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Clinical outcomes of a novel therapeutic vaccine with Tax peptide-pulsed dendritic cells for adult T cell leukaemia/lymphoma in a pilot study

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Summary

Adult T cell leukaemia/lymphoma (ATL) is a human T cell leukaemia virus type-I (HTLV-I)-infected T cell malignancy with poor prognosis. We herein developed a novel therapeutic vaccine designed to augment an HTLV-I Tax-specific cytotoxic T lymphocyte (CTL) response that has been implicated in anti-ATL effects, and conducted a pilot study to investigate its safety and efficacy. Three previously treated ATL patients, classified as intermediate- to high-risk, were subcutaneously administered with the vaccine, consisting of autologous dendritic cells (DCs) pulsed with Tax peptides corresponding to the CTL epitopes. In all patients, the performance status improved after vaccination without severe adverse events, and Tax-specific CTL responses were observed with peaks at 16–20 weeks. Two patients achieved partial remission in the first 8 weeks, one of whom later achieved complete remission, maintaining their remission status without any additional chemotherapy 24 and 19 months after vaccination, respectively. The third patient, whose tumour cells lacked the ability to express Tax at biopsy, obtained stable disease in the first 8 weeks and later developed slowly progressive disease although additional therapy was not required for 14 months. The clinical outcomes of this pilot study indicate that the Tax peptide-pulsed DC vaccine is a safe and promising immunotherapy for ATL.

Keywords: adult T cell leukaemia/lymphoma, tumour vaccine, dendritic cell, human T cell leukaemia virus type-I, cytotoxic T lymphocyte.

Adult T cell leukaemia/lymphoma (ATL) is an aggressive lymphoproliferative disease caused by human T cell leukaemia virus type-I (HTLV-I) infection (Uchiyama *et al*, 1977; Poiesz *et al*, 1980; Hinuma *et al*, 1981). In particular, the acute and lymphoma types of ATL are characterized by a poor prognosis. Although the chronic and smouldering types of ATL exhibit

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milder disease progression, these diseases also result in poor clinical outcome once they have converted to the acute or lymphoma types.

One reason for the poor clinical outcome associated with ATL is rapid progression of the disease at onset, which requires a prompt diagnosis and effective first-line therapy. Currently available first-line therapies for ATL include intensive multi-agent chemotherapy (Tsukasaki *et al*, 2012), interferon-α combined with zidovudine (Gill *et al*, 1995; Hermine *et al*, 1995) and an anti-CCR4 antibody (mogamulizumab) (Ishida *et al*, 2012).

Frequent relapse is another reason for the poor prognosis of ATL, requiring subsequent administration of second-line therapy that can produce a long-lasting anti-ATL effect. Haematopoietic stem cell transplantation (HSCT) has been reported to achieve a long-lasting remission in 30–40% of ATL patients, although it occasionally induces treatment-related mortality in a similar percentage of recipients (Utsunomiya *et al*, 2001; Okamura *et al*, 2005; Hishizawa *et al*, 2010; Ishida *et al*, 2013). In addition to the graft-*versus*-host response (Tanosaki *et al*, 2008), the actions of Tax-specific cytotoxic T lymphocytes (CTLs) have been implicated in the graft-*versus*-ATL effects of HSCT. This is based on our previous finding that ATL patients who obtained complete remission following HSCT often exhibit activation of $CD8^+$ CTLs specific for HTLV-I Tax (Harashima *et al*, 2004).

In untreated ATL patients, Tax-specific CTLs are either undetectable or dysfunctional, if present (Takamori *et al*, 2011). Although ATL patients are in a severe immune suppressive state, the impaired CTL response is not merely a result of general immune suppression in the advanced disease, but also observed in the patients with earlier stages of the disease in a selective manner for HTLV-I-specific responses (Takamori *et al*, 2011). The anti-tumour effects of Tax-specific T cells have been well characterized in animal models, where Tax-coding DNA and Tax-peptide vaccines have been shown to induce T cell immunity, thus eradicating HTLV-I-infected lymphomas in rats (Ohashi *et al*, 2000; Hanabuchi *et al*, 2001).

The efficacy of the vaccine targeting Tax in human ATL patients remains unclear, and no such treatment has ever been attempted as an actual therapy. This is partly because the HTLV-I gene expression levels are believed to be very low *in vivo* (Kurihara *et al*, 2005; Rende *et al*, 2011), and ATL cells occasionally lack the ability to express Tax (Takeda *et al*, 2004). However, our previous finding of the Tax-specific CTL activation in ATL patients following HSCT from uninfected donors indicated the presence of a sufficient level of Tax expression for the CTL response *in vivo* (Harashima *et al*, 2004).

These findings prompted us to attempt to develop a therapeutic anti-ATL vaccine designed to augment a Tax-specific CTL response that may partly reproduce the long-lasting anti-tumour effects of HSCT as second-line therapy for ATL. For the vaccine antigen, we used synthetic oligopeptides corresponding to the major epitopes recognized by Tax-specific CTL identified in our previous studies of post-HSCT ATL patients (Harashima *et al*, 2004, 2005). These epitopes are restricted to HLA-A2, A24 or A11, all of which are common in the Japanese population. For the vaccine adjuvant, we used autologous dendritic cells (DCs) induced from the peripheral monocytes. Although previous reports suggested dysfunctions of DCs in ATL patients (Makino *et al*, 2000; Hishizawa *et al*, 2004), the monocyte-derived DCs obtained from ATL patients retained the ability of antigen presentation in our preliminary experiments. The use of autologous DCs loaded with tumour antigens have been reported in various tumour vaccine trials of different tumours (Nagayama *et al*, 2003; Ueda *et al*, 2004; Linette *et al*, 2005; Fuessel *et al*, 2006; Thomas-Kaskel *et al*, 2006; Wierecky *et al*, 2006).

The present pilot study investigated the safety and efficacy of the Tax peptide-pulsed dendritic cell (Tax-DC) vaccine when administered to augment Tax-specific CTL responses in ATL patients.

Materials and methods

Study design

This clinical study was approved by the institutional ethics committee and registered as UMIN000011423. Three ATL patients possessing HLA-A*02:01, A*24:02 and/or A*11:01, in stable condition at least 4 weeks after the administration of previous therapy, provided their written informed consent and were enrolled in this study, which investigated the safety and efficacy of the Tax peptide-pulsed DC (Tax-DC) vaccine between September 2012 and February 2013.

HTLV-I proviruses in the peripheral blood mononuclear cells (PBMCs) were examined for the potential Tax expression and conservation of targeted CTL epitopes by analysing their nucleotide sequences beforehand. All patients were subcutaneously administered with Tax peptide-pulsed autologous DCs (5×10^6) three times at 2-week intervals (Fig 1A) at Kyushu University Hospital.

Patients

Patient 1 was a 69-year-old male who was diagnosed with acute ATL in August 2011. After receiving four courses of multi-agent chemotherapy, he achieved stable disease (SD). Although additional treatment with lenalidomide was administered for a few weeks, it was discontinued due to the development of thrombocytopenia. The patient was registered to the study in September 2012.

Patient 2 was a 67-year-old female who was diagnosed with acute ATL in December 2011. She presented with remarkable systemic lymphadenopathy and splenomegaly, in addition to an extremely high level of soluble interleukin-2 receptor (sIL2R; 57 815 u/ml). She received four courses of

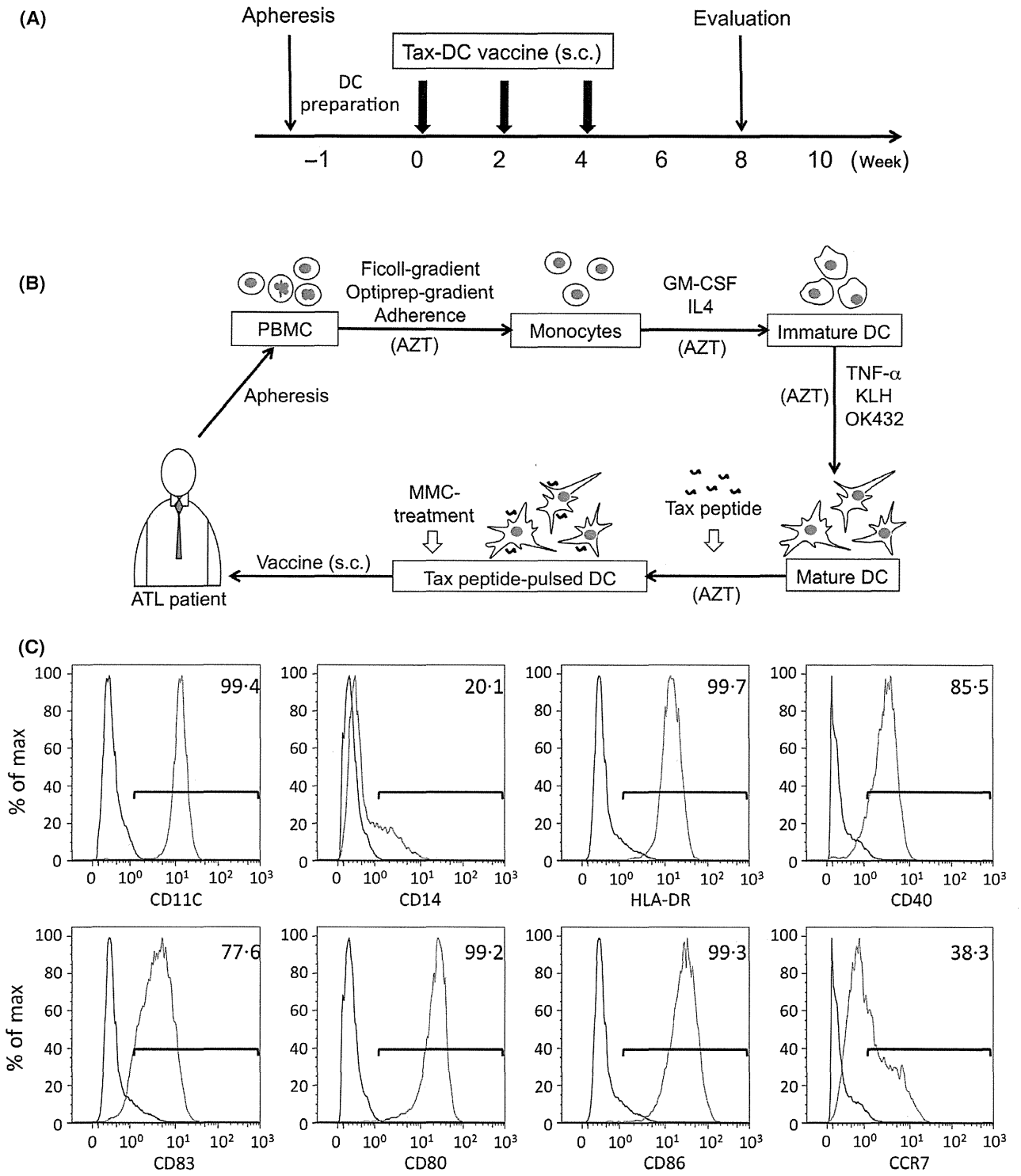


Fig 1. Outline of the Tax-DC vaccine therapy. (A) Schedule for the Tax-DC vaccine therapy. (B) Preparation of the monocyte-derived dendritic cells (DCs). Monocytes were enriched via serial density gradient centrifugation, and the adherent cells were cultured in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin 4 (IL4) for 5 d, followed by 48 h of culture with TNF-α, keyhole limpet haemocyanin (KLH), and OK432. A total of 10 μmol/l of zidovudine (AZT) was added whole throughout the culture. The matured DCs were pulsed with synthetic Tax peptides, treated with Mitomycin C (MMC), and then cryopreserved prior to subcutaneous injection. (C) Representative phenotype of mature dendritic cells prepared from Patient 1 prior to administration, as evaluated using flow cytometry. The red histograms indicate the results of staining with monoclonal antibodies for the indicated molecules, while the black histograms indicate the results of staining with control antibodies. ATL, Adult T cell leukaemia/lymphoma; PBMC, peripheral blood mononuclear cells.

multi-agent chemotherapy and achieved a partial remission (PR). Due to the development of disease recurrence with rapid progression after 2 months, treatment with mogamulizumab and low-dose chemotherapy (sobuzoxane + etoposide) was added. After obtaining a second PR, the patient was registered to the study in November 2012.

Patient 3 was a 56-year-old female diagnosed with acute ATL who presented with severe pneumocystis pneumonia in August 2012. After receiving two courses of multi-agent chemotherapy followed by two courses of mogamulizumab combined with chemotherapy, she achieved a PR. Further intensive treatment was not planned due to the development of severe respiratory dysfunction. The patient was registered to the study in February 2013.

The clinical information of the patients at enrollment is summarized in Table I.

Preparation of Tax peptide-pulsed DCs

Monocyte-derived DCs were generated from apheresis samples collected from the peripheral blood (6 l) of ATL patients at institutional cell processing facilities according to the good manufacturing practice (GMP) standard using a previously reported method, with some modifications (Nagayama *et al*, 2003) (Fig 1B). Briefly, monocytes enriched via serial density gradient centrifugation on Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden) and density-adjusted Optiprep (1·073 g/ml; Axis-Shield PoC, Oslo, Norway) were cultured at 37°C for 2 h, after which the adherent cells were cultured in CellGro DC medium (CellGenix GmbH, Freiburg, Germany) with 1000 iu/ml of granulocyte-macrophage colony-stimulating factor (Leukine; Bayer HealthCare Pharmaceuticals, Seattle, WA, USA) and 100 iu/ml of IL4 (Miltenyi Biotec, Bergisch Gladbach, Germany) for 5 d. The resulting monocyte-derived DCs were matured in the presence of 10 ng/ml of TNF-α (Miltenyi Biotec) and 12·5 μg/ml of keyhole limpet haemocyanin (KLH; Calbiochem, La Jolla, CA, USA) for 48 h, with 0·1 Clinical unit (Klinische Einheit; KE)/ml of OK432 (Picibanil; Chugai Pharmaceutical Co. Ltd., Tokyo, Japan) for the last 24 h. The matured DCs were pulsed with 2 μg/ml of synthetic peptides (NeoMPS; PolyPeptide Laboratories Group, San Diego, CA, USA), including Tax11-19 (LLFGYPVYV) (Kannagi *et al*, 1992) or Tax301-309 (SFHSLHLLY) (Harashima *et al*, 2004) restricted to HLA-A*02:01 or -A*24:02 respectively, and treated with Mitomycin C (MMC; Kyowa Hakko Kirin Co. Ltd., Tokyo, Japan) (50 μg/ml) in order to inactivate the ATL cells potentially contained in the preparation. As DCs are reported to be susceptible for HTLV-I infection (Jones *et al*, 2008), 10 μmol/l of zidovudine (Retrovir, AZT; GlaxoSmithKline, Research Triangle Park, NC, USA) was added whole throughout the culture to avoid *de novo* infection. The peptide-pulsed DCs were then washed and examined for safety by checking for contamination with bacteria, fungi, mycoplasma and/or endotoxins, then cryopreserved until use. The cells (5×10^6) were subsequently thawed and washed prior to administration.

Evaluation of adverse events and the clinical response

Toxic effects were graded according to the Common Terminology Criteria for Adverse Events version 3.0 (http://ctep.cancer.gov/protocolDevelopment/electronic_applications/docs/ctcaev3.pdf). The clinical response was evaluated according to the criteria proposed by the international consensus meetings that led to the modification of the Japan Clinical Oncology Group criteria (Tsukasaki *et al*, 2009). Briefly, complete remission (CR) was defined as the disappearance of all clinical, microscopic and radiographic evidence of disease. PR was defined as a ≥50% reduction in the level of measurable disease without the appearance of new lesions. In addition, the diagnosis of a PR was required to satisfy a 50% or greater reduction in the absolute abnormal lymphocyte count in the peripheral blood. Progressive disease (PD) or relapsed disease was defined as a ≥50% increase from the nadir in the sum of the products of measurable disease or the appearance of new lesions, excluding the skin. Stable disease (SD) was defined as the failure to attain CR/PR nor PD.

The soluble IL2 receptor (sIL2R) level, HTLV-I proviral load and Tax-specific CTL response were monitored in addition to the results of general laboratory tests. Adverse effects

Table I. Patient characteristics at enrollment.

	Patient 1	Patient 2	Patient 3
Age (years)/gender	70/ male	68/ female	57/ female
HLA-A allele	24:02, 31:01	24:02, 26:03	02:01, 11:01
Subtype of ATL	Acute	Acute	Acute
Previous therapy	mEPOCH, lenalidomide	mEPOCH, mogamulizumab + PVP	mEPOCH, mogamulizumab + PVP
Disease status	SD	PR	PR
Interval from previous therapy	2·5 months	1·5 months	2 months
Duration since diagnosis	14 months	11 months	6 months
Complication	Allergic dermatitis	Breast cancer, DM, NASH	Interstitial pneumonia

mEPOCH, modified combination chemotherapy with etoposide + prednisone + vincristine + doxorubicin + carboplatin; PVP, combination chemotherapy with sobuzoxane + etoposide; SD, stable disease; PR, partial remission; DM, diabetes mellitus; NASH, nonalcoholic steatohepatitis.

and the clinical response were monitored and evaluated at 8 weeks after the initiation of the Tax-DC vaccine therapy.

Tax-specific CTL analysis

Phycoerythrin (PE)-conjugated HLA-A*0201/Tax11-19, HLA-A*1101/Tax88-96 and HLA-A*2402/Tax301-309 tetramers were purchased from Medical & Biological Laboratories, Co., Ltd. (Nagoya, Japan). Whole blood samples or PBMCs were stained with PE-conjugated Tax/HLA tetramers, together with fluorescein isothiocyanate (FITC)-conjugated anti-human CD3 and PE/cyanin 5 (Cy5)-conjugated anti-human CD8 monoclonal antibodies (mAbs) (BioLegend, San Diego, CA, USA), then fixed in Becton Dickinson (BD) FACS lysing solution (BD Biosciences, San Jose, CA, USA), followed by analysis on the FACS Calibur system using the CELLQUEST software program (BD Biosciences). For staining intracellular IFN-γ production, PBMCs pre-stained with PE-conjugated Tax/HLA tetramers and anti-human CD8-PE/Cy5 mAb were incubated at 37°C for 6 h in the presence of cognate Tax peptides (10 μmol/l), with brefeldin A (10 μg/ml; Sigma Aldrich, St. Louis, MO, USA) for the last 5 h. The cells were then permeablized using BD Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences) and stained with FITC-conjugated anti-human IFN-γ mAb (4S.B3; BioLegend).

Detection of HTLV-I gene expression

To detect intracellular HTLV-I antigens, cells were serially treated with 4% paraformaldehyde for 10 min and 100% methanol for 10 min on ice, and then stained with Alexa Fluor 488-labelled anti-Tax Lt-4 (Lee *et al*, 1989) or isotype control mAbs followed by flow cytometry.

To quantify HTLV-I *pX* mRNA, total RNA extracted by using Isogen (Nippon Gene, Tokyo, Japan) were treated with DNase (Ambion, Austin, TX, USA), and subjected to quantitative reverse transcription polymerase chain reaction (RT-PCR) with the primer sets specific for HTLV-I *pX* (forward, 5′-CGG ATA CCC AGT CTA CGT GTT TGG AGA CT-3′; reverse, 5′-GAG CCG ATA ACG CGT CCA TCG ATG GGG TCC-3′) and *GAPDH* (forward, 5′-TGA TTT TGG AGG GAT CTC GCT CCT GGA AGA-3′; reverse, 5′-GTG AAG GTC GGA GTC AAC GGA TTT GGT CGT-3′) by using LightCycler Fast Start DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany) after reverse transcription with oligo(dT)20 primers. The *pX* mRNA levels were standardized against *GAPDH* mRNA copy numbers.

Results

Feasibility of the DC preparation in ATL patients

We obtained 4·3–10·6 × 10^7 DCs with 72·2–91·3% purity. The cells exhibited the phenotype of mature DCs ($CD11c^+$, $CD80^+$, $CD86^+$, $CD83^+$, $CD40^+$, $HLA\text{-}DR^+$). The representative results obtained in Patient 1 are shown in Fig 1C. The HTLV-I proviral load of the PBMCs in the input apheresis samples were 114·8, 36·7 and 25·5 copies/1000 cells in the three patients respectively, with final loads in the DCs of 5·9, 5·0 and 10·3 copies/1000 cells, respectively.

Clinical courses after the Tax-DC vaccine therapy in the ATL patients

The clinical outcomes of the Tax-DC vaccine therapy in the three patients are summarized in Table II.

Table II. Clinical responses after the Tax-DC vaccine therapy in the three ATL patients.

Clinical response in 8 weeks after initiation of the vaccine therapy	Patient 1*		Patient 2†		Patient 3‡	
Time at evaluation	Pre-therapy	8 weeks	Pre-therapy	8 weeks	Pre-therapy	8 weeks
KPS (%)	70	90	70	80	70	90
LDH (iu/l)	473	245	250	326	329	268
sIL2R (u/ml)	19 056	1866	806	1462	1739	871
HTLV-I PVL (copies/1000 PBMCs)	114·8	12·4	36·7	14·9	17·7	29·6
Clinical response	–	PR	–	SD	–	PR
Long-term outcomes						
TTNT (months from registration)	25+		15		20+	
Survival (months from diagnosis)	39+		34		26+	

KPS, Karnofsky performance status; LDH, lactate dehydrogenase; HTLV-I PVL, human T cell leukaemia virus type-I proviral load, PBMCs, peripheral blood mononuclear cells; SD, stable disease, PR, partial remission; TTNT, time to next anti-tumour therapy.

*The size of the lymph nodes in Patient 1 repeatedly increased and decreased, especially at time points later than 6 months after initiation of vaccine therapy.

†Patient 2 was considered to have developed a progressive disease at 6 months after the initiation of the vaccine therapy.

‡Patient 3 achieved complete remission at 6 months after the initiation of the vaccine therapy.

Patient 1 was positive for HLA-A*24:02 and vaccinated with Tax 301-309 peptide-pulsed DCs. Following the first administration of the Tax-DC vaccine, he developed a fever (grade 2), dermatitis (grade 2) and diarrhoea (grade 1). The white blood cell count, level of ATL cells in the peripheral blood and LDH level in the serum showed remarkable fluctuation during the vaccination, and then stabilized after the third administration of the vaccine (Fig 2A). In Patient 1, the level of sIL2R, which is a sensitive tumour marker for ATL, decreased from 19 056 to 1866 u/ml (normal range: <570 u/ml) by 8 weeks of therapy (Fig 2B). In addition, his surface lymph nodes decreased in size (Fig 2C), and he achieved a partial remission (PR) that persisted for at least 24 weeks. He returned to his normal life, and his Karnofsky performance status (KPS) improved from 70% to 100%. Although the size of the patient's lymph nodes and the level of sIL2R fluctuated at later time points, he has remained in remission for more than 24 months after the completion of the Tax-DC vaccine therapy, without any additional anti-tumour treatment.

Patient 2 had HLA-A*24:02 and was vaccinated with Tax 301–309 peptide-pulsed DCs. She developed a low-grade fever and dermatitis (grade 2) after each vaccine administration. However, no severe adverse events were observed during her clinical course. At 8 weeks of therapy, she was considered to have achieved SD. Although there was no objective response, an improvement in the KPS was noted. She was subsequently considered to have developed PD 6 months after the initiation of the Tax-DC vaccine therapy. Nevertheless, due to slow progression of the disease and her stable general condition, she was followed without any additional anti-tumour therapy until 14 months after the completion of vaccination. The patient died of infection 23 months after the initiation of the vaccine therapy.

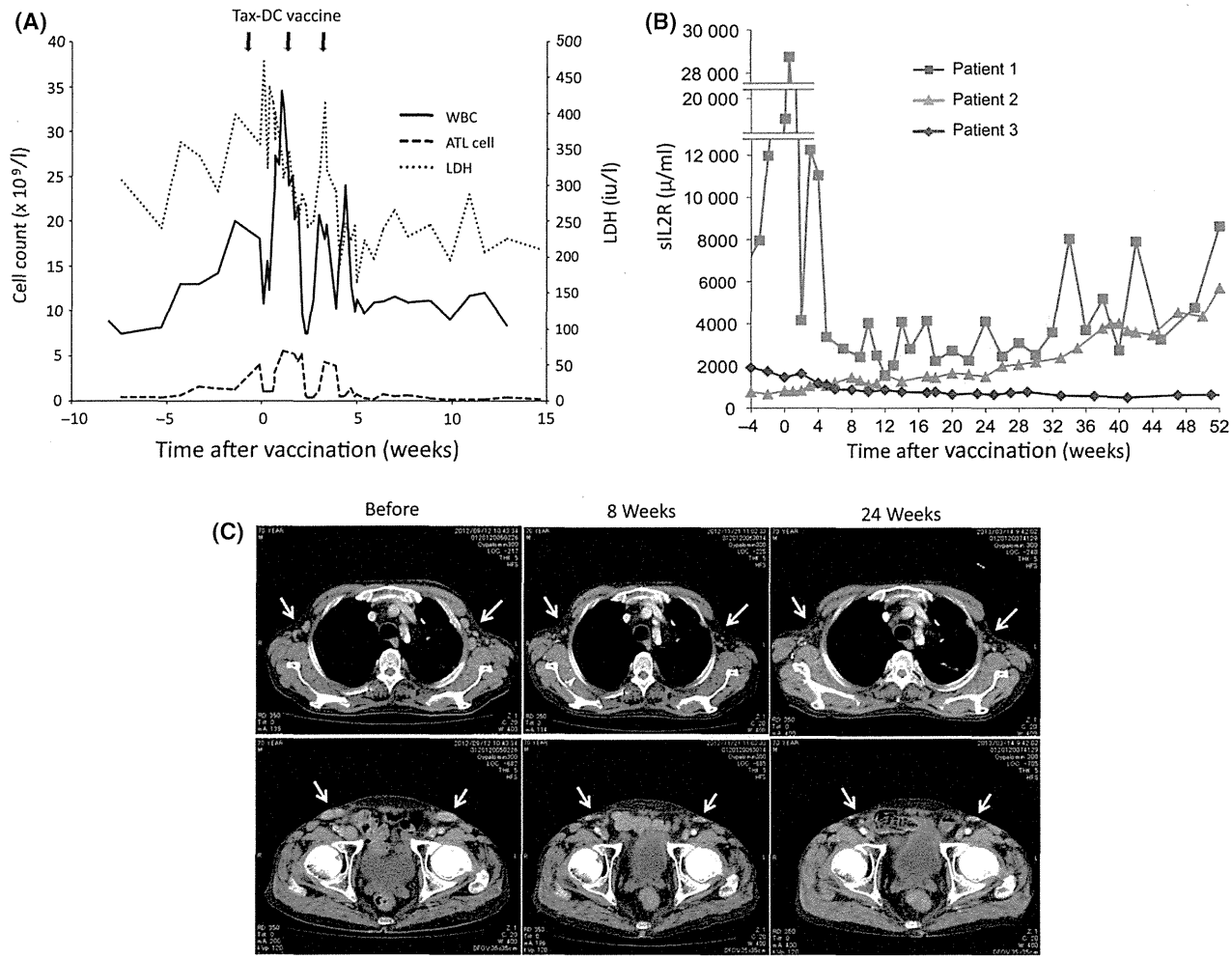


Fig 2. Clinical courses of the patients after the Tax-DC vaccine therapy. (A) Changes in the peripheral white blood cell count (WBC, solid line), ATL cell count (dashed line) and lactate dehydrogenase (LDH) level (fine dotted line) during the initial 15 weeks in Patient 1. The arrows indicate the days of Tax-DC vaccine administration. (B) Kinetics of the sIL2R levels in the sera obtained from Patients 1 (red), 2 (green) and 3 (blue) during the long-term observation period after the initiation of the Tax-DC vaccine therapy. (C) Computerized tomography images of the axillary (top) and inguinal (bottom) lymph nodes (arrows) of Patient 1 before and 8 and 24 weeks after the initiation of the Tax-DC vaccine therapy.

Patient 3 had HLA-A*02:01 and A*11:01. Although peptides of CTL epitopes for both HLA alleles were available, we chose the Tax11-19 peptide for HLA-A2 because HLA-A2 has a higher frequency in Japanese individuals. After each vaccination, the patient developed a low-grade fever and dermatitis (grade 2); however, no other severe adverse events were noted. She achieved a PR with an improvement in the KPS 8 weeks after the initiation of the Tax-DC vaccine therapy. Thereafter, the level of sIL2R returned to normal (Fig 2B). The patient subsequently achieved a CR at 6 months and has remained in this status for more than 19 months after the completion of the Tax-DC vaccine therapy.

Immunological responses after the Tax-DC vaccine therapy

In Patient 1, Tax-specific $CD8^+$ CTLs (HLA-A*24:02/Tax301-309 $tetramer^+$) were detectable prior to vaccination, and their frequency in peripheral $CD8^+$ cells transiently decreased during the Tax-DC vaccine administration, then recovered and maintained a constant level with some fluctuation (Fig 3A). The IFN-γ production from Tax-specific CTL also fluctuated. It is noteworthy that a vigorous proliferative response of Tax-specific CTLs was observed *in vitro* in the PBMC sample obtained at 20 weeks after the initiation of the Tax-DC vaccine therapy (Fig 3B), in which the proportion of HLA-A*24:02/Tax301-309 $tetramer^+$ cells in $CD8^+$ cells increased up to 22·5% within 2 weeks of the culture. A mild proliferative response of CTLs was also observed at 12 weeks. Samples obtained from the same patient prior to vaccination lacked such strong responses, implying a functional improvement in CTLs after the Tax-DC vaccine therapy.

Similar to that observed in Patient 1, a markedly increased level of spontaneous *in vitro* proliferative responses of Tax-specific CTLs was observed in the PBMC samples obtained from Patient 2 at 16 weeks after the initiation of the Tax-DC vaccine therapy, although the CTLs of this patient had exhibited a proliferative response prior to vaccination to a lesser degree (Fig 3B). The IFN-γ producing response of the CTL in this patient slightly improved after vaccination and showed some peaks at later time points.

As the size of the lymph nodes in Patient 2 did not improve within the first 8 weeks, a biopsy of the inguinal lymph node was performed at 9 weeks during the study period. The tumour cells isolated from the lymph node were $CD4^+$ $CD8^+$ $CCR4^+$ (Fig 4A) and possessed HTLV-I proviruses (849·5 copies/1000 cells). However, HTLV-I Tax proteins or mRNA expression was not induced in the lymph node cells after a short-term *in vitro* culture, whereas the viral expression was inducible in the PBMC sample of the same patient before vaccination (Fig 4B,C).

Tax-specific CTLs were below detectable levels prior to vaccination in Patient 3. However, 2 weeks after the initiation of the vaccine therapy with Tax 11–19 peptide-pulsed DCs, $CD8^+$ Tax-specific CTLs became detectable with HLA-A*0201/Tax11-19 tetramers, but not HLA-A*1101/Tax88-96 tetramers (Fig 3A). Although the IFN-γ producing response was barely detectable because of the low CTL frequency, an *in vitro* proliferative response of Tax-specific CTLs was observed in the PBMC samples obtained from Patient 3 most clearly at 16 weeks of the Tax-DC vaccine therapy, upon stimulation with Tax11-19 peptides, but not Tax 88–96 peptides (Fig 3B).

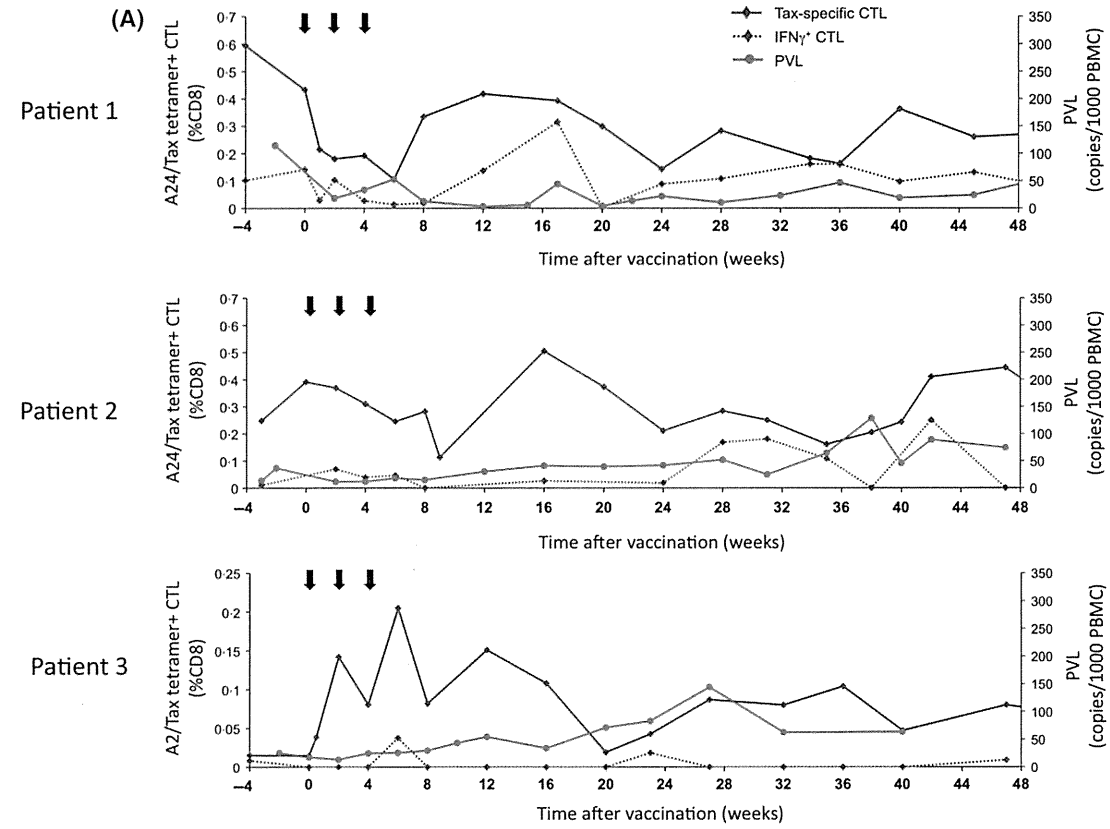
In all three patients, the level of the proviral load in the peripheral blood mostly remained below 100 copies per 1000 PBMCs at least for 1 year after vaccination, with the exception of sporadic small spikes (Fig 3A).

Discussion

Although various therapeutic trials have been conducted, the prognosis of ATL remains dismal. According to the simplified ATL prognostic index (ATL-PI) (Katsuya *et al*, 2012), the median survival time is only 4·6, 7·0 and 16·2 months, while the 2-year overall survival rate is 6%, 17% and 37%, for patients in high-, intermediate- and low-risk groups, respectively. According to the ATL-PI, Patients 1 and 2 were classified as intermediate-risk, while Patient 3 was classified as high-risk. Therefore, it is quite unique and surprising that all three patients remained in a favourable condition, without the need for any additional anti-tumour therapy, for at least 24, 14 and 19 months respectively, after only three administrations of the Tax-DC vaccine. In particular, Patients 1 and 3 obtained PR by 8 weeks after the initiation of the Tax-DC vaccine therapy.

Although these results are exciting, we cannot completely rule out the persisting effects of lenalidomide and/or mogamulizumab, which were previously administered in each patient prior to the Tax-DC vaccine therapy. These previous treatments may also have positively contributed to the present results via their immunomodulatory effects. According to recent reports, mogamulizumab has been shown to decrease the level of $CCR4^+$ regulatory T cells (Ishida & Ueda, 2011), and lenalidomide has immunomodulatory effects indirectly enhancing the activity of natural killer and T cells (Wu *et al*, 2008; De Keersmaecker *et al*, 2012).

The biopsy specimen of a residual surface lymph node from Patient 2 contained HTLV-I proviruses, although the viral expression was not inducible in the isolated cells even after *in vitro* culture (Fig 4). In general, induction of Tax expression after short-term culture is observed in approximately 50% of ATL cases (Kurihara *et al*, 2005). In the other 50% of ATL cases, the ATL cells lack the ability to express Tax, presumably due to the genomic and epigenetic changes in the HTLV-I proviruses (Takeda *et al*, 2004). Given that the viral expression was inducible in PBMCs of Patient 2 obtained prior to vaccination, the absence of viral induction


(A)
Patient 1
Patient 2
Patient 3
Tax-specific CTL
IFNγ+ CTL
PVL
A24/Tax tetramer+ CTL (%CD8)
A2/Tax tetramer+ CTL (%CD8)
PVL (copies/1000 PBMC)
Time after vaccination (weeks)

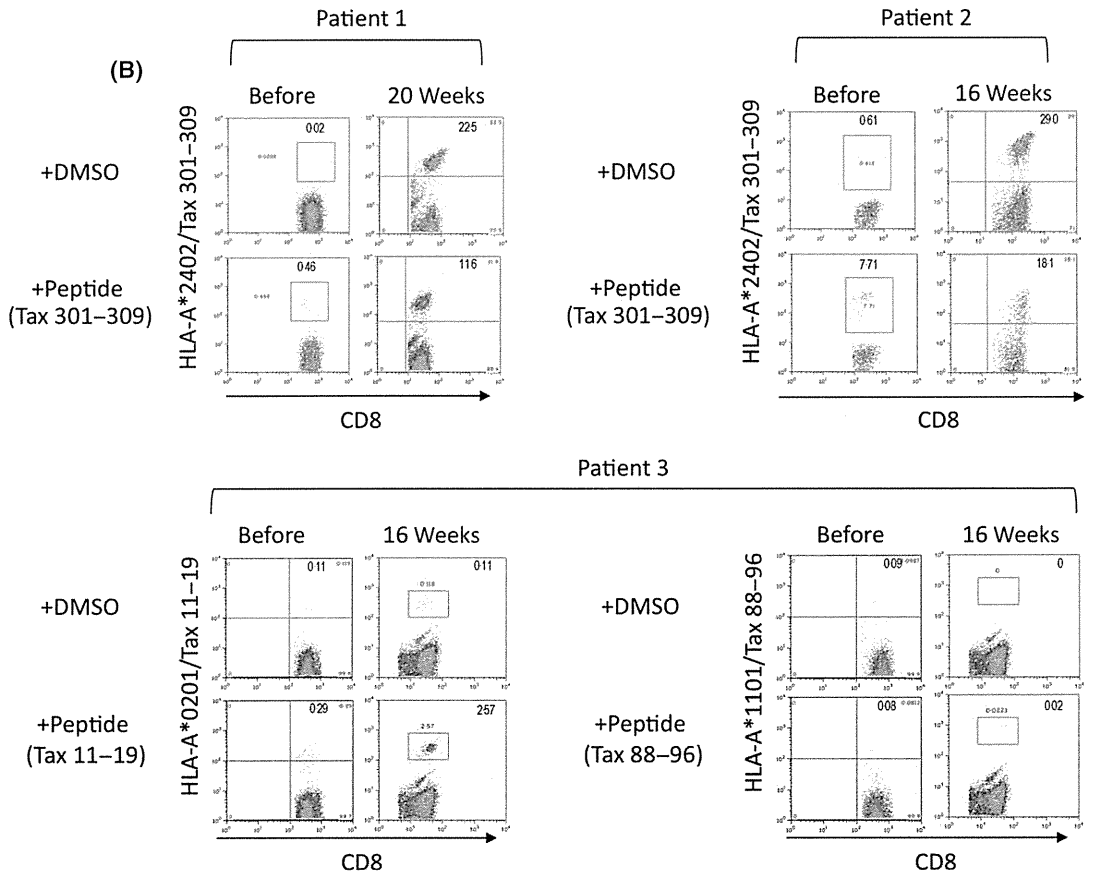

(B)
Patient 1
Patient 2
Patient 3
Before
20 Weeks
16 Weeks
+DMSO
+Peptide (Tax 301–309)
+Peptide (Tax 11–19)
+Peptide (Tax 88–96)
HLA-A*2402/Tax 301–309
HLA-A*0201/Tax 11–19
HLA-A*1101/Tax 88–96
CD8

Fig 3. Immunological responses in the three patients after the Tax-DC vaccine therapy. (A) Long-term kinetics of the Tax-specific cytotoxic T cells (CTLs; % CD8+ cells, blue solid line), and γ-interferon (IFN-γ)-producing Tax-specific CTLs (% CD8+ cells, blue broken line), and human T cell leukaemia virus type-I proviral load (HTLV-I PVL) [copies/1000 peripheral blood mononuclear cells (PBMCs), red] in the peripheral blood of the three patients. Each arrow indicates administration of the vaccine. (B) The proliferative ability of the Tax-specific CTLs was evaluated using flow cytometry following incubation of the PBMCs for 13–15 d *in vitro* with cognate Tax peptide (100 nmol/l) or dimethyl sulfoxide (DMSO) in the presence of 10 u/ml of recombinant human IL2. The cells were stained with HLA/Tax tetramer-PE, anti-human CD8-PE-Cy5 mAb and anti-human CD3-FITC mAb. The values represent the percentage of tetramer$^+$ cells/CD3$^+$ CD8$^+$ cells.

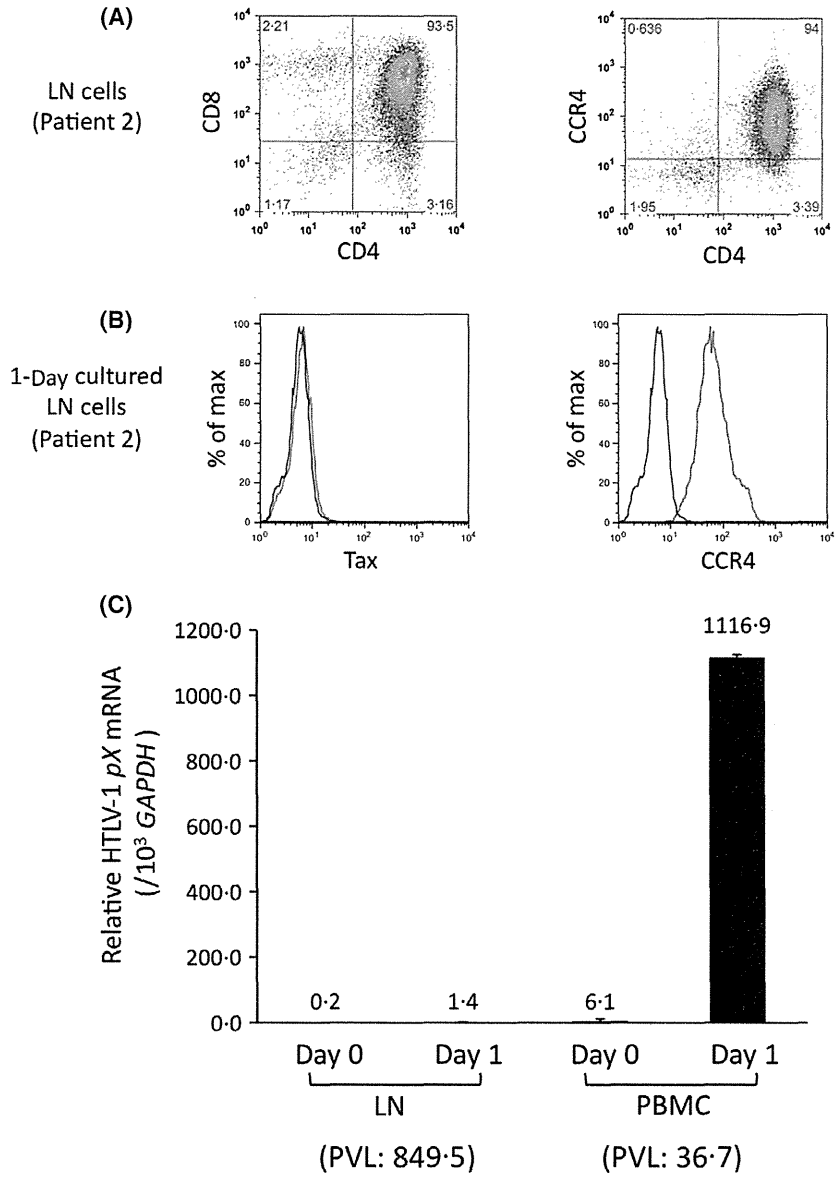


Fig 4. Absence of HTLV-I expression in the lymph node cells obtained from Patient 2. Cells were isolated from a biopsy specimen of the inguinal lymph node (LN) from Patient 2 at 9 weeks after the initiation of the Tax-DC vaccine therapy and subjected for characterization. (A) The cell surface phenotype of the LN cells immediately after isolation was analysed following staining with the indicated mAbs. (B) The intracellular Tax and CCR4 expression levels (red) in the LN cells after a 1-d culture *in vitro* were analysed following fixation of the cells with methanol. The blue histogram indicates the results of control antibody staining. (C) The HTLV-I *pX* mRNA expression levels in the LN cells before and after a 1-d culture *in vitro* were evaluated by quantitative reverse transcription polymerase chain reaction. The viral mRNA expression in peripheral blood mononuclear cells (PBMCs) obtained from the same patient before vaccination was similarly analysed as a positive control. The relative values standardized by *GAPDH* mRNA copy numbers were indicated as the means and standard deviations of duplicate samples. The proviral load (PVL) in the samples (copies/1000 cells) is indicated in parenthesis.

in the lymph node cells suggests that these tumour cells had escaped from Tax-specific CTLs.

Intriguingly, the Tax-specific CTLs demonstrated a vigorous proliferative response *in vitro* in all three patients at approximately 16–20 weeks after the initiation of the Tax-DC vaccine therapy. In particular, in Patients 1 and 2, the CTLs proliferated spontaneously without stimulation (Fig 3B). Similar phenomena have been reported in patients with HTLV-I-Associated Myelopathy/Tropical Spastic Paraparesis (Jacobson *et al*, 1990; Takamori *et al*, 2011) and occasionally in ATL patients post-HSCT (Harashima *et al*, 2005), interpreted to be the result of a normal CTL response against HTLV-I-infected cells *in vivo*. In the present study, although it is unclear whether the Tax-DC vaccine newly induced CTLs or simply activated pre-existing CTLs, Tax-specific CTLs appear to survey infected cells, at least for several months after the Tax-DC vaccine therapy, in responding to the dynamic activity of HTLV-I-infected cells *in vivo*.

In Patient 3, the Tax-specific CTLs emerged after vaccination and exhibited a clear proliferative response that peaked at 16 weeks. This response was preferentially directed toward the HLA-A2-restricted Tax epitope used for the therapy, not the HLA-A11-restricted epitope, suggesting the contribution of the Tax-DC vaccine therapy to CTL induction.

Although active CTL responses were observed in the first several months in all three patients, the responses diminished thereafter. At later time points (6 months or later) the sIL2R levels gradually increased in Patients 1 and 2 (Fig 2B). This finding suggests the need for a boosting vaccination or additional treatment to decrease the degree of immune suppression in order to maintain long-lasting anti-tumour effects.

In conclusion, the Tax-DC vaccine therapy is a safe and feasible treatment for ATL patients in stable condition. The promising clinical outcomes observed in the present study imply that the Tax-DC vaccine therapy has the potential to be an effective second-line treatment for ATL, although the anti-tumour effects of this vaccine therapy must be confirmed in further clinical trials with an increased number of patients. To our knowledge, this is the first clinical report to show the significance of a therapeutic vaccine targeting viral antigens as a new treatment modality for HTLV-I-induced malignancies. Given that Tax-specific CTL responses are impaired in patients with smouldering types of ATL and also in a small subset of asymptomatic HTLV-I carriers (Takamori *et al*, 2011), the vaccine therapy may be beneficial in these populations as well. The present study thus provides important information in a new era of anti-ATL immune therapies with the potential to be extended for prophylaxis of the disease in the future.

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Authorship

Y.S. designed the study, prepared the protocol, administered the Tax-DC therapy in patients and analysed the data. A.H. designed the study, prepared the protocol, established the method of Tax-DC preparation and analysed the data. T.I. administered the Tax-DC therapy in the patients. A.S., R.T., A.T., I.C., T.F., O.M. and T.T. participated in the protocol preparation. M.M. performed the provirus analysis. N.W. and A.T. performed the flow cytometric analysis. S.T. and K.A. supervised the institutional cell processing. M.K. proposed the initial idea and concept, designed the study, prepared the protocol and analysed the data. N.U. and J.O. supervised and coordinated the clinical and basic studies. M.K., Y.S., A.H. and J.O. wrote the manuscript. All co-authors approved the final version of the manuscript.

Disclosure

Tokyo Medical and Dental University holds a patent for the Tax epitope for HLA-A*11:01, of which M. Kannagi and R. Tanosaki are included in the inventors. This epitope was not used for a vaccine in the present study. S. Takaishi receives grants and personal fees from the MEDINET Co. Ltd., outside the submitted work.

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HTLV-1 proviral integration sites differ between asymptomatic carriers and patients with HAM/TSP

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Abstract

Background: HTLV-1 causes proliferation of clonal populations of infected T cells in vivo, each clone defined by a unique proviral integration site in the host genome. The proviral load is strongly correlated with odds of the inflammatory disease HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). There is evidence that asymptomatic HTLV-1 carriers (ACs) have a more effective CD8 + T cell response, including a higher frequency of HLA class I alleles able to present peptides from a regulatory protein of HTLV-1, HBZ. We have previously shown that specific features of the host genome flanking the proviral integration site favour clone survival and spontaneous expression of the viral transactivator protein Tax in naturally infected PBMCs ex vivo. However, the previous studies were not designed or powered to detect differences in integration site characteristics between ACs and HAM/TSP patients. Here, we tested the hypothesis that the genomic environment of the provirus differs systematically between ACs and HAM/TSP patients, and between individuals with strong or weak HBZ presentation.

Methods: We used our recently described high-throughput protocol to map and quantify integration sites in 95 HAM/TSP patients and 68 ACs from Kagoshima, Japan, and 75 ACs from Kumamoto, Japan. Individuals with 2 or more HLA class I alleles predicted to bind HBZ peptides were classified 'strong' HBZ binders; the remainder were classified 'weak binders'.

Results: The abundance of HTLV-1-infected T cell clones in vivo was correlated with proviral integration in genes and in areas with epigenetic marks associated with active regulatory elements. In clones of equivalent abundance, integration sites in genes and active regions were significantly more frequent in ACs than patients with HAM/TSP, irrespective of HBZ binding and proviral load. Integration sites in genes were also more frequent in strong HBZ binders than weak HBZ binders.

Conclusion: Clonal abundance is correlated with integration in a transcriptionally active genomic region, and these regions may promote cell proliferation. A clone that reaches a given abundance in vivo is more likely to be integrated in a transcriptionally active region in individuals with a more effective anti-HTLV-1 immune response, such those who can present HBZ peptides or those who remain asymptomatic.

Keywords: HTLV-1, Human T cell lymphotropic virus-type 1, HBZ, HTLV-1 basic leucine zipper factor, HAM/TSP, HTLV-1-associated myelopathy/tropical spastic paraparesis, Integration site, CD8+ T cell

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Background

Human T cell lymphotropic virus-type 1 (HTLV-1) is estimated to infect over 10 million people [1], and is endemic in sub-Saharan Africa, the south islands of Japan, the Caribbean and parts of South America. HTLV-1 is primarily found in $CD4^+$ T cells, where predominantly only a single copy of the virus integrates into the genome [2]. The virus is almost 100% cell-associated, and the viral burden is defined as the fraction of PBMCs that carry the integrated provirus, termed the proviral load (PVL). Infected cells proliferate in vivo, producing clonal populations of cells, each defined by its unique proviral integration site. The viral regulatory proteins Tax and HTLV-1 basic leucine zipper factor (HBZ) are known to drive proliferation of the infected cells [3-5].

More than 90% of HTLV-1-infected individuals remain lifelong asymptomatic carriers (AC), but 1–6% develop an aggressive malignancy known as adult T cell leukaemia/lymphoma (ATLL). A further 0.25 to 4% develop a chronic inflammatory disease of the central nervous system, HAM/TSP, characterised by a slowly progressive spastic paraparesis with pain and neurogenic bladder disturbance [6]. Risk factors for HAM/TSP include female gender and high PVL [7].

There is strong evidence that the $CD8^+$ T cell response is important in limiting PVL, and reducing the risk of HAM/TSP [8], although innate immunity also plays a role in the host response to HTLV-1 [9]. Certain HLA class I alleles are associated with a reduction in PVL and prevalence of HAM/TSP, in particular HLA-A*02 and Cw*08 in a population from Southern Japan [10]. Tax is the dominant $CD8^+$ T cell target antigen of HTLV-1 [11,12]: Tax escape mutations in the HLA-A2-restricted epitope Tax 11-19 are more frequent in individuals with the HLA-A2 allele [13], and Tax expression is frequently silenced in the expanded clone in ATLL by mutations in *tax* or methylation or deletion of the 5'LTR [14-17]. The rate of lysis of Tax^+CD4^+ cells by $CD8^+$ cells has been inversely correlated with PVL [18], although Tax mRNA is virtually undetectable directly ex vivo. Individuals who remain asymptomatic were shown to have a lower PVL than those with HAM/TSP at a given lysis rate [18], and had a greater $CD8^+$ T-cell lytic efficiency as measured by proportion of Tax-specific CTL which degranulate when exposed to their cognate epitope ex vivo [19].

Unlike Tax, HBZ expression is uniformly maintained in HTLV-1-infected T cells, including ATLL cells [4], and this expression correlates with PVL in both ACs and patients with HAM/TSP [20]. On average, HBZ peptides bind to HLA class I alleles with lower affinity than Tax peptides, and the frequency of HBZ-specific CD8+ T cells [21] is correspondingly lower. HBZ expression may be maintained because it can drive expansion of an infected clone without presenting a strong target to the $CD8^+$ T cell response. The frequency of HLA class I alleles that are predicted to strongly bind HBZ peptides is greater in ACs than patients with HAM/TSP, and is inversely correlated with PVL in each group [21]. These observations suggest that a $CD8^+$ T-cell response to the HBZ protein is protective against HTLV-1-associated inflammatory disease.

The equilibrium abundance in vivo of a particular HTLV-1-infected T-cell clone is the result of the interplay between the proliferation of the clone and counter-selection by the host response, chiefly the $CD8^+$ T cell response. Both factors are governed by the program of proviral expression by the clone. Since the proviral sequence is very stable [22], the chief unique attribute of each HTLV-1-infected T-cell clone is the genomic position of the integrated provirus – the proviral integration site. Specific features of the genomic environment of the HTLV-1 proviral integration site are associated with the frequency of spontaneous reactivation of Tax expression ex vivo [23]. Integration in the same transcriptional orientation as a flanking host gene is associated with suppression of Tax expression: same-sense orientation is more frequent in high-abundance clones, and more frequent in vivo than during in vitro infection, suggesting that this orientation confers a selective advantage by allowing escape from the Tax-specific $CD8^+$ T cell response [23]. There are no published data on the influence of the integration site environment on HBZ expression.

Since the genomic integration site influences proviral expression, we reasoned that the selection pressure exerted by a protective immune response will alter the abundance of clones which have integration site genomic environments with certain characteristics. Our previous reports were neither designed nor powered to examine the relationship between the integration site environment and either disease status or host immunogenetics. In this study, we investigated the differences in integration site environment between Japanese individuals who remained AC and those who developed HAM/TSP, and between those that differed in their capacity to present HBZ peptides on protective HLA class I alleles. We report that integration sites in genes and active regions are significantly more frequent in ACs than in patients with HAM/TSP, even after accounting for clone abundance and PVL. Integration sites in genes are also more frequent in strong HBZ binders.

Results

Possession of HBZ-binding HLA class I alleles was associated with reduced HTLV-1 proviral load, but did not affect oligoclonality of the HTLV-1 infected cell population or total number of clones by load

We have previously shown that in both ACs and patients with HAM/TSP in Kagoshima prefecture (Southern Japan),

Table 1 Total patients analysed and integration sites identified

	Kagoshima						Kumamoto		
	HAM/TSP			AC			AC		
Number of patients in:	Strong HBZ	Weak HBZ	***Total***	Strong HBZ	Weak HBZ	***Total***	Strong HBZ	Weak HBZ	***Total***
Whole cohort	81	148	***229***	92	110	***202***	34	64	***98***
All LM-PCR	42	62	***104***	41	59	***100***	34	64	***98***
LM-PCR QC pass	39	56	***95***	30	38	***68***	24	51	***75***
Number of UIS* with abundance:									
<1 per 10,000 PBMC	7954	12341	***20295***	1451	5485	***6936***	4572	9744	***14316***
1-10 per 10,000 PBMC	10051	12429	***22480***	2178	2515	***4693***	1034	1853	***2887***
>10 per 10,000 PBMC	219	364	***583***	102	67	***169***	42	40	***82***
All			***43358***			***11798***			***17285***

*UIS identified from LM-PCR QC pass samples only.
AC: Asymptomatic carrier, UIS: Unique integration site, QC pass: samples that passed LM-PCR quality controls (QC).

the number of HLA class I A and B alleles predicted to bind HBZ epitopes is inversely correlated with PVL [21]. Here, we extended this analysis with a second southern Japanese AC cohort, from nearby Kumamoto prefecture (Table 1). The predicted ability of individual HLA class I A and B alleles to bind HBZ epitopes was determined using the rank of the top HBZ-binding peptide in the peptide binding prediction software Metaserver, as previously described [21] (Table 2). We then used linear regression to analyse the relationship between the number of HBZ-binding alleles and PVL. In the Kumamoto cohort, as in the two Kagoshima cohorts, there was an inverse correlation between log PVL and the number of HBZ-binding alleles (linear regression: slope = -0.12, Table 3). This correlation was significant in a regression analysis of the combined Kagoshima cohorts ($p = 0.02$) as previously reported, and all three cohorts combined ($p = 0.006$), although it did not reach significance in the smaller Kumamoto cohort alone.

We used our recently developed high-throughput protocol [24] to map and quantify the abundance of unique proviral integration sites in the HAM/TSP cohort and the two AC cohorts (Table 1). Sufficient DNA was not available from all subjects, but the median PVL of the HAM/TSP samples that passed LM-PCR quality controls (QC) was not significantly different from that of the full HAM/TSP cohort (Additional file 1: Figure S1A). In the AC cohorts the median PVL of successfully analysed samples exceeded that of all ACs, because a proportion of ACs have a PVL too low to permit accurate quantification [7] and LM-PCR. Since this reduces the difference in median PVL between the analysed AC and HAM/TSP cohorts, our results represent a conservative estimate of the difference between ACs and patients with HAM/TSP.

Since strong HBZ peptide binding is associated with efficient control of PVL, we tested whether this also altered the frequency distribution of the infected cell population. Individuals were designated strong HBZ binders if they

Table 2 HLA class I alleles divided by HBZ epitope binding status

Strong HBZ binding alleles	Weak HBZ binding alleles
A0201, A0206, A0207, A0210	A0101
A2601, A2602, A2603	A0203
A3001	A0301, A0302
A3303	A1101
	A2402
	A3101
	A3201
B3701	B0702
B4001, B4002, B4006	B1301, B1302
B41	B1501, B1507, B1511, B1518
B4403	B2704
	B3501, B3520, B3532/B3568
	B3802
	B3901
	B4003, B4009/B4027/B40105
	B4601
	B4801
	B5101
	B5201
	B5401
	B5502, B5504
	B5601, B5605
	B5801
	B5901, B5902
	B6701

Table 3 Linear regression of HBZ binding alleles to PVL

Outcome	Predictor	Cohort*	n	Slope	p value	Controlling for:
Log PVL	# HBZ binding alleles	Kagoshima HAM, AC	HAM: 221 AC: 200	-0.12	0.021	Age, sex, disease status
Log PVL	# HBZ binding alleles	Kumamoto AC	AC: 98	-0.12	0.28	
Log PVL	# HBZ binding alleles	Kagoshima HAM, AC & Kumamoto AC	HAM: 229 AC: 300	-0.13	0.0058	Disease status

*Only individuals who had all the relevant information were included in the regression. Age, sex and disease status were included as factors where possible as they may vary with PVL.
Age and sex information was not available for the Kumamoto AC cohort.
PVL: proviral load, AC: Asymptomatic carrier, #: number of.

carried two or more predicted HBZ binding HLA class I alleles. The total number of HTLV-1 proviruses mapped in strong HBZ binders was similar to that in weak HBZ binders in each cohort (Additional file 1: Figure S1B), allowing unbiased estimates of clone structure. We estimated the total number of integration site clones in the blood using our novel in silico approach [25]. The estimated number of clones correlated with PVL in all cohorts (Figure 1A). There was no significant difference in estimated total number of clones in the blood, after stratifying by PVL, between AC and HAM/TSP cohorts, or between strong HBZ binders and weak HBZ binders (Figure 1A). The Oligoclonality Index (OCI) quantifies the diversity in clone abundance in an infected T cell population: an OCI approaching 1 indicates an essentially monoclonal population, whereas an OCI of 0 indicates that all clones have the same abundance. Consistent with our previous observations [24], OCI did not correlate with PVL and did not differ between ACs and HAM/TSP cohorts (Figure 1B); there was also no difference in OCI between strong and weak binders of HBZ in any cohort (Figure 1C).

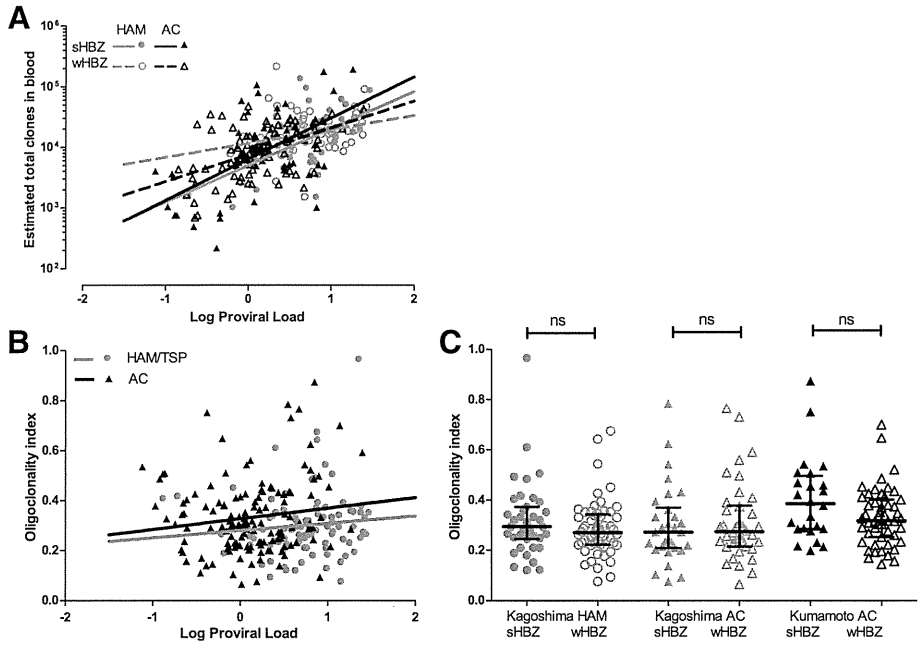

Figure 1 Host disease status and HBZ-binding capacity do not alter total unique HTLV-1-infected clones or oligoclonality. HTLV-1 infected Japanese asymptomatic carriers (AC; triangles) from Kagoshima and Kumamoto prefectures and HAM/TSP patients (circles) from Kagoshima were stratified on the basis of predicted HBZ peptide binding affinity of host HLA class I alleles (strong binders, sHBZ, filled symbols; weak binders, wHBZ, open symbols). Each symbol represents one individual. Genomic DNA samples were processed by sonication-based LM-PCR and samples passing data quality checks were analysed further, as previously described ([23,24]). **(A)** The DivE method was used to estimate the total number of clones in the blood in each subject; the total clone number was positively correlated (Spearman correlation) with proviral load in each cohort both HAM (blue, $p = 0.0004$) and AC (black, $p = 0.0001$)). There was no significant difference in slope of the linear regression line between AC and HAM/TSP cohorts (likelihood ratio test, $p = 0.30$) or between sHBZ (continuous line) and wHBZ (dashed line) in the combined AC and HAM/TSP cohorts (likelihood ratio test, $p = 0.06$) indicating that there is no systematic difference in total clone number by disease status or HBZ binding status. **(B)** The oligoclonality index (OCI), which quantifies the diversity in observed clone abundance in each sample, was not significantly correlated with proviral load in ACs or HAM/TSP (Spearman correlation) and there was no significant difference in slope of the linear regression line between AC and HAM/TSP cohorts. **(C)** The median OCI did not differ significantly by HBZ binding status within individual cohorts (Mann-Whitney U test). Bars denote median +/- interquartile range.

Integration in transcriptionally active areas was positively selected in asymptomatic carriers and was associated with high clonal abundance

Detected integration sites were binned according to their absolute abundance in 10,000 PBMCs (Additional file 1: Figure S1C). In this study, we compared the frequency of transcriptionally active sites between ACs and HAM/TSP patients, after stratifying by host HBZ binding strength and absolute clonal abundance. There was no significant difference in the genomic environment of integration sites between the AC cohorts from Kumamoto and Kagoshima (Additional file 1: Figure S2B, Additional file 1: Figure S3A), which enabled combination of the two cohorts for further statistical analysis. We previously reported that the proportion of integration sites located within a gene and in active genomic regions increased in clones which had a higher absolute abundance; conversely that high abundance clones had a reduced frequency of sites modified with inhibitory epigenetic marks [24]. Here, we also observe significant correlations between clonal abundance and integration in a gene and frequency of epigenetic marks associated with active genomic regions (Figure 2, Additional file 1: Figure S3).

The proportion of integration sites within genes was significantly higher in ACs than in HAM/TSP patients (Figure 2A). The proportion of integration sites within genes did not correlate with PVL (Additional file 1: Figure S2A), suggesting that the difference between ACs and patients

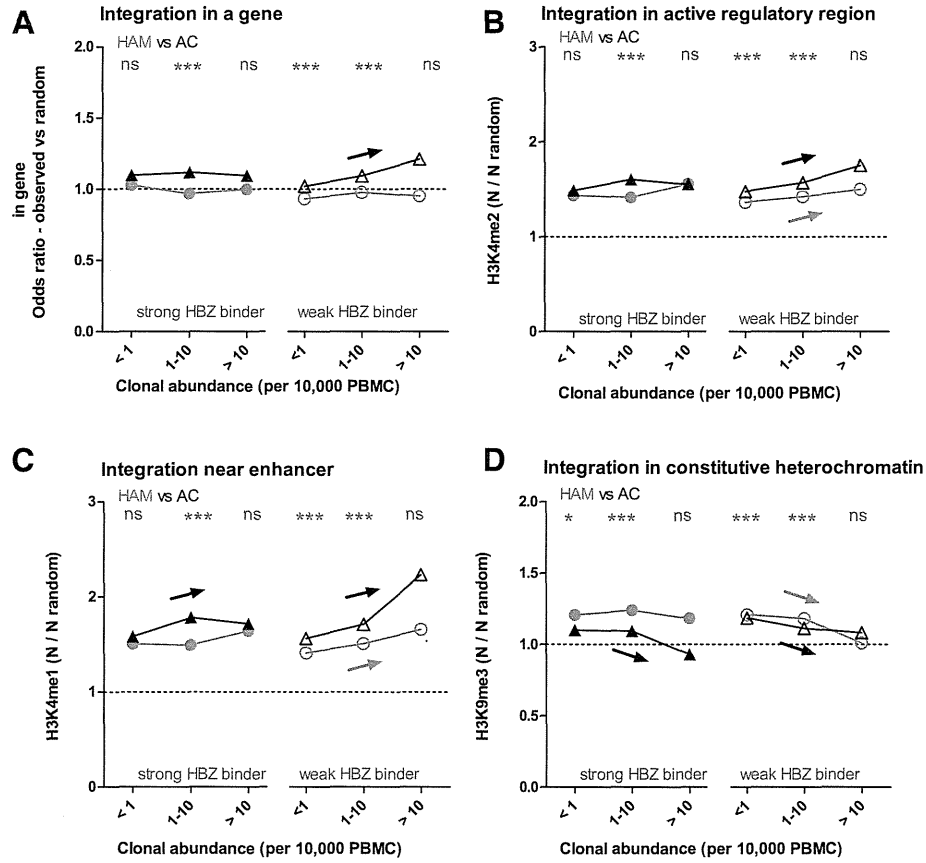

Figure 2 Transcriptionally active integration sites associate with AC status and clone abundance in weak HBZ binders. HTLV-1 unique integration sites from Japanese asymptomatic carriers (AC, black triangles) from Kagoshima and Kumamoto were compared to those from HAM/TSP patients (blue circles, from Kagoshima). Integration sites were stratified on the basis of predicted HBZ peptide binding affinity of host HLA class I alleles (strong binders, sHBZ, filled symbols; weak binders, wHBZ, open symbols) and binned by absolute abundance. Data is expressed versus an in silico generated random integration site dataset. **(A)** AC individuals (Kagoshima and Kumamoto cohorts combined) had a greater proportion of integration sites in genes than HAM/TSP patients (chi-squared test). Percentage of clones with integration sites in genes was correlated with clone abundance only in individuals with HLA class I alleles which could not bind HBZ (arrow, significant chi-squared test for trend). **(B)** Asymptomatic carriers had a higher frequency than HAM/TSP of H3K4me2 marks, enriched in transcriptionally active areas, within 10 Kb of integration sites (Mann-Whitney U test), and **(C)** a higher frequency of H3K4me1 marks, associated with enhancers. Mean epigenetic mark frequency near integration sites in a bin is divided by frequency near random sites. **(D)** In contrast, AC had a lower frequency of H3K9me3 marks, associated with constitutively heterochromatic DNA. Spearman correlation shows a significant (arrow) association between epigenetic mark frequency and log absolute abundance. Statistical comparisons AC vs HAM by Mann-Whitney U test after correction for multiple testing: * $0.05 > p > 0.01$, ** $0.01 > p > 0.001$, *** $p < 0.001$.

with HAM/TSP could not be explained simply by a lower PVL in ACs. There was no significant difference between ACs and patients with HAM/TSP in the proportion of integration sites in the same transcriptional orientation as the nearest host gene (Additional file 1: Figure S2C) or nearby transcriptional start site (Additional file 1: Figure S2D).

Compared to patients with HAM/TSP, integration sites from ACs had higher counts of H3K4me2 and H3K27ac marks within 10 Kb (indicating active regulatory regions and open chromatin; Figure 2B, Additional file 1: Figure S3B) and higher counts of H3K4me1 (associated with enhancers; Figure 2C). Conversely, integration sites had higher counts of H3K9me3 (associated with constitutively repressed chromatin) in patients with HAM/TSP than ACs (Figure 2D). There was no difference in the frequency of H3K27me3 marks, which are associated with facultative heterochomatin (Additional file 1: Figure S3C). Within genes, integration sites marked by H4K20me1 (enriched at 5′ ends of actively expressed gene bodies) were more frequent in ACs (Additional file 1: Figure S3D), but the percentage of integration sites marked by H3K36me3 (enriched at 3′ ends of actively expressed gene bodies) did not consistently correlate with either clone abundance or disease status (Additional file 1: Figure S3E). The increased frequency of active sites and sites in genes in AC individuals was also significant when the characteristics of all integration sites within a patient were averaged, and the cohorts analysed at the patient level (Additional file 1: Figure S4).

Since transcriptional activity of the genome is correlated with gene density, we used a multivariable logistic regression model to simultaneously test the independence of association of integration in a gene, active region and heterochomatin with disease status. This association was tested both at the level of individual integration sites, and using a single value per patient representing the averaged integration sites from that patient. The results showed that integration within a host gene and within active and inhibitory genomic regions were each independently associated with disease status, after controlling for clone abundance and host PVL and predicted HBZ binding affinity (Table 4 and Additional file 1: Figure S3F). Controlling for HBZ affinity using counts of HBZ binding alleles per patient, rather than a strong/weak designation, gave a very similar significance and odds ratio.

We next compared the frequency of transcriptionally active sites between individuals with strong or weak HBZ binding potential. Integration sites from HBZ strong binders were more likely to be in a gene than those from weak binders, even when host disease status and clone absolute abundance were taken into account (Table 5); there was no significant association with frequency of H3K4me2 or H3K9me3 epigenetic marks. There was a similar odds ratio between strong and weak HBZ binders in the percentage of integration sites in a gene per patient, although at this level of individual patients the association did not reach statistical significance.

Discussion

At all levels of clone abundance, ACs had a significantly higher frequency than patients with HAM/TSP of integration sites within host genes and in genomic regions marked by activating epigenetic modifications. This enrichment was associated with disease status per se and was independent of variation in proviral load. The odds ratio of this enrichment in each case was modest (~1.1), however the finding that both integration in a gene and integration in an active genomic region were independently associated with AC status strongly suggests a consistent underlying biological mechanism. The question arises: what are the forces that favour selective survival of these integration sites (i.e. in active transcribed regions) in ACs?

We previously reported that Tax-silenced proviruses were more likely to lie in the same orientation as a flanking host gene or nearby upstream transcriptional start site; we concluded that this effect may be attributed to transcriptional interference [23]. The integration site locations that inhibit Tax expression were also associated with increased clone abundance. Consistent with these two findings, more abundant clones were less likely to express Tax [23]. These observations raised the possibility that a provirus integrated in the same orientation as a host gene might enjoy a selective advantage in individuals with an

Table 4 Proviral integration within genes and active genomic regions are independently associated with disease status

Data	n	Outcome		In gene*	# H3K4me2	# H3K9me3
Integration sites	AC: 29083 HAM/TSP: 43358	AC vs HAM	OR	1.08	1.13	0.88
			p value	4.2×10^{-5}	4.8×10^{-7}	2.1×10^{-6}
Patients	AC: 143 HAM/TSP: 95	AC vs HAM	OR	1.12	1.14	1.0
			p value	0.010	0.011	1.0

Additional factors controlled in multiple logistic regression model: Host HBZ binding status and proviral load, and log absolute clone abundance (per integration site) or log average absolute clone abundance (per patient).
*Percent of integration sites in gene per patient in patient-level analysis.
#: Number of specified epigenetic marks within 10 Kb of integration site. Averaged per patient in patient-level analysis.
OR: Odds ratio, AC: Asymptomatic carrier.

Table 5 Proviral integration within genes and active genomic regions is associated with disease status

Data	n	Outcome		In gene*
Integration sites	sHBZ: 27603 wHBZ: 45439	sHBZ vs wHBZ	OR	1.04
			p value	0.007
Patients	sHBZ: 93 wHBZ: 146	sHBZ vs wHBZ	OR	1.03
			p value	0.2

Additional factors controlled in multiple logistic regression model: Disease status and log absolute clone abundance (per integration site) or log average absolute clone abundance (per patient).
*Percent of integration sites in gene per patient in patient-level analysis.
OR: Odds ratio, sHBZ: strong HBZ binder, wHBZ: weak HBZ binder.

effective immune response because it is less exposed to the strong anti-Tax $CD8^+$ T cell response. However, the integration site environments associated with Tax silencing were not those associated with an increased frequency in ACs compared to HAM/TSP in this study.

A second possibility is that a more efficient cellular and innate immune response in ACs [8] means that a clone needs a greater proliferative capacity to reach a given absolute abundance. Interestingly, we also observe an increase in the percentage of integration sites in a gene amongst integration sites from individuals with strong predicted HBZ binding capacity compared those less likely to bind HBZ epitopes, particularly in the less abundant clones. Increasing numbers of HLA class I alleles able to present HBZ are associated with decreasing PVL, suggesting that there is greater control of infected cells in these patients [21]. Integration sites located in genes and active genomic regions are associated with increased clone abundance ([24], and this study): we postulate that these environments support virus-driven cell proliferation allowing clones to survive under stronger immune control.

Tax is known to drive proliferation of the infected cell; could the clones which selectively survive in ACs express more Tax? We previously observed, in a very small sample of integration sites (n = 40, [26]), that Tax-expressing cells had a higher frequency of integration sites in genes. However, with the advent of high-throughput sequencing, we have recently shown that clones that spontaneously express Tax ex vivo have a minor increase in the frequency of integration in a gene and in regions with activating epigenetic marks ([23] and AM, unpublished observations). Since we have previously observed that more abundant clones are less likely to express Tax [23], Tax expression is unlikely to completely account for the success of these integration site clones. HBZ is also known to drive cell proliferation [4], and integration in a gene or active region may also promote increased expression of HBZ. To definitively determine, however, whether transcriptionally active regions promote increased HBZ expression will require high-throughput sorting and integration site analysis of HBZ expressing clones directly ex vivo: this is currently precluded by the difficulty in sorting cells based on detection of HBZ protein in naturally-infected PBMCs. The role of the integration site in driving proliferation via either Tax or HBZ expression is also complicated by the effect of Tax and HBZ on the expression or function of each other.

A recent study in primary infection with BLV, a related retrovirus [27], has shown that early in infection, integration is favoured in transcriptionally active areas but is strongly selected against by the host immune response. Yet in subsequent chronic infection, abundant clones have a higher frequency of integration sites in transcriptionally active areas. Similarly, in HTLV-1, the effectiveness of the initial host response against expressed viral proteins is likely to define PVL set point, selecting against highly active clones. Clones in heterochomatin may also represent a dead end for the virus, because it may never be re-expressed. During lifelong chronic infection, however, surviving clones with integration sites in 'intermediate' transcriptionally active areas may have a proliferative advantage, although other factors (including clone TCR specificity or immune escape by Tax silencing or timing of viral expression [16,23,28]) will also contribute to the relative success of a clone. These transcriptionally active clones are more common in ACs than HAM/TSP, plausibly because they compete against the effective host response in ACs, or less likely, because they are selectively lost (by an unknown mechanism) in HAM/TSP.

There are other differences between HAM/TSP patients and AC individuals, in addition to the effectiveness of the T-cell immune response, which could alter the selection of proviral integration site during chronic infection. For example, in HAM/TSP, proliferation of HTLV-1-infected T cells may be maintained by IL-2 [29] and IL-15 [30], which may reduce the advantage conferred by integration sites that increase expression of proliferation-inducing factors such as HBZ.

Our results reflect systematic differences in the characteristics of HTLV-1 integration sites that persist in vivo between HAM/TSP patients and ACs. We propose that these differences are not themselves causative of the disparate clinical outcomes, but rather they reflect an underlying difference between patients with HAM/TSP and ACs in the efficiency of host-mediated

control of HTLV-1 replication, for which there is extensive evidence [8].

Two previous studies compared the integration site environment between ACs and patients with HAM/TSP and found no differences, or a borderline significant ($p = 0.049$) difference [24,26]. The difference between these reports and the present study may be attributable to the differences in sample size ($N = 238$ in present study, cp. $N = 40$ and 24 respectively in [24,26]), the quantitative nature and greater sensitivity of the present high-throughput method, or in the ethnicity of the study population (uniformly southern Japanese vs. predominantly Caribbean). The incidence of HAM/TSP is much lower in the Japanese population (studied here) than in individuals of Caribbean origin in the previous studies (0.25 vs 3% [31,32]). In the current study, we excluded differences in LM-PCR efficiency and mean clone abundance as causes of the observed differences between patients with HAM/TSP and ACs.

Conclusions

The expression levels of Tax and HBZ influence both the rate of proliferation and the rate of CTL killing of each infected T-cell clone, and thus its equilibrium abundance in vivo. The balance between the relative strength of these opposing effects differ between the two genes: *tax* encodes the dominant target of the CTL response and is often silenced in abundant clones despite its capacity to support proliferation, but HBZ expression is typically maintained and is a poorer target for the CTL response. We have previously reported features of the integration site that influence Tax expression but there are no studies directly linking integration site and HBZ expression. We show that integration sites within genes and active genomic regions are more frequent in AC clones compared to equivalently-sized clones from HAM/TSP patients, and in individuals who have HLA class I allelesable to effectively present HBZ peptides to CTL. We postulate that integration in a transcriptionally active area may elevate HBZ and/or Tax expression and increase the equilibrium abundance of a clone. The increased frequency of integration sites observed in transcriptionally active genomic regions in ACs is consistent with the conclusion that greater proliferation is required to reach a given clonal abundance under the selection pressure exerted by an effective anti-HTLV-1 immune response.

Methods

Subjects

Kagoshima cohort. The study population has been previously reported [7,10]. Subjects consisted of 229 patients with HAM/TSP and 202 HTLV-1-infected asymptomatic carriers randomly selected from blood donors; all were of Japanese ethnic origin and residing in Kagoshima, Kyushu, Japan [7,10].

Kumamoto cohort. The study population consisted of 98 HTLV-1-infected asymptomatic carriers from blood donors in Kumamoto Prefecture, Kyushu, Japan.

Research was carried out in compliance with the Helsinki Declaration. The study was approved by the Faculty of Medical and Pharmaceutical Sciences Ethics Review Board, University Hospital, Kumamoto, Japan (Ethics 149). All patients gave written, informed consent for the study and for publication of anonymized results.

HTLV-1 proviral load measurements

HTLV-1 DNA was amplified by quantitative PCR in a ABI7900HT FAST real time PCR system using FastSYBR Green (Applied Biosystems) reagents with the Tax-specific primers SK43 and SK44. A control region in B-actin was also amplified using ActF and ActR primers. The rat cell line TARL-2, which contains one integrated copy of the HTLV-1 provirus, was used to generate a standard curve. The sample copy number was interpolated from the standard curve and PVL was expressed as number of infected cells per 100 PBMCs. Proviral load data for the Kagoshima HAM/TSP and AC cohorts, with TARL-2 as pX region control, were as previously described ([7]). SK43: 5′CGGATACCCAGTCTACGTGT, SK44: 5′GAGCCGA TAACGCGTCCATCG, ActF: 5′TCACCCACACTGTGCC CATCTATGA, ActR: 5′ CATCGGAACCGCTACTTGCC GATAG.

HLA class I alleles

HLA class I typing of the Kagoshima cohort was reported previously ([10]). HLA typing of the Kumamoto cohort was done by Luminex reverse SSOP at the Hammersmith Hospital, London, UK to 2 digit resolution with 'strings' of possible 4 digit resolution alleles. For each individual's string of possible alleles, the most frequent 4-digit allele in the Japanese population (represented by a study of 1018 Japanese individuals [33]) was identified as the most likely allele. If there were multiple possible alleles with a population frequency >3%, all these frequent alleles were retained as possibilities for the individual. If no allele subtype in the string was represented in the population study, all alleles in the string were retained.

Epitope binding prediction

We used the Metaserver algorithm (detailed in [21]) to predict HLA class I epitopes. Metaserver combines predicted TAP transport, proteasomal cleavage and HLA–peptide binding from NetCTL and NetMHC to predict peptide binding to HLA Class I A and B alleles present in the Kagoshima and Kumamoto cohorts. For each HLA class I A or B allele, all HTLV-1 peptides were ranked by binding score. The rank of the top HBZ peptide was