obtained from peripheral blood by separation on Ficoll/Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation at 400 × *g* for 30 min, followed by washing 3 times with 1% FCS RPMI-1640 at 200 × *g* centrifugation for 10 min to remove residual platelets. The fresh PBMCs were used for tetramer assay and *ex vivo* expansion of anti-HTLV-1 CD8+ CTL. The remaining PBMCs were cryopreserved in liquid nitrogen until examination as described previously (Kozako et al., 2006).

2.9. Induction of HTLV-1 Tax-specific CD8+ T-cells from ACs and ATL patients

Aliquots of PBMCs (1×10^6 cells) were used for *in vitro* expansion of HTLV-1-specific CD8+ T-cell clones in cultures with each antigen in RPMI-1640 medium supplemented with the following reagents: 100 units/ml penicillin, 0.1 mg/ml streptomycin, 0.1 mM non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.05 mM 2-mercaptoethanol, 50 units/ml of recombinant human interleukin-2, and 10% heat-inactivated fetal calf serum (RPMI-1640-CM). All culture conditions were the same as described elsewhere (Kozako et al., 2006). The cultured PBMCs were examined using the HTLV-1/HLA tetramer assay described below (Kozako et al., 2006).

2.10. Flow cytometric assay of cell mediated cytotoxicity

Cytotoxic activity of peptide-specific CTLs was evaluated using the T2-A2 cell line, *HLA-A*0201*-transfected, transporter associated with antigen processing-deficient (T × B) cell hybrid T2 cell line, which was maintained in RPMI-1640 supplemented with 10% FCS, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 2 mM L-glutamine, as targets by double staining (5 and 6)-carboxy fluorescein diacetate succinimidyl ester (WAKO, Japan) and Annexin V-PE-Cy5 (Medical and Biological Laboratories, Nagoya, Japan) (Aubry et al., 1999). Briefly, T2-A2 cells were incubated at 26 °C for 16 h, then incubated with/without HLA-A*0201-restricted HTLV-1 Tax peptide (LLFGYPVYV: 10 μM) or HLA-A*0201-restricted CMV pp65 peptide (NLVPMVATV: 10 μM) for 2 h at 26 °C followed by CFSE labeling. CFSE-labeled target cells were washed 3 times and

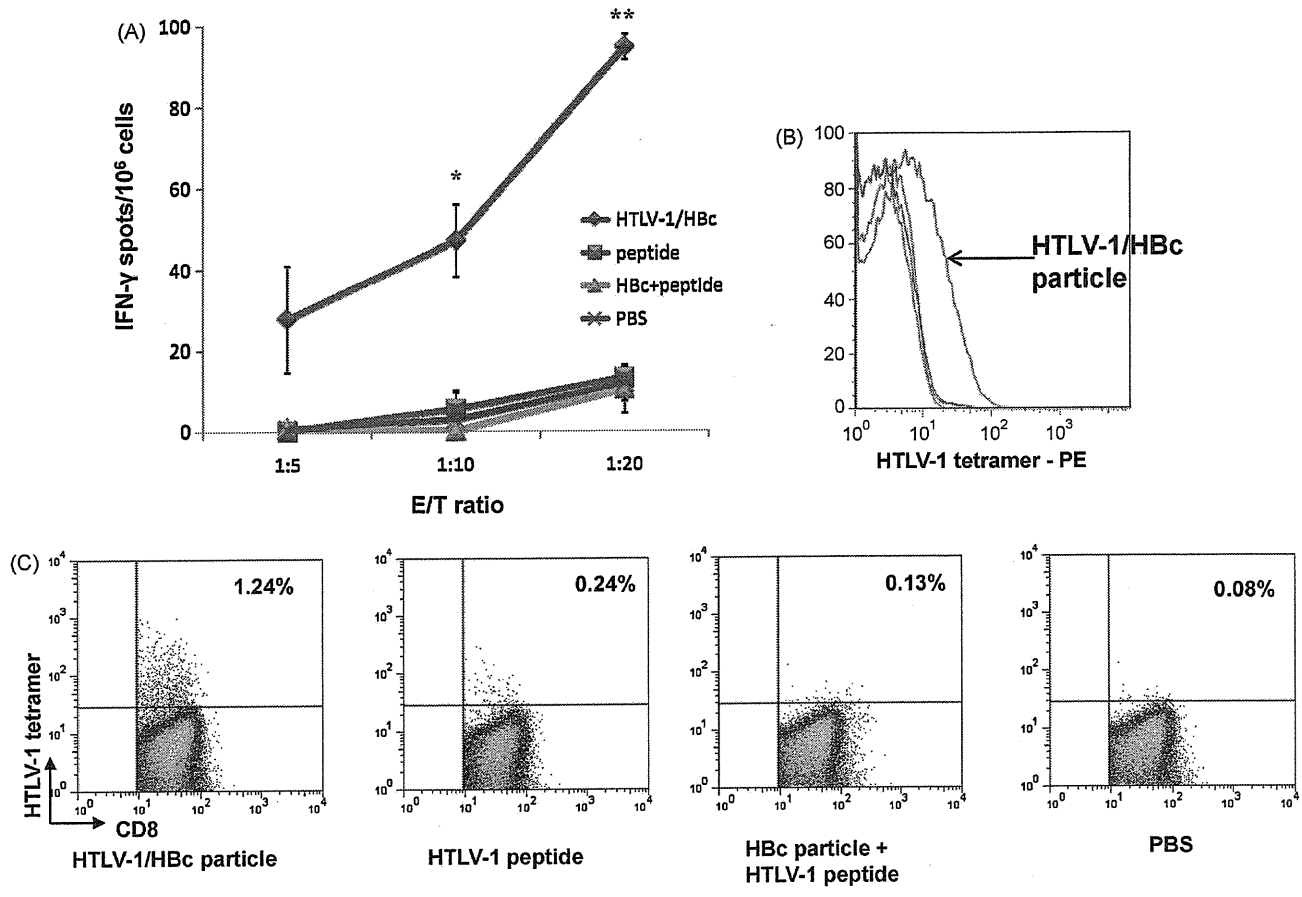

Fig. 2. Induction of cellular immunity by intradermal immunization with HTLV-1/HBc chimeric particle. (A) HLA-A*0201-transgenic mice were intradermally immunized twice with HTLV-1/HBc chimeric particle (20 μg), HTLV-1 peptide (1 μg: LLFGYPVYV), HTLV-1 peptide (1 μg) plus HBc particle (20 μg), or phosphate buffered saline (PBS) at days 0 and 14. Seven days after the last immunization, the spleens and inguinal lymph nodes were collected. The inguinal lymph node cells (2×10^6/well) were stimulated with Tax11–19 peptide *in vitro*. Then 6 days later, the frequency of cells producing IFN-γ per 5, 10, 20×10^4 inguinal lymph node cells upon stimulation with syngenic BM-DC (1×10^4/well), pulsed with or without each peptide, was determined by ELISPOT assay. IFN-γ spots are expressed as the number of peptide-loaded to peptide-unloaded target cells. $^*P < 0.05$, $^{**}P < 0.01$ vs PBS group. The experiments were performed in triplicates. Results represent means ± S.D. HTLV-1-specific CD8+ T-cell induction from spleen cells (B) and inguinal lymph node cells (C) by intradermal immunization with HTLV-1/HBc chimeric particle. Inguinal lymph node cells and spleen cells were harvested 7 days after the last immunization and stimulated with HTLV-1 peptide for 32 days. HTLV-1-specific CD8+ cells were analyzed by flow cytometry with anti-CD8-FITC and HTLV-1/HLA-A*0201 tetramer-PE. Numbers in the upper right quadrants represent the percentages of tetramer + CD8+ T-cells in CD8+ T lymphocytes.

seeded in a 96-well plate at a concentration of 1×10^4 cells/well. CTLs were added at 1:1, 5:1, 10:1 and 50:1 effector:target cell ratio and incubated for 4 h. All tests were performed in triplicate. Cytotoxicity (%) = [(ET − T0)/(100 − T0)] × 100; ET = Annexin V positive rate in the CFSE positive cells when target cells were co-cultured with effector cells. T0 = Annexin V positive rate in the CFSE positive cells when target cells were not co-cultured with effector cells.

2.11. Statistical analysis

Data obtained by FCM and ELISPOT assay were analyzed by two-tailed Student's *t*-test. A *P*-value < 0.05 was considered statistically significant. Statistical analyses were made using the StatView 5.0 software package (Abacus Concepts, Calabasas, CA).

3. Results

3.1. Expression of HTLV-1 Tax/HBc chimeric particle

We constructed an HTLV-1/HBc chimeric particle by inserting HTLV-1 Tax-epitope obtained from *P. pastris* His+ transformants. Protein was prepared by purification from yeast cell extracts as described previously (Shiosaki et al., 1991). HTLV-1/HBc chimeric protein expressed in *P. pastris* was collected in the fraction of 46–48% (w/w) sucrose. These results were nearly identical to the HBc protein (42–44%) (Miyanohara et al., 1986; Shiosaki et al., 1991). HTLV-1/HBc chimeric protein was collected in the high concentration fraction. The HTLV-1/HBc chimeric protein was 28 kDa, while the HBc protein was 21 kDa in Western blot analyses (Fig. 1B). As confirmed by electron microscopy, the chimeric particle from the yeast maintained the capacity to fold correctly, and spontaneously assembled into structured capsid particles with a diameter of 36 nm (Fig. 1C).

3.2. HTLV-1 Tax/HBc chimeric particle is immunogenic in the absence of adjuvant in vivo

The immune responses were investigated in HLA-A*0201-transgenic mice after intradermal immunization with HTLV-1 Tax/HBc chimeric particle. To determine the induction of humoral and cellular immunity, female mice were intradermally immunized twice at intervals of 14 days with HTLV-1 Tax/HBc chimeric particle, Tax peptide alone or HBc particle + Tax peptide. Seven days after the last immunization, inguinal lymph node cells from the mice immunized with these antigens were examined for their ability to induce IFN-γ-producing cells by ELISPOT assays. Immunization of HLA-A*0201-transgenic mice with the chimeric particle resulted in the efficient induction of IFN-γ-producing cells (Fig. 2A).

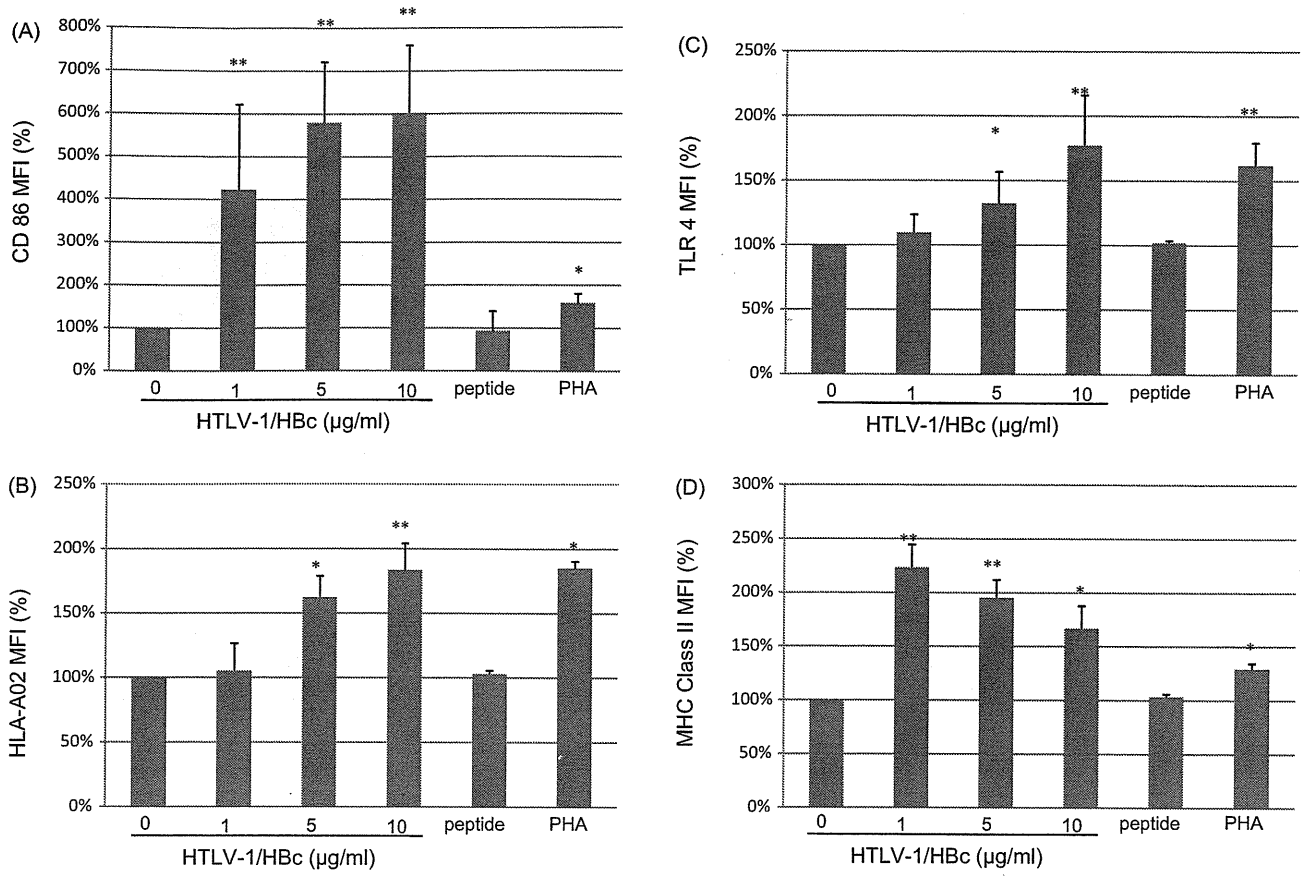

Fig. 3. Maturation of DCs induced by HTLV-1/HBc chimeric particle. Maturation of DCs induced by HTLV-1/HBc chimeric particle is illustrated by the expression of CD86 (A), HLA-A02 (B), TLR4 (C) and MHC class II (D) on the surface of DCs after incubation with antigens. The iDCs were incubated with the indicated concentrations of HTLV-1/HBc chimeric particle: 10 μg/ml of HTLV-1 peptide or 10 μg/ml of phytohemagglutinin (PHA) at 37 °C. Data are expressed as the mean fluorescence intensity (MFI) for each molecule compared to unpulsed (0 μg/ml) iDC controls. Results represent means ± S.D. for four independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ vs unpulsed iDC controls.

This induction of IFN-γ-producing cells correlated well with effector cell increase, and was significantly higher than observed for either immunization with Tax peptide alone, or uncombined but simultaneous administration of HBc particle with Tax peptide.

We then measured HBc specific antibody levels in serum by ELISA. Mice immunized with HTLV-1 Tax/HBc chimeric particle or HBc particle had high levels of anti-HBc IgG in their sera, while mice immunized with Tax peptide had no anti-HBc IgG. IgG concentrations for HTLV-1 Tax/HBc chimeric particle and HBc particle displayed sigmoid kinetics (data not shown).

3.3. *HTLV-1 Tax/HBc chimeric particle induces HTLV-1-specific CD8+ T-cells*

To examine HTLV-1 Tax-specific CD8+ cell induction, spleen cells and inguinal lymph node cells from mice immunized with HTLV-1 Tax/HBc chimeric particle were stimulated with Tax peptide *in vitro*. HTLV-1 Tax-specific CD8+ cells from spleen and inguinal lymph nodes were detected by tetramer assay. The induction of HTLV-1 Tax-specific CD8+ cells from spleen (Fig. 2B) and inguinal lymph nodes (Fig. 2C) was observed after immunization with HTLV-1 Tax/HBc chimeric particle.

3.4. *Maturation of DCs through uptake of chimeric particle*

Dendritic cell maturation is associated with increased expression of several cell surface markers, including the co-stimulatory molecules CD86 and MHC class II. To determine whether phenotypic maturation of DCs was mediated by chimeric particle uptake, iDCs were incubated with HTLV-1/HBc chimeric particle for 48 h, and the expression of surface molecules was measured by FCM. Upon exposure of these DCs to the chimeric particle, the expression of CD86 and HLA-A02 was increased in a dose-dependent manner (Fig. 3A and B). As a positive control, PHA-pulsed DCs displayed a marked increase in maturation markers as well, while HTLV-1 epitope peptide did not upregulate these surface markers. HTLV-1/HBc chimeric particle also upregulated the expression of TLR4 in a dose-dependent manner compared to unpulsed control iDCs (Fig. 3C). In addition, the chimeric particle resulted in a marked increase in MHC class II expression at 1 μg/ml, but which trended downwards at concentrations equal to or greater than 5 μg/ml chimeric particle (Fig. 3D).

3.5. *Induction of HTLV-1 Tax-specific CD8+ T-cells from ATL patients and ACs and cytotoxic activity of induced CTL*

PBMCs from ACs or ATL patients were cultured with or without 20 μM of Tax11–19 peptide (LLFGYPVYV) or 0.5 μM of HTLV-1/HBc chimeric particle. An increase in the proportion of Tax11–19 tetramer positive cells was evident for AC cells exposed to peptide or chimeric particle (5 of 6, representative data shown in Fig. 4A), with similar levels of induction. In contrast, we observed no increase in the proportion of Tax11–19 positive cells for ATL patient cells ($n = 5$ patients) exposed to either Tax11–19 peptide or chimeric particle alone, although a combination of both peptide and chimera elicited modest increases (in 2 of 2 ATL patient sam-

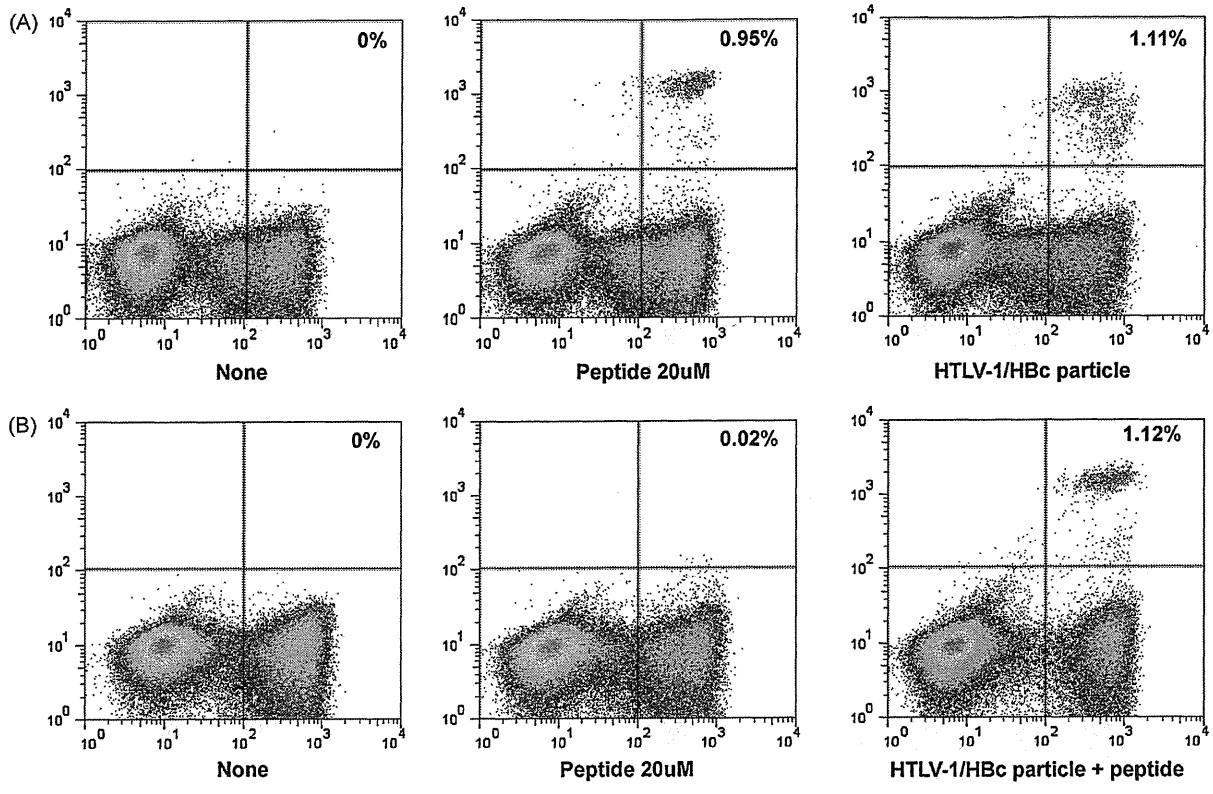

Fig. 4. Induction of HTLV-1 Tax-specific CD8+ T-cells from ACs and ATL patients. Freshly isolated PBMCs from ACs (A) and ATL patients (B) were cultured without peptide (left), with peptide (middle), with HTLV-1/HBc particle (right in A) or with peptide + HTLV-1/HBc particle (right in B). Numbers in the upper right quadrants represent the percentages of tetramer + CD8+ T-cells in T lymphocytes.

ples, representative data shown in Fig. 4B). Induction of tetramer positive cells was highly variable among asymptomatic carriers, with increases sometimes evident even in the absence of peptide or chimera; conversely, peptide or chimera sometimes resulted in no induction (data not shown).

Furthermore, these HTLV-1-specific CD8+ cells induced apoptosis of HTLV-1 epitope peptide-pulsed T2-A2 cells (Fig. 5). The T-cells efficiently lysed T2-A2 target cells pulsed with the peptide expressing Annexin V, whereas only low background lysis was observed in the absence of Tax peptide, or for CMV peptide-loaded T2-A2 cells. These results demonstrated that the HTLV-1/HBc chimeric particle-induced CTL response was MHC class I restricted, specifically lysing cells presenting the appropriate peptide.

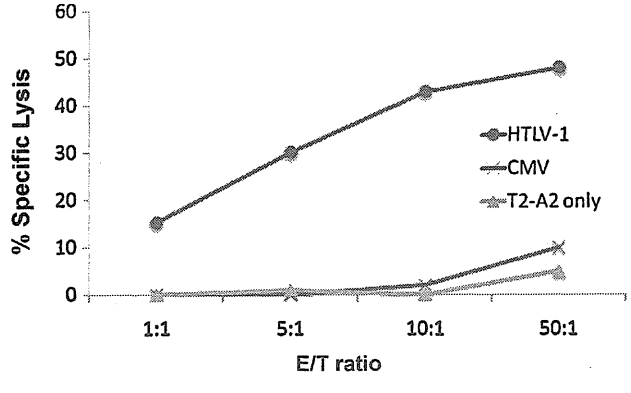

Fig. 5. Cytotoxic activity of induced HTLV-1-specific CD8+ T-cells. Using HTLV-1 peptide and CMV peptide-loaded and unpulsed T2-A2 cells as target cells, specific cytotoxic activity was evaluated by flow cytometric assay of cell mediated cytotoxicity. All tests were performed in triplicate and at effector:target ratios of 1:1, 5:1, 10:1 and 50:1.

4. Discussion

Viral-like particles have been demonstrated to prime CTL and antibody responses *in vivo* (Qian et al., 2006; Storni et al., 2002). HBc particles with a diameter of 33 nm have been synthesized by the yeast glyceraldehyde-3-phosphate dehydrogenase promoter and terminator, *Escherichia coli* K802d, *E. coli* BL21 cell and the yeast strain AH22 (Imamura et al., 1987; Shiosaki et al., 1991; Storni et al., 2002; Zhang et al., 2007). Here, we obtained HTLV-1/HBc chimeric particle inserting HTLV-1 epitope from *P. pastris* His+ transformants. As confirmed by electron microscopy, the chimeric product maintained the capacity to correctly fold and self-assemble into structured capsid particles with a diameter of 36 nm (Fig. 1B). These results indicate that the HTLV-1/HBc chimeric particle, using a different expression system, behaved as anticipated.

Immunization of HLA-A*0201-transgenic mice with the chimeric particle resulted in the efficient induction of IFN-γ-producing cells. This induction of IFN-γ-producing cells correlated well with effector cell increase, and was significantly higher than observed for either immunization with Tax peptide alone, or uncombined but simultaneous administration of HBc particle with Tax peptide. These results may be attributable to an adjuvant effect on the immune response by HBc capsids. It is known, for example, that T helper epitopes in the HBc protein augment CTL development and enhance CD8+ memory T-cell survival. Others have reported on the efficient processing of lymphocytic choriomeningitis virus-derived p33/HBc chimera via cross-presentation, although only weak CTL responses were induced in C57BL/6 mice (Storni et al., 2002). Thus, while VLPs alone are inefficient at inducing CTL responses, they become very potent vaccines when combined with antigen presenting cell (APC) activating substances like anti-CD40 mAbs or nonmethylated CG

motif-rich DNA (CpGs). We demonstrated the efficient induction of IFN-γ-producing cells and HTLV-1-specific CD8+ cells from spleens and inguinal lymph nodes by the HTLV-1/HBc chimeric particle in HLA-A*0201-transgenic mice. These results may be due to the efficient processing of the HTLV-1/HBc chimeric particle by DCs for MHC class I associated presentation in this human mouse model.

Dendritic cell maturation is intricately involved in the processing and presentation of antigens to T-cells through increased expression of MHC class I, MHC class II, and CD86 co-stimulatory molecules (Banchereau and Steinman, 1998). Exposure of bone marrow-derived iDCs to the HTLV-1/HBc chimeric particle increased the expression of CD86 and HLA-A02 in a dose-dependent manner, in contradistinction to peptide only stimulation. Expression levels of co-stimulatory molecules and TLR4 in the chimeric particle-pulsed DCs were significantly greater than for PHA-pulsed DCs. These results suggest that the uptake of HTLV-1/HBc chimeric particle by iDCs supports the phenotypic and functional maturation of DCs without adjuvant, likely via TLR4 signaling. It is known that VLPs can enhance activation and maturation of DCs (Da Silva et al., 2007), but there has been no report on whether HBc particles increase co-stimulatory molecule expression in HLA-A*0201-transgenic mice. The present study therefore demonstrates that the HTLV-1/HBc chimeric particle is able to activate APCs, such as macrophages and DCs, which in turn have the ability to cross-present VLPs and other particulate antigens.

HTLV-1 carriers showing insufficient T-cell responses to HTLV-1, despite and especially in the presence of high viral loads, may be categorized in the high-risk group for ATL. HTLV-1-specific CTLs evinced vigorous proliferation in *ex vivo* cultures of PBMCs from post-hematopoietic stem cell transplantation (HSCT) patients, but not from pre-HSCT patients (Harashima et al., 2004). Our results in this study show that although HTLV-1-specific CD8+ T-cell response in ATL cells *ex vivo* was not induced by Tax peptide only, CTL response to peptide could be augmented by HTLV-1/HBc particle, leading to specific lysis of cells presenting the appropriate peptide. Harashima et al. (2004) have also reported a similar difficulty in inducing CD8+ cells from ATL patients. Indeed, CD8+ T-cell response can be quite varied among patients, as seen for positive responses even in the absence of peptide, possibly due to reaction to self-antigen, or no response to antigen stimulation in our assay system, suggesting possible immune exhaustion due to relative antigen excess. Induction of an adequate HTLV-1-specific cellular immune response may significantly reduce HTLV-1 proviral load as has been reported in a squirrel monkey model of HTLV-1 infection (Kazanji et al., 2006). Imperatively, therefore, protection against ATL development in chronic HTLV-1 carriers may be afforded by the induction of HTLV-1-specific CTLs. The efficient induction of HTLV-1-specific CTL by HTLV-1/HBc chimera particle could also be adapted to prevent the relapse of ATL in post-HSCT patients.

In this study, we have demonstrated that the HTLV-1/HBc chimeric particle enhanced DC maturation in HLA-A*0201-transgenic mice. These activated DCs, in turn, strongly induced the HTLV-1-specific CD8+ T-cell response without adjuvant. These results suggest that HTLV-1/HBc chimeric particle without adjuvant is capable of inducing strong cellular immune responses via effective dendritic cell maturation, and is potentially useful as an effective therapeutic vaccine model against tumors and infectious diseases by substituting the epitope peptide.

Conflict of interest

No potential conflicts of interest relevant to this article.

Acknowledgements

Grant supports: This work was supported in part by a Grant-in-Aid (to NA and TK) from the Japanese Ministry of Health, Labor, and Welfare, and by the Kagoshima University for Frontier Science Research Center Program (to NA).

We thank Mr. Aikawa, Mr. Shoji and Mrs. Higashi for technical assistance, and Mr. Toji for providing tetramer reagents.

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