Ⅳ．研究成果の刊行物・別刷

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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

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of ATL exhibit 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

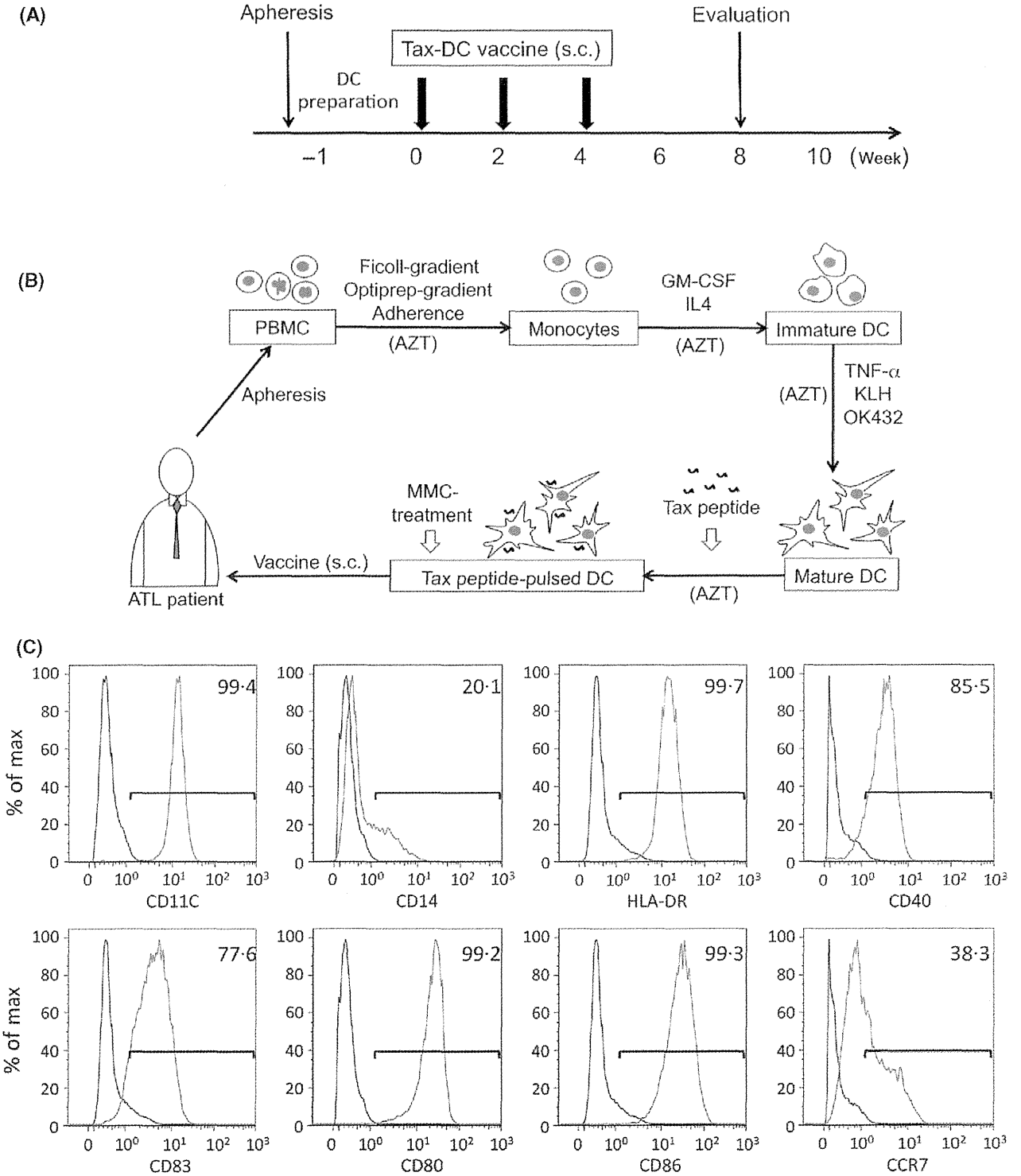

Fig 1. Outline of the Tax-DC vaccine therapy. (A) Schedule for the Tax-DC vaccine therapy. (B) Preparation of the monocyte-derived dendritic cells (DCs). Monocytes were enriched via serial density gradient centrifugation, and the adherent cells were cultured in the presence of granulocyte-macrophage 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.

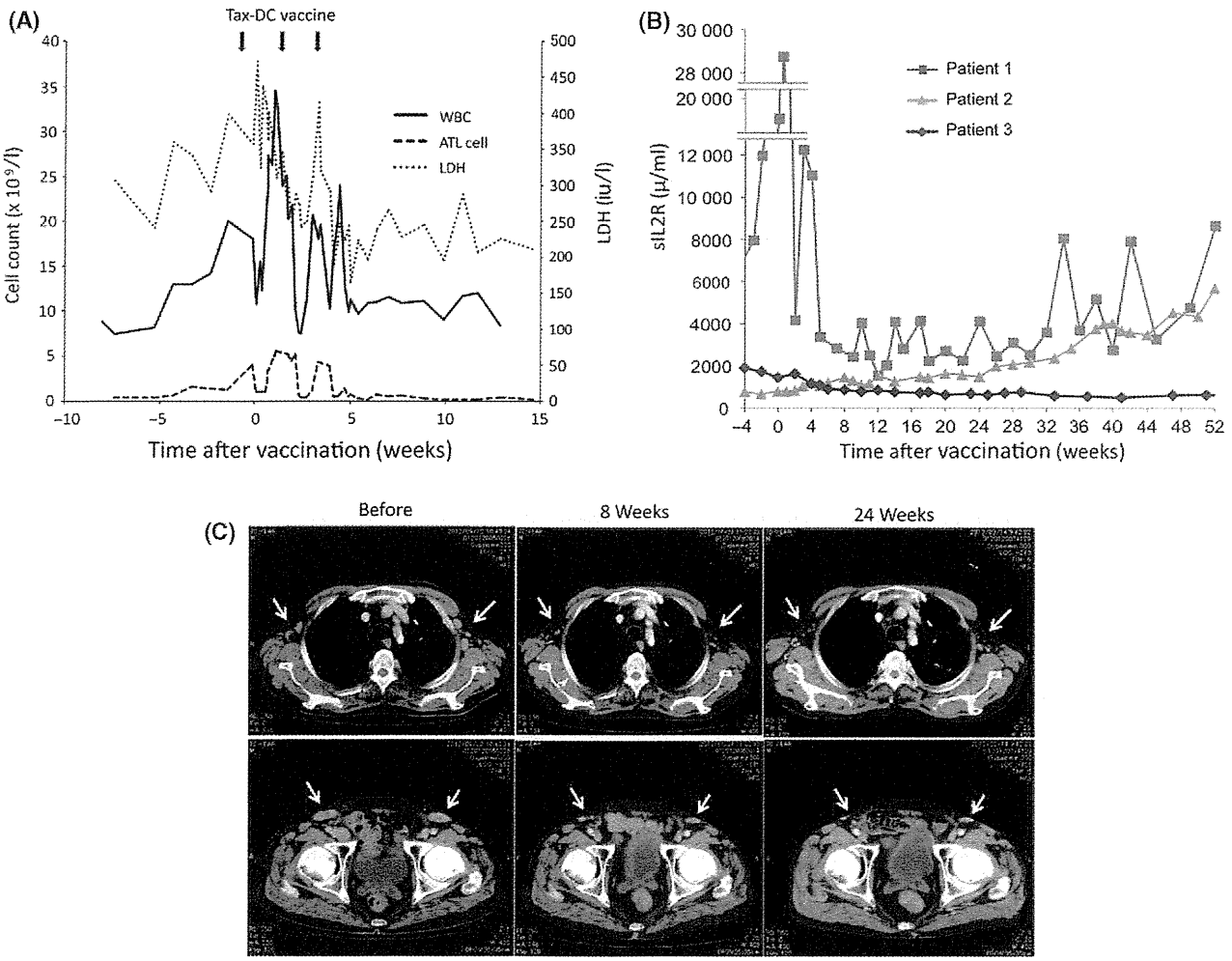

Fig 2. Clinical courses of the patients after the Tax-DC vaccine therapy. (A) Changes in the peripheral white blood cell count (WBC, solid line), ATL cell count (dashed line) and lactate dehydrogenase (LDH) level (fine dotted line) during the initial 15 weeks in Patient 1. The arrows indicate the days 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

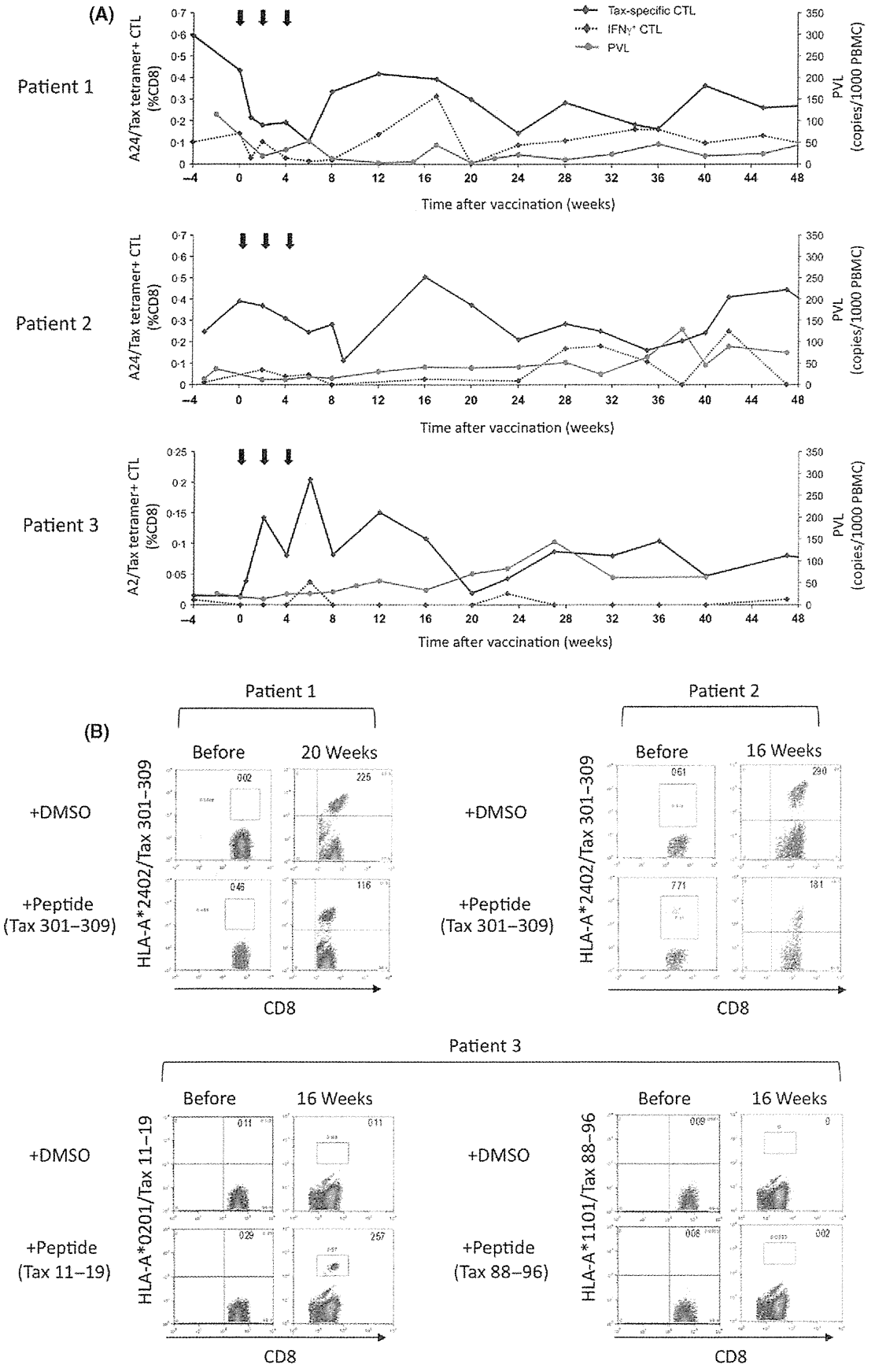

Fig 3. Immunological responses in the three patients after the Tax-DC vaccine therapy. (A) Long-term kinetics of the Tax-specific cytotoxic T cells (CTLs; % CD8+ cells, blue solid line), and γ-interferon (IFN-γ)-producing Tax-specific CTLs (% CD8+ cells, blue broken line), and human T cell leukaemia 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.

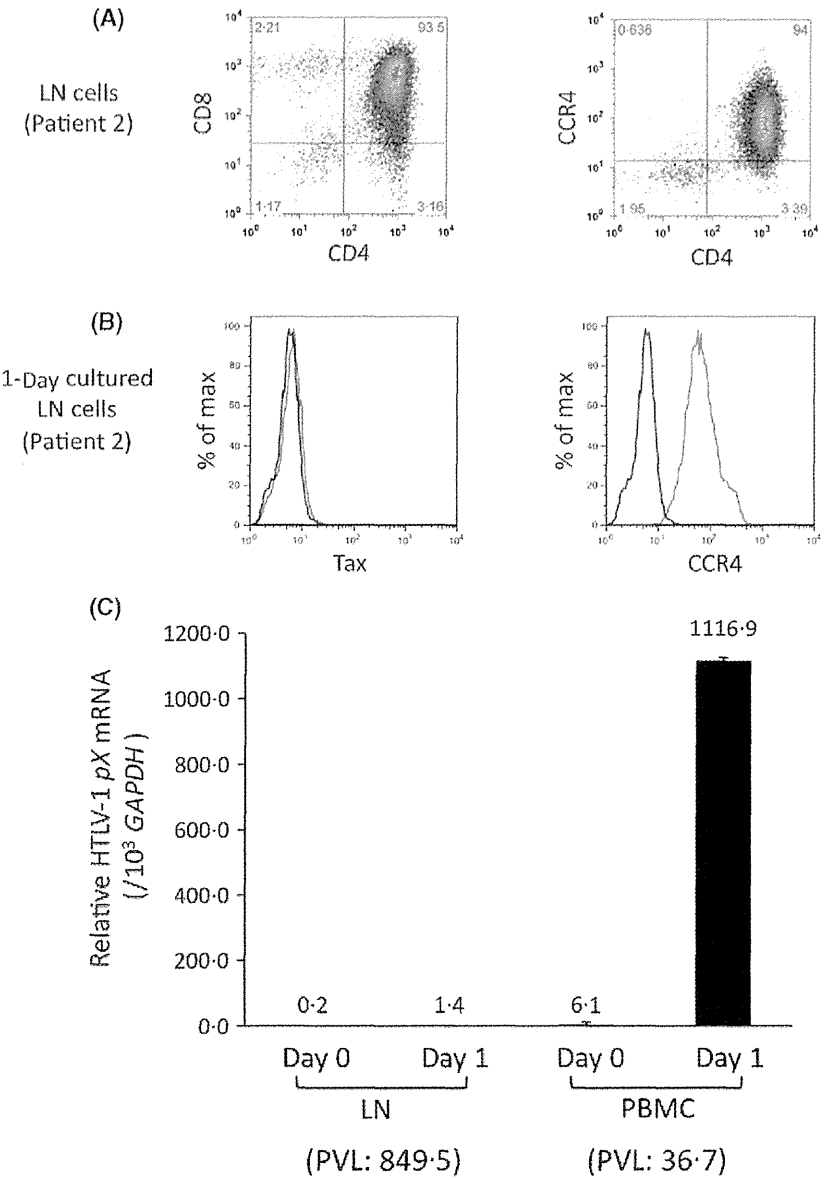

Fig 4. Absence of HTLV-I expression in the lymph node cells obtained from Patient 2. Cells were isolated from a biopsy specimen of the inguinal lymph node (LN) from Patient 2 at 9 weeks after the initiation of the Tax-DC vaccine therapy and subjected for characterization. (A) The cell surface phenotype 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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LETTER TO THE EDITOR

Involvement of double-stranded RNA-dependent protein kinase and antisense viral RNA in the constitutive NFκB activation in adult T-cell leukemia/lymphoma cells

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The constitutive activation of NFκB has an important role in the leukemogenesis of adult T-cell leukemia/lymphoma (ATL) caused by human T-cell leukemia virus type-1 (HTLV-1).[1] Although HTLV-1 Tax is known to activate NFκB, ATL cells exhibit NFκB activities even in the absence of the Tax expression, the mechanism of which has long been a puzzling question.[2] The activation of the non-canonical NFκB pathway associated with the upregulation of NFκB-inducing kinase (NIK)[3] and downregulation of miR31 targeting NIK[4] has been demonstrated in Tax-negative ATL cells. However, it is unclear how HTLV-1 induces these changes.

We herein report that double-stranded RNA (dsRNA)-dependent protein kinase (PKR) is involved in the NFκB activity in Tax-negative ATL cells. PKR is a serine/threonine kinase that is activated by dsRNA and mediates the translational regulation of viral replication, as well as NFκB signaling via IκB kinase, NIK and so on.[5]

We also report that antisense HTLV-1 transcripts containing the long terminal repeat (LTR) region are constitutively expressed in ATL cells and involved in the NFκB activity. HTLV-1 LTR contains Rex-responsive elements (RexRE) consisting of the R and part of the U3 regions predicted to form multiple stem-loop structures.[1,6] An early report suggested that the T7 promoter-transcribed sense RNA at the RexRE region potentially activates interferon (IFN)-stimulated genes, such as 2′, 5′, oligoadenylate synthetase and PKR, *in vitro*.[7] However, the significance of the antisense RNA at the LTR region in ATL cells has not been previously reported.

We previously found that IFN-α suppresses the HTLV-1 Tax expression partly via PKR.[8] The involvement of PKR in the IFN-α-mediated suppression of *de novo* HTLV-1 infection has also been reported.[9] In our previous study, we noticed that PKR is spontaneously upregulated in some HTLV-1-infected cell lines and suppresses the viral expression to some extent.[8] Enhanced expression of IFN-stimulated genes has also been reported in primary ATL cells.[10] Suppression of the viral expression may favor viral persistence to evade host immunity. In addition, PKR mediates NFκB signaling. We therefore investigated whether PKR is involved in NFκB activation in ATL cells.

We first found that a chemical PKR inhibitor (an imidazolo-oxindole derivative, C16) (Supplementary Methods) significantly suppressed the NFκB reporter activity in ATL-derived ED40515(−) and MT-1 cell lines lacking Tax expression (Figure 1a). The PKR inhibitor also suppressed the expression of NIK and various NFκB-responsive genes, including IκB-α and CD25 (Figures 1b and c and Supplementary Figure 1A), associated with decreases in the NFκB protein levels (phospho-p100, p52 and pospho-p65) (Figure 1d), indicating the involvement of PKR in NFκB activation in these cells. The NFκB activity in an Epstein–Barr virus-transformed B-cell line was not markedly affected by the PKR inhibitor (Supplementary Figure 1B). As PKR has previously been shown to inhibit HTLV-1 gene expression,[8,9] PKR thus appears to have a role in both the NFκB activity and a low viral expression, which are both characteristics of ATL cells *in vivo*.

As PKR is known to be activated by dsRNA, we searched for candidate RNAs derived from the HTLV-1 genome in ED40515(−) and MT-1 cells using quantitative RT-PCR to differentially detect sense and antisense HTLV-1 RNA (Supplementary Methods and Supplementary Figure 2). In the ED40515(−) cells, sense RNA was hardly detectable, whereas antisense RNA was detected with the primer sets for the R, U5 and Gag, but not pX, regions (Figure 1e and Supplementary Figure 2). The MT-1 cells expressed antisense RNA in all regions tested, with smaller amounts of sense RNA. In contrast, the HTLV-1 producer MT-2 cells possessed an overwhelming amount of sense RNA.

In addition, we found similar antisense RNA in primary ATL cells derived from four acute ATL patients (Figure 1f). Antisense RNA, especially at the R region, was detected in all ATL cases tested, as similarly observed in the ED40515(−) and MT-1 cells. The samples from two patients also expressed antisense RNA containing the *Gag* region. Sense RNA was detected sporadically. The mRNA of HTLV-1 basic leucine zipper factor (HBZ), a known antisense HTLV-1 product,[11,12] was detected in all ATL-derived cell lines and primary ATL samples tested.

The 5′ rapid amplification of cDNA ends (5′ RACE) method revealed that the antisense transcripts containing the R region in ED40515(−) cells initiated from the 3′ cellular flanking region (Figure 2a). An analysis of the nucleotide sequence using the UVA FASTA Server demonstrated that the 3′ cellular flanking sequence is identical to a portion of the *Homo sapiens* 12 BAC RP11-946P6 complete sequence.

The RT-PCR analysis using a primer set specific for the 3′ cellular flanking region showed that the antisense RNA initiating from the 3′ cellular flanking region extended at least to the *U3*, but not *pX*, region, suggesting that the antisense RNA contains most of the LTR (Figure 2b). These results differed from those for *HBZ* mRNA, which initiates from several points in the *R* and *U5* regions of the 3′ LTR and does not include the U3 region in a spliced form.[11]

In the primary ATL cells, the 5′ RACE analysis indicated that the antisense transcripts were also transcribed from the 3′ cellular flanking region and occasionally from the *Gag* region (Supplementary Figures 3A–C). Interestingly, a previous report indicated that HTLV-1 proviruses in persistently infected cells *in vivo* are preferentially integrated into transcriptionally active genomic regions,[13] possibly as a result of *in vivo* selection.

We next assessed the effects of the antisense transcripts containing the LTR region on the NFκB activity using the RNA interference method in ED40515(−) cells. Although the siRNAs targeting the antisense R region (si-R) reduced the antisense RNA only partially via electroporation, even under selected conditions, they significantly suppressed the NFκB reporter activity and expression levels of NIK and some NFκB-responsive genes, as well as NFκB proteins in both the canonical and noncanonical pathways (Figures 2c–h and Supplementary Figures 4A–D).

Interestingly, siRNAs targeting antisense *U5* (si-U5) neighboring the R region failed to reduce the antisense RNA detected by the

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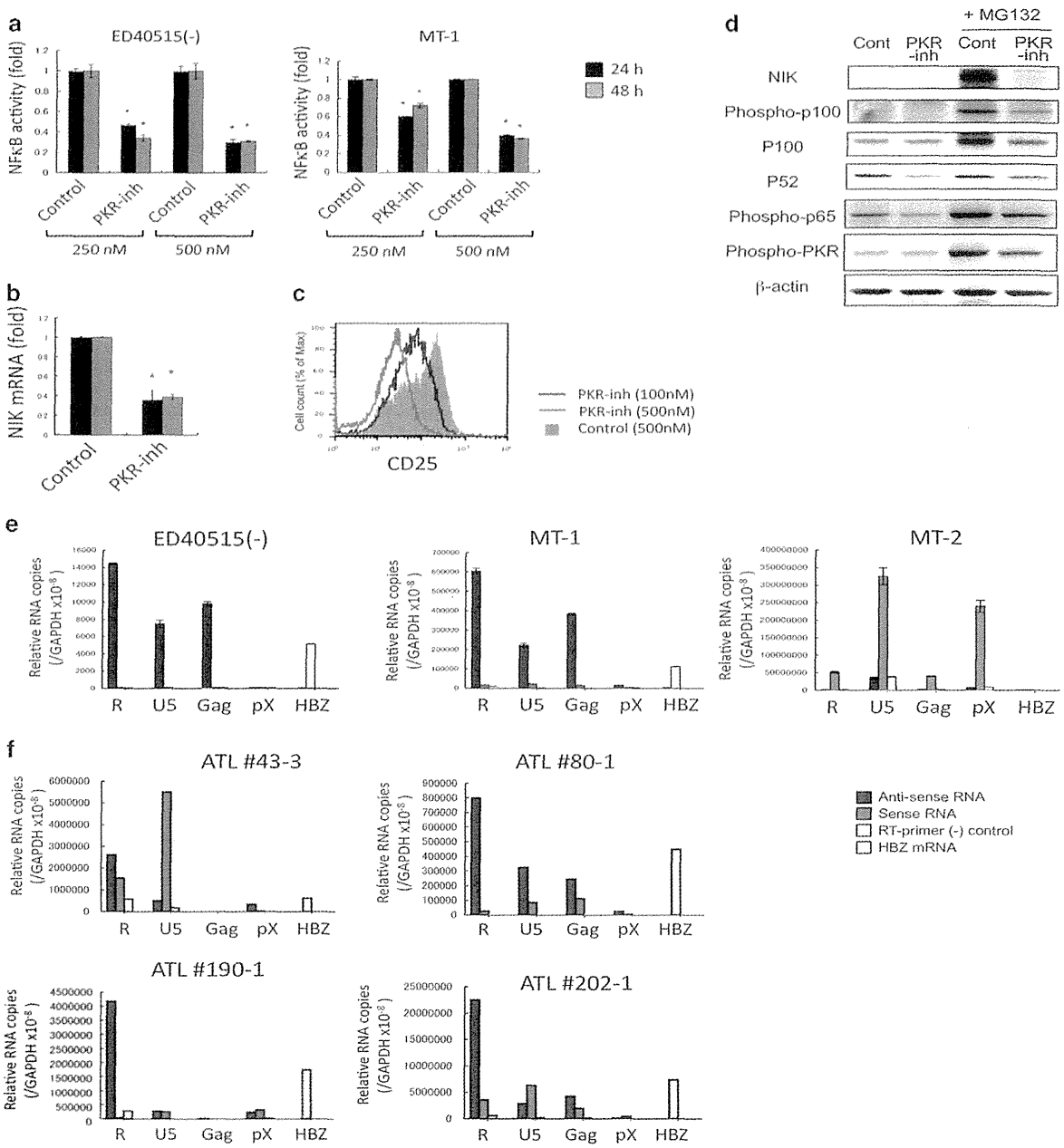
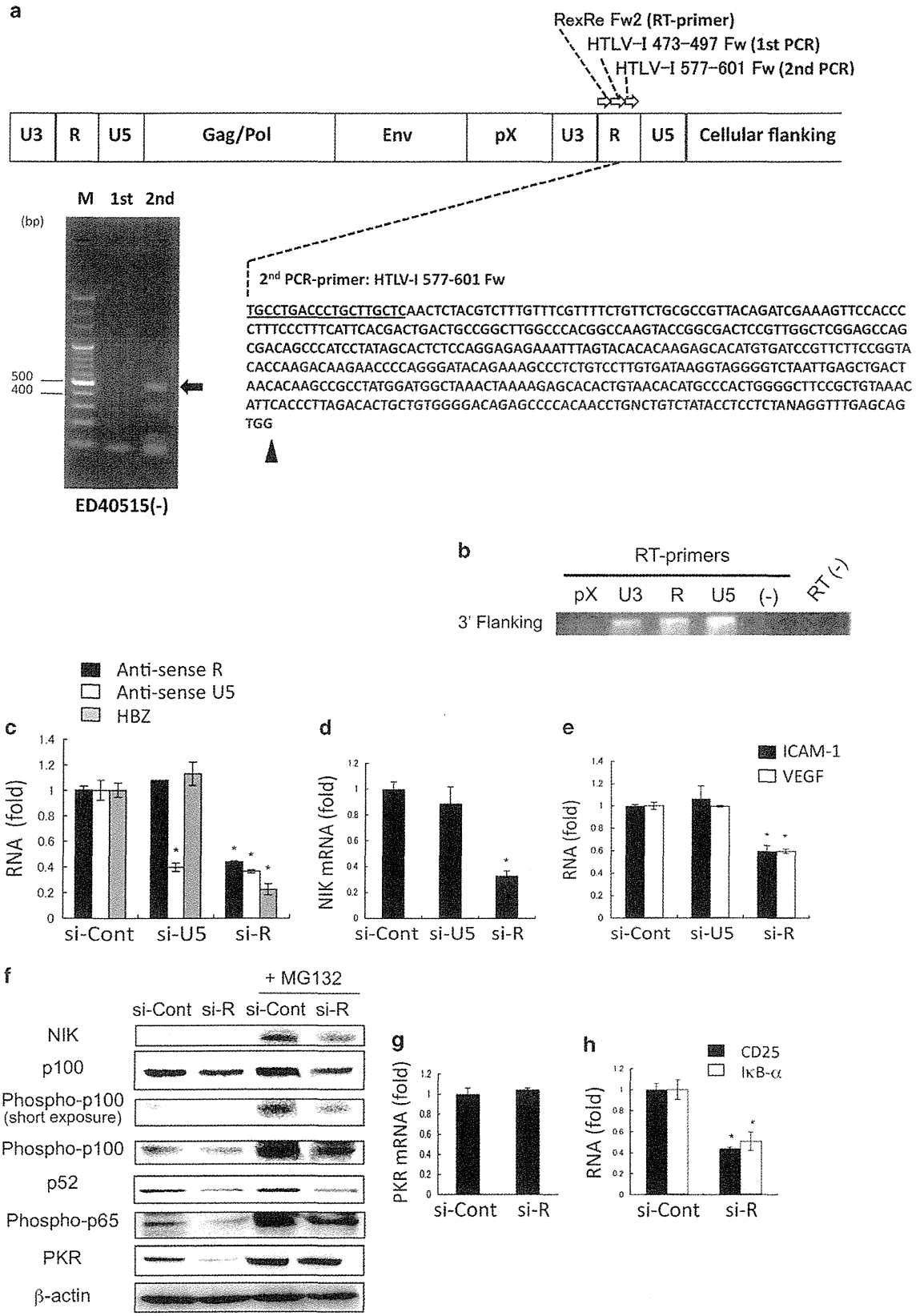


Figure 1. Suppression of NFκB activity by a PKR-inhibitor and the presence of antisense RNA at the HTLV-1 LTR in Tax-negative ATL cells. (**a**) ED40515(−) (left) and MT-1 (right) cells containing reporter genes for NFκB and the thymidine kinase (TK) promoter were incubated with a chemical PKR inhibitor (PKR-inh) or its negative control (control) (250 and 500 nM) for 24 (black) and 48 h (gray). The relative NFκB activity standardized with the TK-promoter activity is indicated as the mean and s.d. of duplicate samples. $^*P < 0.05$. (**b**) The expression levels of NIK mRNA were evaluated by quantitative RT-PCR (RT-qPCR) in the ED40515(−) cells treated with the control or PKR inhibitor (500 nM) for 24 (black) and 48 h (gray). The relative RNA expression standardized with the GAPDH mRNA copy number were indicated as the mean and s.d. of duplicate samples. $^*P < 0.05$. (**c**) The surface CD25 expression levels were evaluated using flow cytometry in the ED40515(−) cells in the presence of 500 nM of the control (gray) or 100 nM (blue) or 500 nM (red) of the PKR inhibitor 72 h after incubation. (**d**) An immunoblotting analysis for NIK, phosphorylated-p100, p100, p52, phosphorylated-p65, phosphorylated-PKR and β-actin proteins in the cell lysates of ED40515(−) cells cultured in the presence of the control or PKR inhibitor (500 nM) for 72 h, with or without MG132-treatment for the last 3 h. (**e**) Total RNA was extracted from ED40515(−), MT-1 and MT-2 cells, and the levels of the antisense RNA (blue) and sense RNA (red) were measured by qPCR with primer sets at the indicated region of HTLV-1 genome, following RT with either forward or reverse primer alone, respectively, as described in the Supplementary Methods and Supplementary Figure 2. The background amplification without RT primers (yellow) and the amounts of HBZ mRNA (white) were also measured. The RT-qPCR results are standardized according to the 10^8 GAPDH mRNA copy number and indicated as the mean and s.d. of duplicate samples. The samples without reverse transcriptase did not yield any measurable PCR products. (**f**) The levels of antisense (blue) and sense (red) HTLV-1 RNAs, the background amplification without RT-primers (yellow) and the amount of HBZ mRNA (white) in the primary ATL cells from four patients (#43-3, #80-1, #190-1, #202-1) were analyzed by RT-qPCR as described above. For the clinical samples, the assay was performed on single aliquots owing to the scarcity of the sample amounts. Similar results were obtained in at least two independent experiments.



R-specific primers, whereas si-U5 successfully knocked down the antisense RNA detected by the *U5*-specific primers (Figure 2c). In contrast, si-R knocked down the antisense transcripts detected by all sets of *R-*, *U5-* and *HBZ*-specific primers used. The reason for this observation is unclear, although this phenomenon might be attributed to the effects of the multiple stem-loop structure or partial truncation of the transcripts. The siRNA targeting the *U3* region (si-U3) partly suppressed the NFκB activity in the

Figure 2. Antisense transcripts at the LTR region are critical for the NFκB activation and the stabilization of PKR in ED40515(−) cells. (**a**) The initiating points of the antisense RNA in the ED40515(−) cells were analyzed using the 5′ RACE PCR method, as described in the Supplementary Methods. The locations of the RT and forward primers for the first and second PCR cycles are indicated (white arrows). The PCR products were visualized following electrophoresis (lane 1: marker, lane 2: 1st PCR, lane 3: 2nd PCR), and the nucleotide sequences of the major product (black arrow) were determined. The nucleotide sequences starting from the second PCR primers (underlined) until the initiation site of the antisense transcripts (▲) are indicated. The blue characters represent the 3′ cellular flanking region. (**b**) The antisense RNAs were detected in the total RNA of ED40515(−) cells by RT-PCR using the primer pairs specific for 3′ cellular-flanking region (627–651 Fw and ED40515(−) cellular Rev), following RT with forward primers at pX (pX2), U3 (LTR6–26), R (RexRE fw2) and U5 (577–601Fw) regions. PCR products were visualized by ethidium bromide staining following electrophoresis on a 2% agarose gel. (**c**) ED40515(−) cells were transfected with si-Cont, siRNA targeting antisense *U5* (si-U5) (mixture of si-LTR-628 and 733), or si-RNA targeting anti-sense *R* (si-R) (mixture of si-R409, 475, 500 and 571) regions, and the antisense transcripts were quantified via RT-qPCR using the primer sets for the *R* (black), *U5* (white) and *HBZ* (gray) regions 48 h after transfection. (**d** and **e**) The mRNA levels of NIK (**d**), ICAM-1 (black), VEGF (white) (**e**) in the RNA samples prepared in **c** were quantified via RT-qPCR. The relative RNA expression standardized with the GAPDH mRNA copy number were indicated as the mean and s.d. of duplicate samples. $^*P < 0.05$. (**f**) The immunoblotting analysis for NIK, p100, phosphorylated-p100, p52, phosphorylated-p65, PKR and β-actin proteins in the ED40515(−) cells transfected with si-Cont or si-R. The cell lysates were prepared after 48 h of incubation with or without MG132 (10 μM) for the last 3 h. (**g** and **h**) The total RNA was extracted from the ED40515(−) cells 48 h after transfection with si-Cont or si-R (the same cell samples prepared in **f**), and the mRNA levels of PKR (**g**), CD25 (black) and IκB-α (white) (**h**) were analyzed using RT-qPCR. Similar results were obtained in at least two independent experiments. $^*P < 0.05$.

ED40515(−) cells (Supplementary Figure 4E), suggesting that the *U3* region is included in the antisense RNA responsible for the NFκB activity.

It is of note that the PKR expression was markedly decreased by si-R at the protein level only, not the RNA level (Figures 2f and g). Furthermore, the PKR protein levels in the cells transfected with si-R were restored by a proteasome inhibitor MG132 (Figure 2f), implying that the antisense RNA contributed to the stabilization of PKR proteins, presumably by acting as dsRNA. A previous report showed that the stability of PKR is markedly enhanced following autophosphorylation mediated by the dimerization of PKR induced in the binding of dsRNA.[14]

Although *HBZ* mRNA partly overlaps with the antisense R region,[11] the siRNA targeting *HBZ* (si-HBZ) did not affect the expression of the antisense transcripts at the R region or the mRNA of NIK, VEGF, ICAM1 and IκB-α (Supplementary Figures 4F–H), consistent with the previous finding that HBZ does not activate NFκB.[15] The si-HBZ, however, exceptionally suppressed the CD25 expression, suggesting the contribution of HBZ to the CD25 expression via NFκB-independent mechanisms.

In the present study, using Tax-negative ATL-derived cells we demonstrated (i) the suppression of the NFκB activity by a PKR inhibitor, (ii) the presence of antisense RNA including the LTR region of HTLV-1, (iii) the suppression of the NFκB activity by siRNA targeting the antisense *U3* and R regions and (iv) the destabilization of PKR proteins by the knockdown of the antisense transcripts. These results strongly suggest that the antisense RNA of the LTR region is involved in the constitutive activation of NFκB in ATL cells, at least via the activation of PKR. This phenomenon partly explains the long unsolved question, namely how and which viral components, other than Tax, constitutively activate NFκB in ATL cells. The antisense transcripts at the LTR region might also contribute to the persistence of HTLV-1 *in vivo*. However, the effect of PKR inhibition observed in the present study was partial, thus suggesting the presence of additional pathways. The possible involvement of other pattern recognition molecules was not excluded. Our results indicate the existence of a link between HTLV-1 leukemogenesis and the host antiviral responses, which provides new insight into the disease mechanisms and therapeutic strategies for ATL.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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HTLV-1 induces a Th1-like state in CD4+CCR4+ T cells

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Human T-lymphotropic virus type 1 (HTLV-1) is linked to multiple diseases, including the neuroinflammatory disease HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP) and adult T cell leukemia/lymphoma. Evidence suggests that HTLV-1, via the viral protein Tax, exploits CD4+ T cell plasticity and induces transcriptional changes in infected T cells that cause suppressive CD4+CD25+CCR4+ Tregs to lose expression of the transcription factor FOXP3 and produce IFN-γ, thus promoting inflammation. We hypothesized that transformation of HTLV-1–infected CCR4+ T cells into Th1-like cells plays a key role in the pathogenesis of HAM/TSP. Here, using patient cells and cell lines, we demonstrated that Tax, in cooperation with specificity protein 1 (Sp1), boosts expression of the Th1 master regulator T box transcription factor (T-bet) and consequently promotes production of IFN-γ. Evaluation of CSF and spinal cord lesions of HAM/TSP patients revealed the presence of abundant CD4+CCR4+ T cells that coexpressed the Th1 marker CXCR3 and produced T-bet and IFN-γ. Finally, treatment of isolated PBMCs and CNS cells from HAM/TSP patients with an antibody that targets CCR4+ T cells and induces cytotoxicity in these cells reduced both viral load and IFN-γ production, which suggests that targeting CCR4+ T cells may be a viable treatment option for HAM/TSP.

Introduction

The flexibility of the CD4+ T cell differentiation program that underlies the success of the adaptive immune response has recently been implicated in the pathogeneses of numerous inflammatory diseases (1–3). The majority of CD4+ T lymphocytes belong to a class of cells known as Th cells, so called because they provide help on the metaphorical immune battlefield by stimulating the other soldiers — namely, B cells and cytotoxic T lymphocytes — via secretion of various cytokines. Interestingly, there is also a minority group of CD4+ T cells with quite the opposite function: Tregs actively block immune responses by suppressing the activities of CD4+ Th cells as well as many other leukocytes (4). Tregs are credited with maintaining immune tolerance and preventing inflammatory diseases that could otherwise occur as a result of uninhibited immune reactions (5). Thus, the up- or downregulation of certain CD4+ T cell lineages could disrupt the carefully balanced immune system, threatening bodily homeostasis.

The plasticity of CD4+ T cells, particularly Tregs, makes CD4+ T cell lineages less clean-cut than they may originally appear. CD4+ T cells are subdivided according to various lineage-specific chemokine receptors and transcription factors they express, as well as the cytokines they produce (6). Th1 cells, for example, can be identified by expression of CXC motif receptor 3 (CXCR3) and T box transcription factor (T-bet; encoded by *TBX21*) and are known to secrete the proinflammatory cytokine IFN-γ (6). While both have been known to express CC chemokine receptor 4 (CCR4) and CD25, Th2 cells and Tregs can usually be distinguished from each other by their expression of GATA-binding protein 3 (GATA3) and forkhead box p3 (FOXP3), respectively (6, 7). CCR4 is coexpressed in the majority of CD4+FOXP3+ cells and in virtually all CD4+CD25+FOXP3+ cells, making it a useful — albeit not fully specific — marker for Tregs (8, 9). FOXP3 is a particularly noteworthy marker because its expression is said to be required for Treg identity and function (10). In fact, *Foxp3* point mutations are reported to cause fatal multiorgan autoimmune diseases (11). Even partial loss of FOXP3 expression can disrupt the suppressive nature of Tregs, representing one of several pathways by which even fully differentiated Tregs can reprogram into inflammatory cells (12). There have been several reports of Tregs reprogramming in response to proinflammatory cytokines such as IL-1, IL-6, IL-12, and IFN-γ (12, 13); it is thought that this reprogramming may have evolved as an adaptive mechanism for dampening immune suppression when protective inflammation is necessary (12). However, this same plasticity can lead to pathologically chronic inflammation, and several autoimmune diseases have been associated with reduced FOXP3 expression and/or Treg function, including multiple sclerosis, myasthenia gravis, and type 1 diabetes (14, 15).

Of the roughly 10–20 million people worldwide infected with human T-lymphotropic virus type 1 (HTLV-1), up to 2%–3% are affected by the neurodegenerative chronic inflammatory dis-

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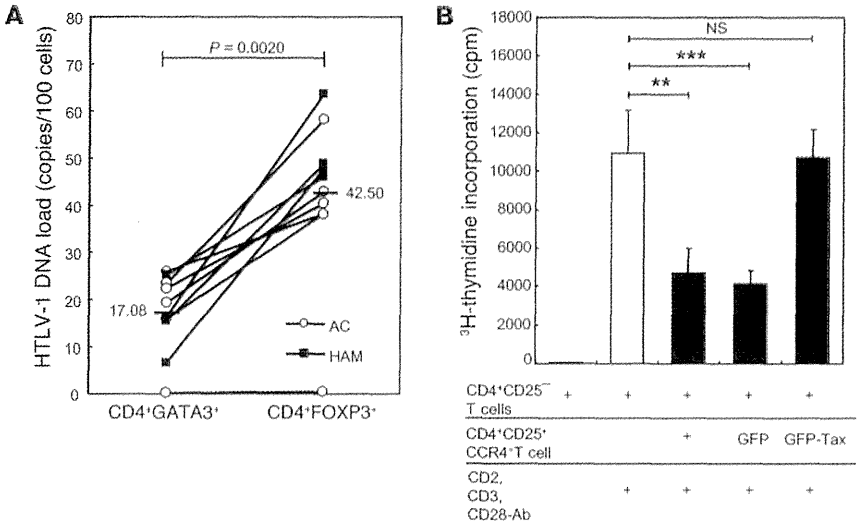

Figure 1. HTLV-1 mainly infects Tregs and inhibits their regulatory function. (**A**) Higher HTLV-1 proviral DNA load in CD4$^+$FOXP3$^+$ cells (Tregs) compared with CD4$^+$GATA3$^+$ cells ($P = 0.0020$, Wilcoxon test) from asymptomatic carriers (AC; $n = 6$) and HAM/TSP patients ($n = 4$). PBMCs were FACS sorted, and proviral load was measured using quantitative PCR. Horizontal bars represent the mean value for each set. (**B**) Loss of regulatory function in Tax-expressing CD4$^+$CD25$^+$CCR4$^+$ cells (Tregs). CD4$^+$CD25$^-$ T cells from an HD were stimulated with CD2, CD3, and CD28 antibodies and cultured alone or in the presence of equal numbers of CD4$^+$CD25$^+$CCR4$^+$ T cells, GFP lentivirus–infected HD CD4$^+$CD25$^+$CCR4$^+$ T cells, or GFP-Tax lentivirus–infected HD CD4$^+$CD25$^+$CCR4$^+$ T cells. As a control, CD4$^+$CD25$^-$ T cells alone were cultured without any stimulus. Proliferation of T cells was determined using ^{3}H-thymidine incorporation by adding ^{3}H-thymidine for 16 hours after 4 days of culture. All tests were performed in triplicate. Data are mean ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$, ANOVA followed by Tukey test for multiple comparisons.

ease HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP). The main other condition associated with the retrovirus is adult T cell leukemia/lymphoma (ATLL), a rare and aggressive cancer of the T cells. HAM/TSP represents a useful starting point from which to investigate the origins of chronic inflammation, because the primary cause of the disease — viral infection — is so unusually well defined. HAM/TSP patients share many immunological characteristics with FOXP3 mutant mice, including multiorgan lymphocytic infiltrates, overproduction of inflammatory cytokines, and spontaneous lymphoproliferation of cultured CD4$^+$ T cells (16–18). We and others have proposed that HTLV-1 preferentially infects CD4$^+$CD25$^+$CCR4$^+$ T cells, a group that includes Tregs (7, 19). Samples of CD4$^+$CD25$^+$CCR4$^+$ T cells isolated from HAM/TSP patients exhibited low FOXP3 expression as well as reduced production of suppressive cytokines and low overall suppressive ability — in fact, these CD4$^+$CD25$^+$CCR4$^+$FOXP3$^-$ T cells were shown to produce IFN-γ and express Ki67, a marker of cell proliferation (19). The frequency of these IFN-γ–producing CD4$^+$CD25$^+$CCR4$^+$ T cells in HAM/TSP patients was correlated with disease severity (19). Finally, evidence suggests that the HTLV-1 protein product Tax may play a role in this alleged transformation of Tregs into proinflammatory cells in HAM/TSP patients: transfecting Tax into CD4$^+$CD25$^+$ cells from healthy donors (HDs) reduced FOXP3 mRNA expression, and Tax expression in CD4$^+$CD25$^+$CCR4$^+$ cells was higher in HAM/TSP versus ATLL patients despite similar proviral loads (19, 20). Therefore, we hypothesized that HTLV-1 causes chronic inflammation by infecting CD4$^+$CD25$^+$CCR4$^+$ T cells and inducing their transformation into Th1-like, IFN-γ–producing proinflammatory cells via intracellular Tax expression and subsequent transcriptional alterations including but not limited to loss of endogenous FOXP3 expression.

In this study, we first sought to discover the detailed mechanism by which Tax influences the function of CD4$^+$CD25$^+$CCR4$^+$ T cells. We used DNA microarray analysis of CD4$^+$CD25$^+$CCR4$^+$ T cells from HAM/TSP patients to identify *TBX21*, known as a master transcription factor for Th1 differentiation, as a key intermediary between Tax expression and IFN-γ production. We demonstrated that Tax, in concert with specificity protein 1 (Sp1), amplified *TBX21* transcription and subsequently IFN-γ production. Next, we established the presence of Th1-like CD4$^+$CCR4$^+$ T cells in the CSF and spinal cord lesions of HAM/TSP patients. The majority of these CD4$^+$CCR4$^+$ T cells coexpressed CXCR3 as well as T-bet and IFN-γ. Finally, we investigated the therapeutic potential of an anti-CCR4 monoclonal antibody with antibody-dependent cellular cytotoxicity (ADCC) (21). Applying this antibody in vitro diminished the proliferative capacity of cultured PBMCs and reduced both proviral DNA load and IFN-γ production in cultured CSF cells as well as PBMCs. In conclusion, we were able to elucidate a more detailed mechanism for the pathogenesis of HAM/TSP and use our findings to suggest a possible therapeutic strategy.

Results

HTLV-1 preferentially infects Tregs and alters their behavior via Tax. Experiments were conducted to determine which among CD4$^+$CD25$^+$CCR4$^+$ T cells were infected by HTLV-1, and how the infection influenced their functionality. Analysis of fluorescence-activated cell sorting (FACS)-sorted PBMCs obtained from asymptomatic carriers ($n = 6$) as well as HAM/TSP patients ($n = 4$) revealed that Tregs (CD4$^+$FOXP3$^+$) carried much higher proviral loads than Th2 cells (CD4$^+$GATA3$^+$) ($P = 0.0020$; Figure 1A). As it is well established that each infected cell contains only 1 copy of the HTLV-1 provirus (22, 23), these results indicate that a larger proportion of FOXP3$^+$ than GATA3$^+$ CD4$^+$ T cells are infected. As expected, proliferation of CD4$^+$CD25$^-$ cells after stimulation, as measured by ^{3}H-thymidine incorporation, was suppressed upon coculture with CD4$^+$CD25$^+$CCR4$^+$ cells, including Tregs ($n = 3$, $P < 0.01$; Figure 1B). However, after being transduced with lentiviral vector expressing GFP-Tax, the CD4$^+$CD25$^+$CCR4$^+$ cells no longer suppressed cell proliferation; conversely, cells transduced with the control vector expressing only GFP retained full suppressive function ($P < 0.001$; Figure 1B).

The HTLV-1 protein product Tax induces IFN-γ production via T-bet. Experiments were conducted to determine if and how Tax affects IFN-γ production in infected T cells. First, the existence of a functional link between *Tax* and *IFNG* was established by using the