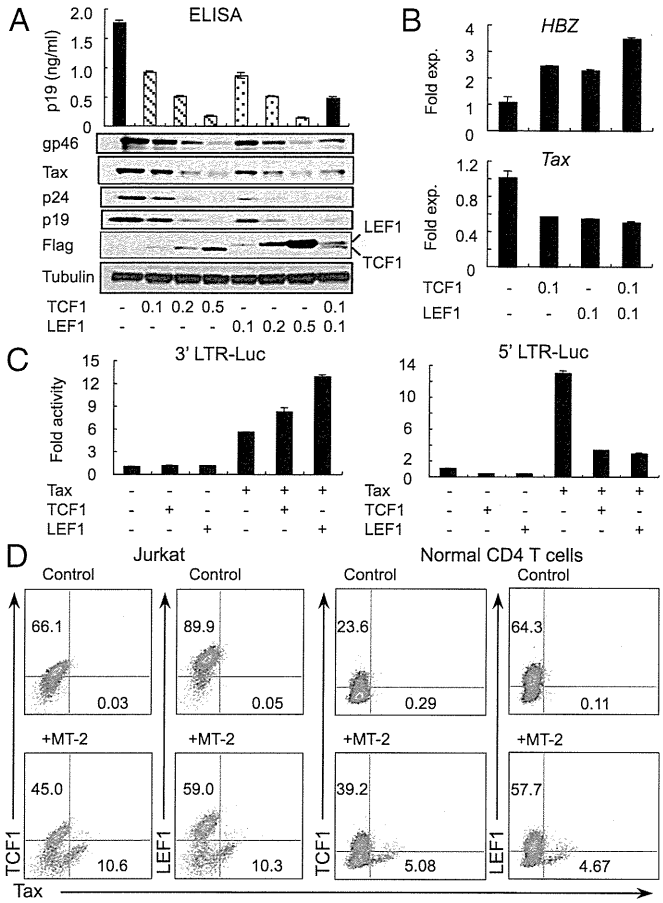

Fig. 3. TCF1 and LEF1 each inhibit HTLV-1 replication by antagonizing Tax. (*A*) TCF1 and LEF1 each inhibits HTLV-1 production (*Upper*) and protein expression (*Lower*). pX1MT-M (0.5 μg) was transfected with or without TCF1 or LEF1 into 293FT cells. 48 h later, supernatants were collected for p19 ELISA and cells were lysed for Western blot. (*B*) TCF1 and LEF1 each inhibit Tax transcription (*Lower*) but not HBZ transcription (*Upper*). pX1MT-M (0.5 μg) was transfected with or without TCF1 or LEF1 into 293FT cells. 44 h later, RNA was extracted for qPCR analysis. (*C*) TCF1 and LEF1 each slightly enhance Tax-mediated 3′ LTR-Luc (*Left*) activation, whereas they significantly suppress 5′ LTR activation (*Right*). Reporter assays were performed in Jurkat cells. (*D*) Jurkat or normal human CD4 T cells were either cultured alone (*Upper*) or cocultivated with lethally irradiated (150 Gy) MT-2 cells (*Lower*) at a 2:1 ratio. 48 h later (when MT-2 cells were all dead), cells were stained for intracellular Tax and TCF1 or LEF1. Numerals indicate percentages of gated populations. Fold exp. indicates fold expression.

by TCF1/LEF1, we performed reporter assays with the complete 5′ and 3′ LTR sequences. Tax mildly activated the 3′ LTR, and this activation was enhanced by TCF1 or LEF1 (Fig. 3*C*). This observation explains why HBZ transcription increased in the presence of TCF1/LEF1 (Fig. 3*B*). Consistent with the WT-Luc result (Fig. 2*A*), TCF1 or LEF1 significantly suppressed Tax-induced 5′ LTR activation (Fig. 3*C*). To evaluate the effect of TCF1/LEF1 upon HTLV-1 de novo infection, we cocultivated Jurkat or normal CD4 T cells with lethally irradiated MT-2 cells. Tax expression was detected predominantly in the TCF1/LEF1 low-expressing fraction (Fig. 3*D*), suggesting that TCF1/LEF1 restricts HTLV-1 de novo viral expression and its replication.

Tax Down-Regulates TCF1 and LEF1 via STAT5a. Antigen encounter or T-cell activation were reported to trigger TCF1/LEF1 down-regulation (15). We confirmed that phorbol myristate acetate (PMA)/ionomycin (P/I) stimulation down-regulate TCF1 and LEF1 in Jurkat and primary CD4 T cells (Fig. 4*A* and Fig. S5*A*). Therefore, we suspected that reduced expression of TCF1 and LEF1 in HTLV-1–infected cells is also caused by Tax, which is known to activate T cells (3). As expected, Tax induced the expression of the same activation markers as P/I stimulation (Fig. S5*B*), and suppressed the expression of TCF1 and LEF1 in Jurkat cells (Fig. 4*A*). Furthermore, cadmium-induced Tax expression in JPX-9, a modified Jurkat line that expresses Tax under a metallothionein promoter (20), also down-regulated TCF1 and LEF1 (Fig. 4*B*). However, Tax (Fig. S5*C*) did not inhibit transcription from the TCF1 and LEF1 promoters. To see whether the NFκB, NFAT, or AP1 pathways, the three major TCR downstream pathways, are involved in TCF1/LEF1 down-regulation (21), we activated them by electroporation of the corresponding transcription factors into Jurkat (Fig. S5*D*). However, neither single nor combined activation of these pathways clearly suppressed TCF1 or LEF1 expression (Fig. S5*E*). JAK/STAT signaling, a major cytokine pathway of T cells that becomes active following T-cell activation (22), has been found to be constitutively active in HTLV-1–infected T cells (23). Because STAT proteins are transcription factors that activate this pathway (24), we examined the effect of STAT5a, which is reported to be a target of Tax (25). First, we confirmed that STAT5a expression was induced upon P/I stimulation and Tax expression (Fig. 4*C*). Then we overexpressed either the wild type or the constitutively active form of STAT5a in Jurkat cells, and found significantly decreased expression of TCF1 and LEF1 (Fig. 4*D*).

Higher Expression of TCF1 and LEF1 Is Associated with Low STLV-1 Proviral Load in Vivo. The above results suggest that Tax function and HTLV-1 replication are impaired in TCF1/LEF1 high expressing cells, most likely in thymocytes that express higher levels of TCF1/LEF1. To analyze the relationship between TCF1/LEF1 expression and proviral load (PVL) in vivo, a model of HTLV-1 infection was required. We have reported that

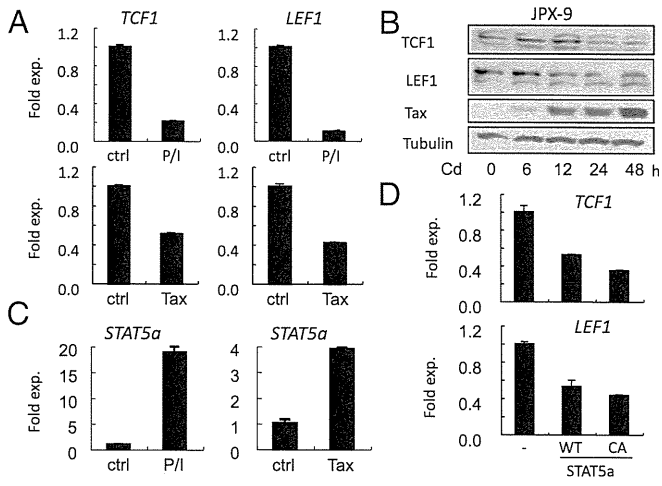

Fig. 4. Tax down-regulates the expression of TCF1 and LEF1 via STAT5a. (*A*) P/I stimulation (*Upper*) or Tax overexpression (*Lower*) inhibits TCF1/LEF1 transcription in Jurkat. For P/I stimulation, cells were treated with 50 ng/mL of PMA and 500 ng/mL of ionomycin (P/I) for 5 h and then subjected to RNA extraction and qPCR analysis. Overexpression of Tax was achieved by electroporation and 24 h later, RNA was extracted for qPCR. (*B*) Tax induction in JPX-9 down-regulates the expression of TCF1 and LEF1. JPX-9 was cultured in RPMI supplemented with 20 μM of cadmium (Cd) to induce Tax expression. At indicated time points, cells were lysed for Western blot analysis. (*C*) P/I stimulation or Tax overexpression induces STAT5a expression in Jurkat. P/I stimulation and Tax overexpression were performed as in *A*. (*D*) Overexpression of STAT5a down-regulates TCF1 and LEF1. Jurkat was transfected with wild type (WT) or constitutively active (CA) STAT5a by electroporation. 24 h later, RNA was extracted for qPCR. Fold exp. indicates fold expression.

STLV-1 encoded Tax and STLV-1 bZIP factor (SBZ) possess functions similar to those of HTLV-1 Tax and HBZ, and an STLV-1–infected Japanese macaque developed T-cell lymphoma (26), indicating that STLV-1–infected Japanese macaques can serve as a suitable model of HTLV-1 infection. STLV-1 Tax is highly homologous to HTLV-1 Tax (26). Similar to HTLV-1 Tax, it also has a typical PDZ-binding motif (ETDV) in its C-terminal end. We sorted various T-cell subsets from an STLV-1–infected Japanese macaque (Fig. S6) and found that CD4+CD8+ thymocytes (T-DP) showed the highest expression levels of TCF1 and LEF1 (Fig. 5*A*) whereas their PVL was the lowest (Fig. 5*B*). This result is consistent with our hypothesis that TCF1 and LEF1 inhibit viral expansion through impairing both the function and expression of Tax (Fig. 2 and 3). CD4+ thymocytes (T-CD4) were about twofold higher in TCF1/LEF1 expression (Fig. 5*A*) than their counterparts in the periphery (P-CD4). However, the PVL of P-CD4 T cells was 10-fold higher than that of T-CD4 T cells (Fig. 5*B*). Similar measurements were made in thymic (T-CD8) and peripheral CD8 T cells (P-CD8) (Fig. 5*A* and *B*). Interestingly, only a 1.3-fold increase of PVL in P-CD8 over T-CD8 was observed, in contrast to a 10-fold increase in P-CD4 over T-CD4 (Fig. 5*B*). Along with the fact that thymic CD8 and CD4 T cells had similar PVLs, this observation implies a much smaller expansion of infected CD8 T cells in the periphery than of CD4 T cells, an observation in agreement with a previous report showing that HTLV-1's in vivo tropism is a consequence of predominant expansion of peripheral CD4 over CD8 T cells (27).

Next we compared the levels of transcriptional activity from the 5′ and 3′ LTRs of the provirus in STLV-1–infected cells. We did this by normalizing either Tax or SBZ transcription to PVL. Recall that TCF1/LEF1 regulate transcription of these genes in opposing manners (Fig. 3*B*). The 5′ LTR was clearly more active in peripheral CD4 or CD8 T cells than their thymic counterparts (Fig. 5*C*). In contrast, transcription from the 3′ LTR was more active in thymocytes, although the differences were not so big as with the 5′LTR (Fig. 5*D*). Memory (CD45RA-) CD4 T cells from the spleen of the STLV-1–infected Japanese macaque showed lower TCF1 and LEF1 expression but much higher PVL than naïve (CD45RA+) CD4 T cells (Fig. 5 *E* and *F*), which is in agreement with the fact that HTLV-1–infected cells have mostly a memory phenotype (28).

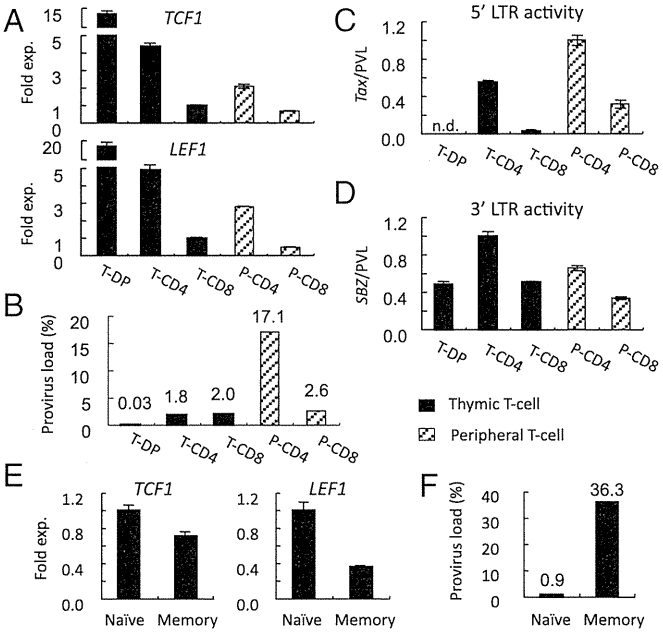

Fig. 5. TCF1 and LEF1 expression correlate negatively with STLV-1 proviral load in vivo. (*A*) TCF1 (*Upper*) and LEF1 (*Lower*) transcription in sorted CD4/CD8 double positive (T-DP), CD4 single positive (SP) thymocytes (T-CD4), CD8 SP thymocytes (T-CD8) and peripheral CD4 (P-CD4) and CD8 (P-CD8) SP T-cells from an STLV-1–infected Japanese macaque, determined by qPCR. (*B*) Genomic DNAs of sorted T cells were analyzed for STLV-1 proviral load. Numerals indicate number of virus copies in 100 cells. (C) Relative 5′ LTR activity of infected T cells. Tax mRNA expression was normalized to PVL of the same subset to represent relative transcription efficiency from 5′ LTR. (*D*) Relative 3′ LTR activity of infected T cells determined by normalizing SBZ mRNA expression to PVL. (*E*) TCF1 (*Left*) and LEF1 (*Right*) mRNA expression in sorted CD3+CD4+CD45RA+ (naive) and CD45RA- (memory) T cells from the STLV-1–infected Japanese macaque, determined by qPCR. Fold exp. indicates fold expression. (*F*) Genomic DNAs of naïve and memory T cells were analyzed for STLV-1 proviral load. Numerals indicate number of virus copies in 100 cells.

Discussion

During coevolution between virus and the host, host cells acquire many restriction factors that suppress viral replication (29, 30). HTLV-1 is derived from STLV-1 in monkeys, just like HIV-1 is derived from SIV. Many restriction factors have been reported for HIV-1 (31). However, restriction factors for HTLV-1 have not been studied extensively. It has been reported that APOBEC3G suppresses replication of HTLV-1 whereas Gag protein inhibits incorporation of APOBEC3G into the virion (32). Recently, SAMHD1 has been reported to suppress replication of HTLV-1 in monocytes (33). Tax is indispensable for HTLV-1 replication because expression of most viral genes, including all HTLV-1 structural genes, depends on transcription from the 5′ LTR that is activated by Tax. Moreover, Tax also plays a key role in dysregulating the cellular environment toward one which favors viral propagation, such by activation and transformation of an infected T cell (2). It is presumed that the T-cell tropism of HTLV-1 is more likely determined by postinfection events triggered by the virus because viral receptors are expressed in a wide variety of host cells (34). This study suggests that TCF1 and LEF1 are factors that restrict the tropism of this virus to peripheral T cells. In thymocytes expressing high levels of TCF1 and LEF1, these factors impair the functions of Tax, likely hindering not only viral replication but also the proliferation of the infected cells.

Restriction of tropism to peripheral T cells is likely a useful adaptation for HTLV-1. If HTLV-1 could replicate efficiently in the thymus, it might cause serious damage to the host immune system and thus the host. Furthermore, this virus is transmitted via breast-feeding or sexual transmission through infected T cells, so infected T cells must enter breast milk or semen. Most T cells in breast milk are peripheral T cells with an effector/memory phenotype (35). Restriction by TCF1/LEF1 would explain viral tropism to peripheral T cells and facilitate transmission of the virus.

Neoplasm of immature T cells has not been reported in HTLV-1–infected individuals. However, transgenic expression of Tax in the thymus induced immature T-cell lymphomas (36). These findings suggest that overexpression of Tax is oncogenic even for thymocytes, but that Tax expression or functions are normally impaired in the thymus of infected individuals. This study presents a mechanism for how thymocytes are relatively resistant to HTLV-1 infection and leukemogenesis in vivo, by identifying TCF1 and LEF1 as antagonists for Tax. We discovered an unexpected Wnt-independent role of TCF1 and LEF1 as Tax antagonists and demonstrated that this antagonism renders thymocytes less permissive for HTLV-1 replication compared with peripheral T cells.

The roles of TCF1/LEF1 have been well established in the thymus; they are indispensable in driving T-cell development (37). Nevertheless, their functions in the periphery remain

unknown. Recent studies showed that down-regulation of TCF1/LEF1 always occurs in activated or differentiated peripheral T cells (38). HTLV-1 may exploit this down-regulation to achieve its expansion, because down-regulation of TCF1/LEF1 allows Tax to execute its functions. A previous report also indicated that preactivated primary T cells are easier to transform by HTLV-1 (39). Down-regulation of TCF1/LEF1 upon T-cell activation/differentiation would allow Tax expression and subsequent HTLV-1 expansion.

Down-regulation of TCF1/LEF1 also occurs as T cells develop or differentiate, from DP to SP in the thymus (Fig. 5*A*), or from naïve to memory in the periphery (Fig. 5*E*). Therefore, our results also imply an interesting possibility that HTLV-1 might achieve its expansion as infected T cells differentiate or even by driving differentiation of infected T cells to reduce TCF1/LEF1 expression. Indeed, a recent report using humanized mice showed altered T-cell development upon HTLV-1 infection in that the mature SP population, instead of immature DN or DP, becomes dominant in the thymus (40). This finding suggests that thymocytes are propelled to develop by HTLV-1 or the virus selectively expands in the more differentiated subsets. Similarly, in a previous study of peripheral T cells, we demonstrated that HTLV-1–infected T cells were mostly memory cells and the number of naïve cells was significantly decreased (28). Our current results also reveal the preferential infection of CD4 effector/memory T cells by STLV-1. However, to clarify the roles of T-cell development/differentiation in contributions of HTLV-1 expansion, further studies are needed.

STLV-1–infected Japanese macaque has been demonstrated to be a suitable model for HTLV-1 infection (26). It also served as an ideal model to analyze the impact of the antagonism of TCF1/LEF1 against Tax in vivo. However, due to the complexity of viral infections in vivo, other factors such as the susceptibility to viral infections, postinfection mitotic potential and cytotoxic T-cell killing efficiency might affect the consequence of an infection in a specific T-cell subset. Indeed, the tropism of the virus for peripheral CD4 T cells over peripheral CD8 T cells does not appear to be explained by TCF1/LEF1 levels. More detailed investigations in STLV-1–infected Japanese macaques are expected to clarify these points in the future.

TCF1/LEF1 regulate the HTLV-1 5′ and 3′ LTR activities in opposing manners via their interplay with Tax (Fig. 3*C*). This may result in distinct expression levels of Tax and HBZ in vivo in different T-cell subsets or during various stages of infection. Interestingly, valproate, a histone deacetylase inhibitor, was reported to induce Tax expression while suppressing that of HBZ (41). These intriguing observations that the HTLV-1 5′ and 3′ LTR are regulated in opposite ways by multiple mechanisms, in addition to frequently observed contradictory functions of Tax and HBZ, may suggest a complex but fine-tuned viral pathogenesis. For instance, although activation of NF-κB pathway has been considered a critical function of Tax for cellular transformation (2, 3), the recent studies have reported that hyperactivation of NF-κB pathway induces cellular senescence whereas HBZ suppresses this action of Tax, thereby enabling clonal expansion (42). This study shows that TCF1/LEF1 inhibit Tax-mediated NF-κB activation by direct binding to Tax. Furthermore, TCF1/LEF1 inhibit various functions of Tax, whereas HBZ selectively modulates signaling pathways (43, 44). Thus, Tax and HBZ collaboratively function for clonal expansion and viral replication, whereas TCF1/LEF1 inhibit functions of Tax by direct interaction, which leads to suppression of viral replication and proliferation of infected cells.

In summary, we here identify TCF1 and LEF1 as previously unidentified Tax antagonists that likely restrict viral expansion in the thymus. The critical interplay of TCF1 and LEF1 with Tax during HTLV-1 infection may shed light on how HTLV-1 achieves its tropism and persistence in peripheral T cells in vivo.

Materials and Methods

Primary Samples Ethics Statement. The experiments using primary samples in this study were conducted according to the principles expressed in the Declaration of Helsinki. This study was approved by the Institutional Review Board of Kyoto University (approval numbers G310 and E2005). All ATL patients and healthy individuals provided written informed consent for the collection of samples and subsequent analysis. A Japanese macaque used in this study was 3 y old and naturally infected with STLV-1. The monkey was reared in the Primate Research Institute, Kyoto University. All animal studies were conducted in accordance with the protocols of experimental procedures approved by the Animal Welfare and Animal Care Committee of the Primate Research Institute (approval number 2011–095).

Cell Lines. ATL-derived T-cell lines (HPB-ATL-2, HPB-ATL-T, ATL-43T-, ATL-43T+, ATL-55T+, ED, MT-1, and TL-Om1), HTLV-1–transformed T-cell lines (ATL-35T, Hut102, MT-2, and MT-4) were used in this study. Jurkat, CEM, Hut78, SupT1, Molt4, and Kit225 are HTLV-1–negative T-cell lines. All T-cell lines were maintained in RPMI supplemented with 10% (vol/vol) FBS, whereas Kit225, ATL-43T+, and ATL-55T+ were maintained in the media supplemented with 100 U/mL of recombinant IL-2. 293FT (Life Technologies) is a subline of HEK293, which originated from a human embryonic kidney cell.

Plasmids. Expression vectors for TCF1, LEF1, and Tax were described (16, 45). Flag-CREB was made by subcloning the CREB coding sequence into pCAG-Flag. WT-Luc and 5′ LTR-Luc were kind gifts from J. Fujisawa, Kansai Medical University, Osaka. pX1MT-M was a generous gift from D. Derse, National Cancer Institute, Frederick, MD. NFκB-Luc and AP1-Luc were purchased from Stratagene. 3′ LTR-Luc was described (19).

Antibodies. Rabbit monoclonal antibodies for TCF1 (C63D9) and LEF1 (C12A5) were purchased from Cell Signaling Technology. HRP conjugated mouse anti-HA (12A5) antibody was purchased from Sigma. Mouse monoclonal antibodies against HTLV-1 gp46, p24, and p19 were purchased from Zeptometrix. Mouse monoclonal antibodies for FLAG (M2), Myc (9E10), α-tubulin (DM1A), and Tax (MI73) were described (16). For flow cytometric analysis of cell surface markers, APC-Cy7 anti-CD3 (SP34-2), PerCP-Cy5.5 anti-CD4 (OKT4), V500 anti-CD8 (RPA-T8), and PE anti-CD45RA (5H9) were used. PerCP-Cy5.5 anti-CD4 (OKT4) was purchased from Biolegend, whereas the others were from BD.

Detection of Tax and TCF1/LEF1 by Flow Cytometry. Intracellular staining for Tax and TCF1/LEF1 was performed using the kit from eBioscience. DyLight 649 conjugated donkey anti-rabbit IgG and FITC conjugated goat anti-mouse IgG were purchased from Biolegend. Normal mouse IgG was purchased from Santa Cruz and used for blocking nonspecific binding.

ELISA. Supernatants from cultured cells were centrifuged at 1,710 × *g* for 5 min to remove debris and then diluted and quantified for p19 by ELISA (Zeptometrix) according to manufacturer's instructions.

Sorting by FACS Aria II. See Fig. S6 for details.

Electroporation, real-time PCR, knockdown, Western blot, coimmunoprecipitation, and reporter assays were performed as described (16).

ACKNOWLEDGMENTS. We thank Drs. J. Fujisawa and D. Derse for providing reagents and Dr. L. Kingsbury for proofreading. We appreciate the help from Dr. Tani-ichi for cell sorting. This study was supported by a Grant-in-aid for Scientific Research on Innovative Area from the Ministry of Education, Science, Sports, and Culture of Japan (to M.M.) (22114003), and a grant from the Japan Leukemia Research Fund (to M.M.). This study was conducted by the Cooperation Research Program of the Primate Research Institute, Kyoto University.

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Human T-cell leukemia virus type 1 Tax oncoprotein represses the expression of the BCL11B tumor suppressor in T-cells

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Key words
Adult T cell leukemia, BCL11B, HBZ, HTLV-1, Tax

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Funding Information
Ministry of Education, Culture, Sports, Science and Technology of Japan, Promotion of Niigata University Research Project, National Institutes of Health (CA100730).

Received September 28, 2014; Revised January 7, 2015; Accepted January 17, 2015

Cancer Sci **106** (2015) 461–465

doi: 10.1111/cas.12618

Human T-cell leukemia virus type 1 (HTLV-1) is the etiological agent of adult T cell leukemia (ATL), which is an aggressive form of T-cell malignancy. HTLV-1 oncoproteins, Tax and HBZ, play crucial roles in the immortalization of T-cells and/or leukemogenesis by dysregulating the cellular functions in the host. Recent studies show that HTLV-1-infected T-cells have reduced expression of the BCL11B tumor suppressor protein. In the present study, we explored whether Tax and/or HBZ play a role in downregulating BCL11B in HTLV-1-infected T-cells. Lentiviral transduction of Tax in a human T-cell line repressed the expression of BCL11B at both the protein and mRNA levels, whereas the transduction of HBZ had little effect on the expression. Tax mutants with a decreased activity for the NF-κB, CREB or PDZ protein pathways still showed a reduced expression of the BCL11B protein, thereby implicating a different function of Tax in BCL11B downregulation. In addition, the HTLV-2 Tax2 protein reduced the BCL11B protein expression in T-cells. Seven HTLV-1-infected T-cell lines, including three ATL-derived cell lines, showed reduced BCL11B mRNA and protein expression relative to an uninfected T-cell line, and the greatest reductions were in the cells expressing Tax. Collectively, these results indicate that Tax is responsible for suppressing BCL11B protein expression in HTLV-1-infected T-cells; Tax-mediated repression of BCL11B is another mechanism that Tax uses to promote oncogenesis of HTLV-1-infected T-cells.

Human T-cell leukemia virus type 1 (HTLV-1) is a causative agent of adult T-cell leukemia (ATL), which is characterized as an aggressive mature T-cell leukemia. HTLV-1 has two oncoproteins, Tax and HTLV-1 bZIP factor (HBZ), both of which play crucial roles in persistent HTLV-1 infection and leukemogenesis.[1–3] Tax by itself immortalizes primary human T-cells *in vitro*, and *tax*-transgenic mice develop various malignancies, including mature T-cell leukemia. To achieve these effects, Tax interacts with several cellular proteins involved in cell cycle regulation,[4,5] apoptosis,[6] genomic instability[7,8] and DNA repair,[6] and modulates their functions. For instance, Tax interacts with various transcription regulatory factors, including cAMP-responsive element-binding protein/activating transcription factor (CREB/ATF) and IKK, through which Tax activates the expression of a number of cellular genes.

Unlike Tax, HBZ is dispensable for T-cell immortalization by HTLV-1, but it was required for persistent HTLV-1 infection in an *in vivo* animal model.[9] In addition, HBZ-transgenic mice develop mature T-cell lymphoma.[10] HBZ also has multiple activities. For example, HBZ prevents apoptosis by promoting the anti-apoptotic function of FoxO3a.[11] HBZ upregulates the expression of the hTERT telomerase subunit gene,[12] and inhibits the transcriptional activation of cellular genes mediated by c-Jun, CREB/ATF and RelA.[13–15]

BCL11B is a transcriptional regulatory protein containing C2H2-type zinc fingers, and it is required for normal T-cell development.[16] Moreover, BCL11B has been shown to act as a tumor suppressor gene in T-cell acute lymphoblastic leukemia (T-ALL). Genetic alterations of BCL11B, such as by chromosomal rearrangements, have been identified in several T-ALL patients.[17] Intriguingly, HTLV-1-infected T-cells, including ATL cells, have been reported to have reduced BCL11B protein expression,[18] but the mechanism remains to be elucidated. In the present study, we show that the HTLV-1 oncoprotein Tax is sufficient to downregulate BCL11B expression in T-cells.

Materials and Methods

Cell lines and culture condition. SLB-1, HUT-102, MT-2 and MT-4 are HTLV-1-transformed human T-cell lines. TL-OmI, KK-1 and KOB are HTLV-1-positive ATL patient-derived cell lines. Jurkat and MOLT-4 are HTLV-1-negative T-cell lines.

These human T-cell lines were cultured in RPMI1640 medium supplemented with 10% FBS, 4 mM L-glutamine and antibiotics (RPMI/10%FBS). Recombinant human IL-2 (Takeda Chemical Industries, Osaka, Japan) was added at 0.5 nM to the cultures of KK-1 and KOB cells. 293T cells, which are highly transfectable kidney-derived cells, were cultured in DMEM supplemented with 10% FBS, 4 mM L-glutamine and antibiotics.

Plasmids. CSII-EF-Tax-IRES and CSII-EF-Tax-IRES-sHBZ were the IRES-mediated bicistronic lentiviral vectors for Tax and Tax together with spliced-HBZ (sHBZ), respectively. CSII-EF-IRES-GFP was used as a control vector. The lentiviral expression vectors for HTLV-1 Tax, its mutants (TaxΔC, TaxM22, Tax703, Tax[225–232]) and HTLV-2 Tax2B have been described previously.[19,20] Tax(TTG) has a mutation from A to T at the initiation codon of *tax* gene, and, thus, it expresses the Tax transcript, but not its protein. Tax(TTG) cDNA was constructed by introducing mutation with the PCR. Next, the Tax(TTG) cDNA was cloned into the pENTR/D-TOPO plasmid (Invitrogen, Carlsbad, CA, USA), and then the cDNA was transferred into lentiviral vector CSII-EF-IRES-GFP-RfA[20] through an LR recombination reaction using LR clonase (Invitrogen). The expression vector pHβPr-1-neo was used for the transient expression of Tax, Tax2B and its mutant proteins (TaxΔC, TaxM22, Tax703) in Jurkat cells in order to perform a lucifearase assay as described previously.[21,22] Tax (225–232) protein is defective for the activation of the non-canonical NF-κB/p100/p52 pathway, but it is active for the canonical NF-κB pathway,[19] and the expression vector was constructed by inserting the Tax(225–232) gene into the expression vector pHβPr-1-neo. The transcriptional activity reporter plasmids, κB-luc, CRE-luc and pGK/β-gal, have all been described previously.[22,23]

Lentivirus transduction. Recombinant lentiviruses were generated by transfecting pCAG-HIVgp, pCMV-VSV-G-RSV-Rev and the respective lentiviral vectors into 293T cells using FuGENE HD (Roche Diagnostic, Mannheim, Germany). Seventy-two hours after transfection, the culture supernatants were harvested, and were infected into Jurkat or MOLT-4 cells (4×10^5) at a final volume of 2 mL of RPMI/10%FBS containing 8 μg/mL polybrene.

Quantitative real-time RT-PCR. Total RNA was isolated from cells using the NucleoSpin RNA II Kit (MACHEREY-NAGEL; TaKaRa, Shiga, Japan), and RNA was reverse-transcribed using the PrimeScript RT reagent kit (TaKaRa). BCL11B cDNA fragments were amplified and analyzed by the real-time PCR assay performed using SYBR Green Real-Time PCR Mix (TOYOBO, Osaka, Japan) with a 25 μL reaction volume. The quantity of BCL11B mRNA was normalized to the quantity of GAPDH mRNA.

Western blot analysis. The western blot analyses were carried out as described previously.[24] The primary antibodies used at the indicated dilutions were anti-BCL11B (1/1000),[16] anti-BCL11B (1/1000) (Cell Signaling Technology, Beverly, MA, USA), anti-Tax (1/2000) (Taxy7),[25] anti-Tax2,[26] anti-α-tubulin (DM1A) (1/1000) (Calbiochem, San Diego, CA, USA) and α-HBZ (1/1000).[9] Horseradish peroxidase (HRP)-conjugated rat anti-mouse IgG kappa light chain (BD Biosciences, San Jose, CA, USA) and HRP-conjugated goat anti-rabbit IgG (sc-2054; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as secondary antibodies (1/10 000). Immunoreactive bands were visualized with the Enhanced Chemiluminescence Detection System (Amersham Pharmacia Biotech, Orsay, France).

Luciferase assay. Jurkat cells (2.0×10^5) in 12-well plates were cotransfected with the expression vector pHβPr-1-neo encoding Tax, TaxΔC, TaxM22, Tax703, Tax(225-232) or Tax2B, together with pGK/β-gal and either κB-luc or CRE-luc using TransFectin (Bio-Rad Technologies) according to the manufacturer's instructions. At 48 h after transfection the cell lysates were prepared, and the luciferase and β-galactosidase activities in the lysates were measured using both the Luciferase Assay System (Promega, Fitchburg, WI, USA) and the Galacto-Light System (Applied Biosystems, Foster City, CA, USA), respectively.

Statistical analysis. Differences in the gene expression levels between the cell lines were analyzed using unpaired Student's *t*-tests, and were presented as the means ± SD.

Results

Kurosawa *et al.*[18] showed that the BCL11B expression levels in HTLV-1-infected cells were downregulated relative to those in HTLV-1-infected cells. To elucidate the mechanism responsible for this, we measured the expression of BCL11B in various human T-cell lines, including HTLV-1-transformed and ATL-derived cell lines by performing western blot analyses and RT-PCR assays. Consistent with the previous study, the amounts of BCL11B protein and mRNA in the HTLV-1-transformed and ATL-derived cell lines were lower than those in HTLV-1-uninfected cell lines (Fig. 1). The extent of BCL11B mRNA downregulation in HTLV-1-transformed cells, except for the MT-4 cells, was higher than that in the ATL cells. While the ATL-derived cells expressed the HBZ protein at a level equivalent to that in HTLV-1-transformed cells, they expressed no or very little Tax protein (Fig. 1a), which is typical of ATL-derived cell lines. Therefore, the present results indicate that Tax, either alone or together with HBZ, regulates BCL11B expression in HTLV-1-infected cells.

To assess this possibility, we transiently expressed Tax and the spliced form of HBZ (sHBZ) in an HTLV-1-uninfected T-cell line (Jurkat) expressing a relatively high amount of BCL11B by using lentiviral vectors (Fig. 2a,b). Tax reduced the amount of BCL11B protein in the Jurkat cells (Fig. 2b), whereas sHBZ had little effect on its expression amount (Fig. 2a). Tax also reduced the amount of BCL11B protein in another human T-cell line, MOLT-4 (Fig. 2c). The RT-PCR analysis elucidated that Tax represses BCL11B expression at the mRNA level in Jurkat cells (Fig. 2d). These results indicated that Tax represses the expression of BCL11B in HTLV-1-infected T-cells.

To explore the mechanism by which Tax regulates BCL11B repression, we transiently expressed several Tax mutants in Jurkat cells using lentiviral vectors. Similar to the results obtained for wild type Tax, four Tax mutants (TaxΔC, TaxM22, Tax703 and Tax[225–232]) reduced the expression of the BCL11B protein in Jurkat cells, whereas Tax(TTG), a Tax mutant without an initiation codon, did not show such reduction. Moreover, HTLV-2 Tax2 also reduced the expression of the BCL11B protein. A luciferase reporter assay showed that Tax, TaxΔC and Tax(225–232) equivalently activated the promoter activity under the control of κB-enhancer as well as the CRE (c-AMP responsive element) enhancer in the HTLV-1 promoter (Fig. 3c,d). In contrast, TaxM22 only slightly stimulated the NF-κB-dependent transcription. Tax703 activated CRE, but it was less effective than Tax. It should also be noted that Tax(225–232) has a selective defect for non-

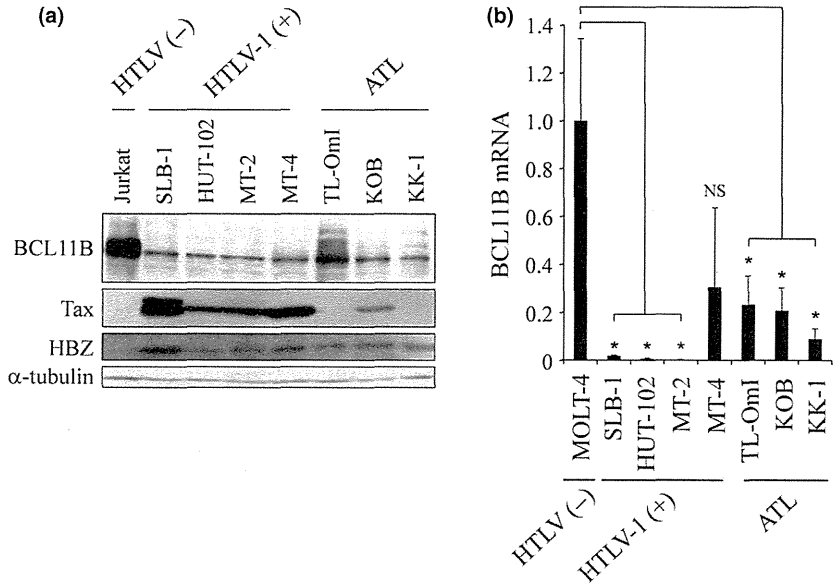

Fig. 1. The downregulation of BCL11B expression in HTLV-1-infected T-cell lines. (a) Cell lysates were prepared from the indicated human T-cell lines, and the expression levels of BCL11B, Tax, HBZ and α-tubulin in the cell lysates were measured by western blot analysis. (b) Total RNA was prepared from the indicated human T-cell lines, and the amounts of BCL11B mRNA were measured by quantitative real-time RT-PCR. The quantity of BCL11B mRNA was normalized to the quantity of GAPDH mRNA. $*P < 0.05$. ATL, adult T cell leukemia; HTLV-1, human T-cell leukemia virus type 1; NS, not significant.

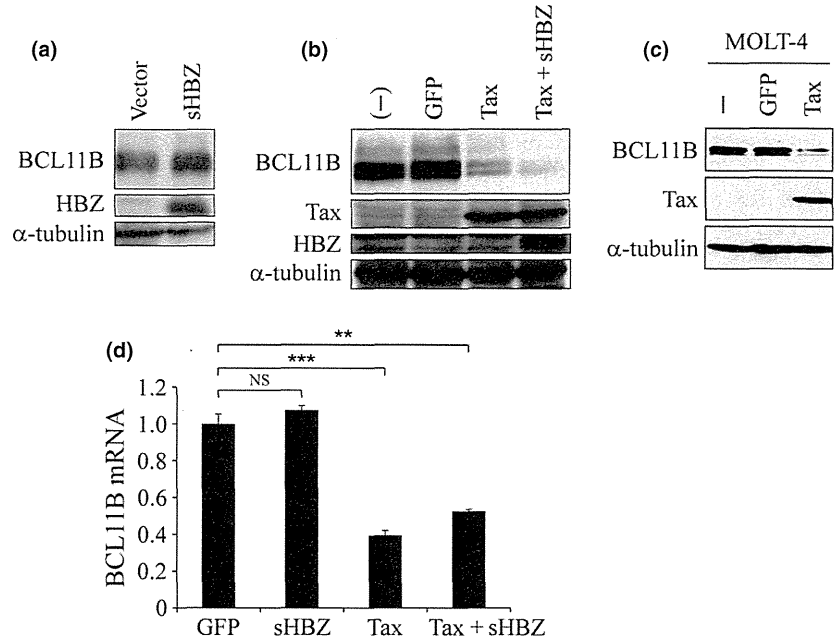

Fig. 2. Tax represses BCL11B expression in T-cells. (a–c) Cell lysates were prepared from Jurkat (a, b) or MOLT-4 cells (c) infected with the indicated lentiviruses, and the expression of BCL11B, Tax, HBZ and α-tubulin in the cell lysates was examined by western blot analysis. A lentivirus expressing the GFP protein (GFP) was used as a control. (d) Total RNA was prepared from Jurkat cells infected with the indicated lentiviruses, and the amounts of BCL11B mRNA in the cells were measured by quantitative real-time RT-PCR. The quantity of BCL11B mRNA was normalized to the quantity of GAPDH mRNA. $**P < 0.01$; $***P < 0.001$. NS, not significant.

canonical κB-dependent transcriptional activation,[19] and TaxΔC has a deletion of the PDZ-domain binding motif (PBM).[27] These results suggested that the κB-dependent and CRE-dependent transcriptional activation, as well as PBM-mediated signaling of Tax, are dispensable for the repression of BCL11B.

Discussion

The previous study showed that both HTLV-1-transformed T-cells and ATL-derived cell lines have reduced expression of BCL11B.[18] In this study, we found that Tax is a major factor associated with the BCL11B downregulation in HTLV-1-transformed T-cells, and this downregulation mainly occurred at the transcriptional level. Together with the known roles of BCL11B in normal T-cell development and as a tumor suppressor in T-cell leukemia, the present study suggests that the downregulation of BCL11B by Tax plays a role in T-cell transformation by HTLV-1 and persistent HTLV-1 infection.

How does the Tax-induced downregulation of BCL11B alter the phenotypes of HTLV-1-infected cells? Kurosawa *et al.*[18] showed that the exogenous expression of BCL11B in two ATL-derived cell lines reduced their growth, but the expression did not affect the growth of Jurkat cells (a reduced level of the BCL11B protein is associated with adult T-cell leukemia/lymphoma). These results suggest that the downregulation of BCL11B can specifically promote the growth of HTLV-1-infected cells.

Like HTLV-1 Tax, the HTLV-2 Tax2 protein repressed the expression of the BCL11B protein (Fig. 3a). Tax2 is essential for the immortalization of primary human T-cells by HTLV-2 *in vitro*.[28] Thus, the BCL11B repression by Tax2 proteins may also play a role in HTLV-2 immortalization of T-cells. Tax and Tax2 share many activities, including NF-κB

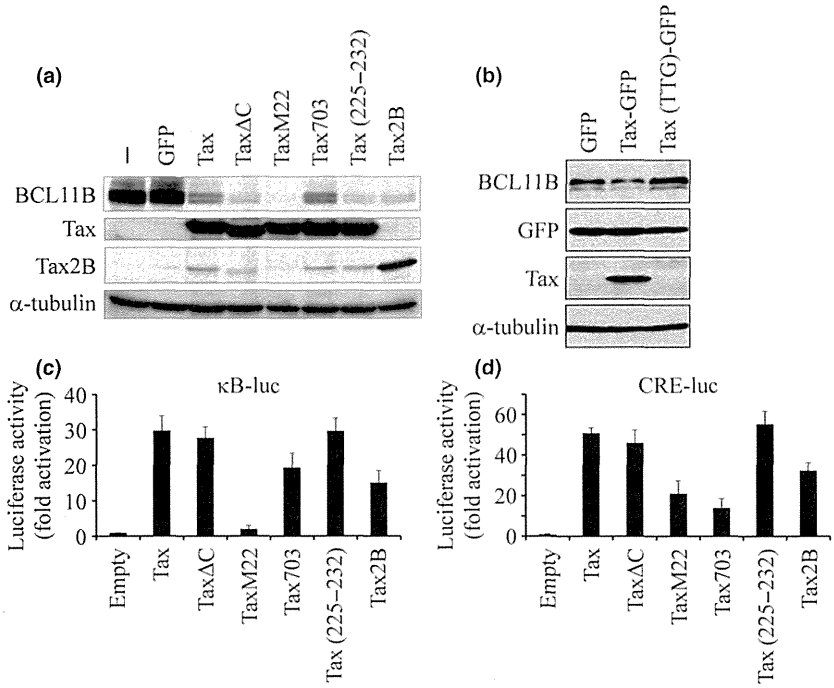

Fig. 3. Tax mutants also reduced the expression of BCL11B in T-cells. (a, b) Cell lysates were prepared from Jurkat cells infected with the indicated lentiviruses, and the expression levels of BCL11B, Tax, Tax2B, GFP and α-tubulin in the cell lysates were measured by western blot analysis. A lentivirus expressing the GFP protein (GFP) was used as a control. Tax and Tax(TTG) lentiviruses coexpressed Tax and GFP protein by a IRES-mediated bicistronic transcript (b). (c, d) Jurkat cells (2.0×10^5) were cotransfected with the indicated expression vector encoding Tax or its mutant together with pGK/β-gal and either κB-luc (c) or CRE-luc (d) using TransFectin. At 48 h after transfection, the cell lysates were prepared, and then the luciferase and β-galactosidase activities in the lysates were measured. The luciferase activity was normalized by the activity of β-galactosidase, and the fold activity indicates the normalized luciferase activity of Tax-transfection relative to that of the control plasmid transfection.

activation.[29] The four Tax mutants examined herein still repressed the expression of the BCL11B protein in T-cells (Fig. 3). Therefore, a further analysis is required to determine how Tax represses the expression of BCL11B in T-cells.

The BCL11B gene shows T lineage-specific expression, including CD4-positive T cells. Li *et al.*[30] identified the long-range tissue-specific transcriptional regulatory elements in the BCL11B gene, and showed that both the promoter region containing a transcription initiation site and the 1.9 kb region 850 kb downstream of the BCL11B gene are required for T lineage-specific expression. The 1.9 kb region in the BCL11B gene contained several putative transcription factor binding sites, and these TCF-1 binding sites were required for the T lineage-specific expression of BCL11B in the reporter assay.

While the three ATL cell lines possessed reduced amounts of BCL11B expression, one out of three ATL cell lines expressed a low amount of Tax protein, indicating that the downregulation of BCL11B expression in ATL cells is generally Tax-independent. Kurosawa *et al.*[18] failed to detect DNA methylation of the BCL11B locus. Therefore, the mechanism underlying BCL11B downregulation in ATL cells remains to be elucidated.

Several leukemogenic events have been shown to be initially set in motion by Tax in HTLV-1-infected cells *in vivo*, but once such cells become transformed, then a Tax-independent mechanism may subsequently continue the leukemic process. For instance, primary ATL cells and ATL-derived cell lines without Tax expression demonstrate constitutive NF-κB activation, which is critical for the growth of leukemic cells and is a later stage step in the development of leukemia. Therefore, the downregulation of BCL11B by Tax in HTLV-1-infected cells sets the stage for development of ATL. Our data reveal a novel mechanism of Tax in repressing the expression of a tumor suppressor, BCL11B, in order to promote the continuous proliferation of HTLV-1-infected cells. Taken together, our findings support the idea that the downregulation of BCL11B plays a role in ATL development.

Acknowledgments

The authors thank Renaud Mahieux, William Hall, Hiroyuki Miyoshi and Toshio Kitamura for providing us with reagents. We thank Takeda Pharmaceutical Company for providing recombinant human IL-2. We also express our gratitude to Misako Tobimatsu for technical assistance. This work was supported in part by a grant-in-aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by a Grant for the Promotion of Niigata University Research Project to MF and by a grant from the National Institutes of Health (CA100730) to PLG.

Disclosure Statement

The authors have no conflict of interest to declare.

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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

Leukemia advance online publication, 30 January 2015; doi:10.1038/leu.2015.1

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

Accepted article preview online 8 January 2015

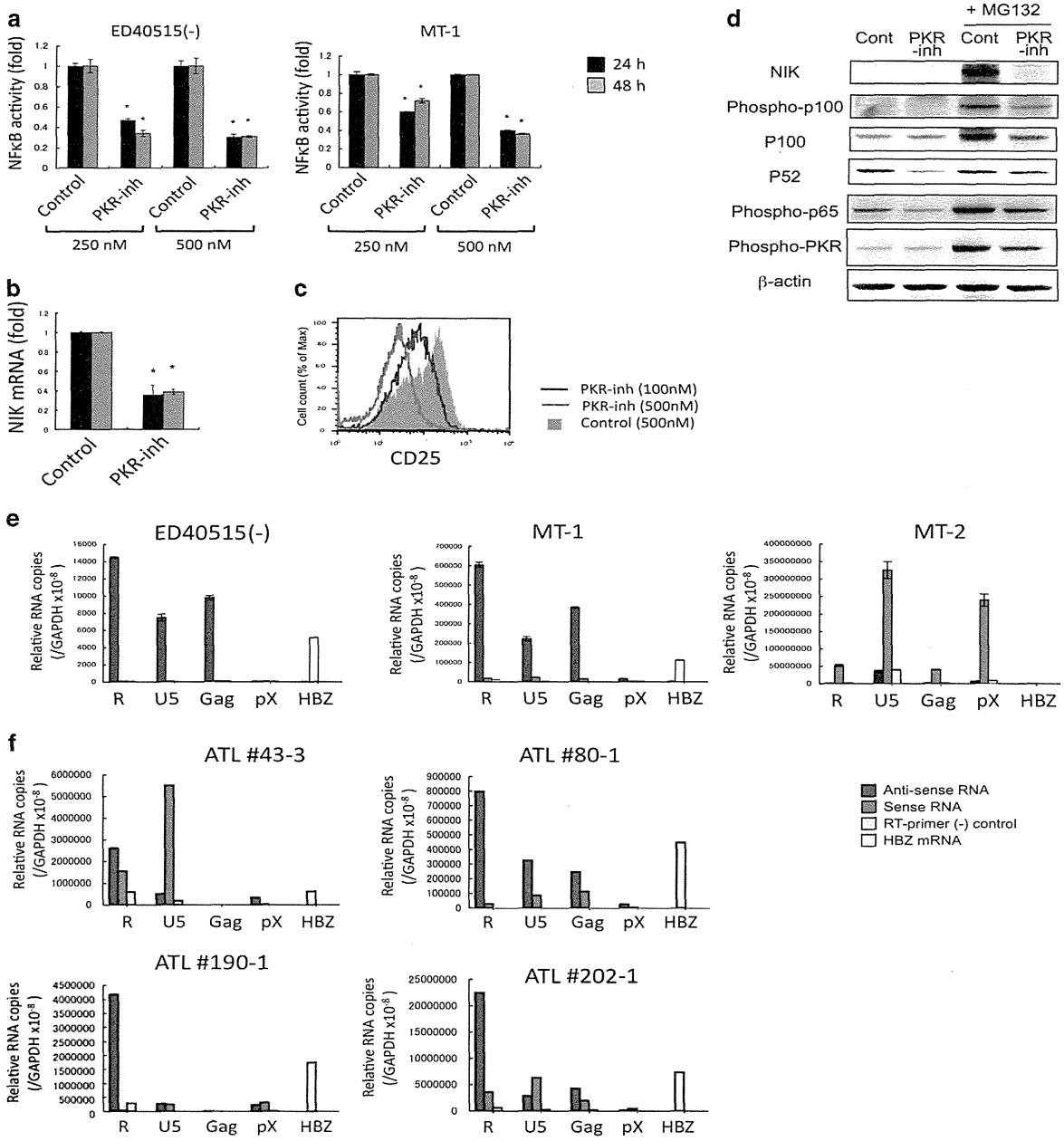
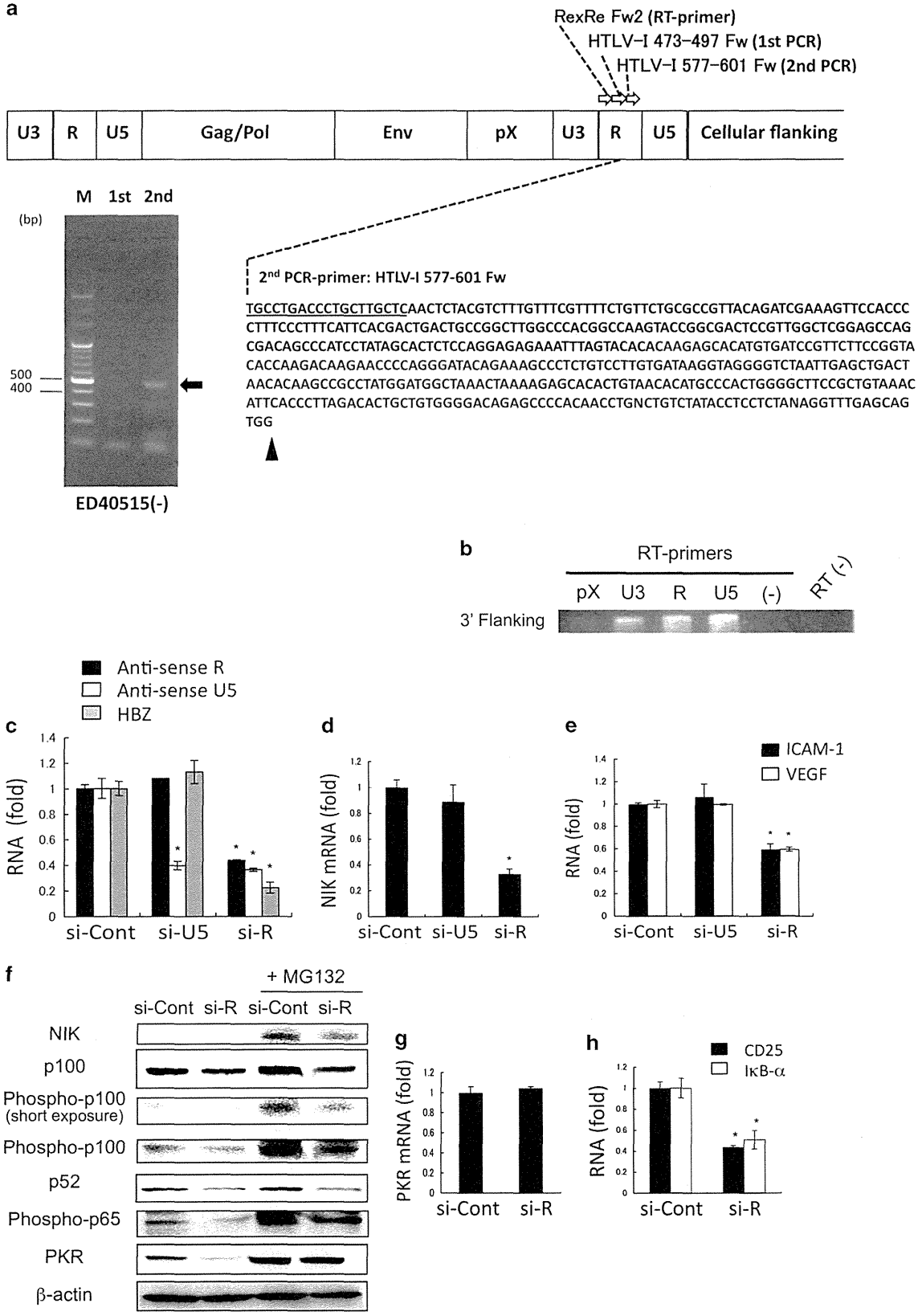


Figure 1. Suppression of NFκB activity by a PKR-inhibitor and the presence of antisense RNA at the HTLV-1 LTR in Tax-negative ATL cells. (**a**) ED40515(−) (left) and MT-1 (right) cells containing reporter genes for NFκB and the thymidine kinase (TK) promoter were incubated with a chemical PKR inhibitor (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.

ACKNOWLEDGEMENTS

This work was supported by Scientific Support Programs for Cancer Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan and the Ministry of Health, Labour and Welfare of Japan. We thank Dr Michiyuki Maeda (Kyoto University) for permission to use the ED40515(−) cells.

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

bjh research paper

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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Received 13 November 2014; accepted for publication 17 December 2014

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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

First published online 22 January 2015
doi: 10.1111/bjh.13302

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

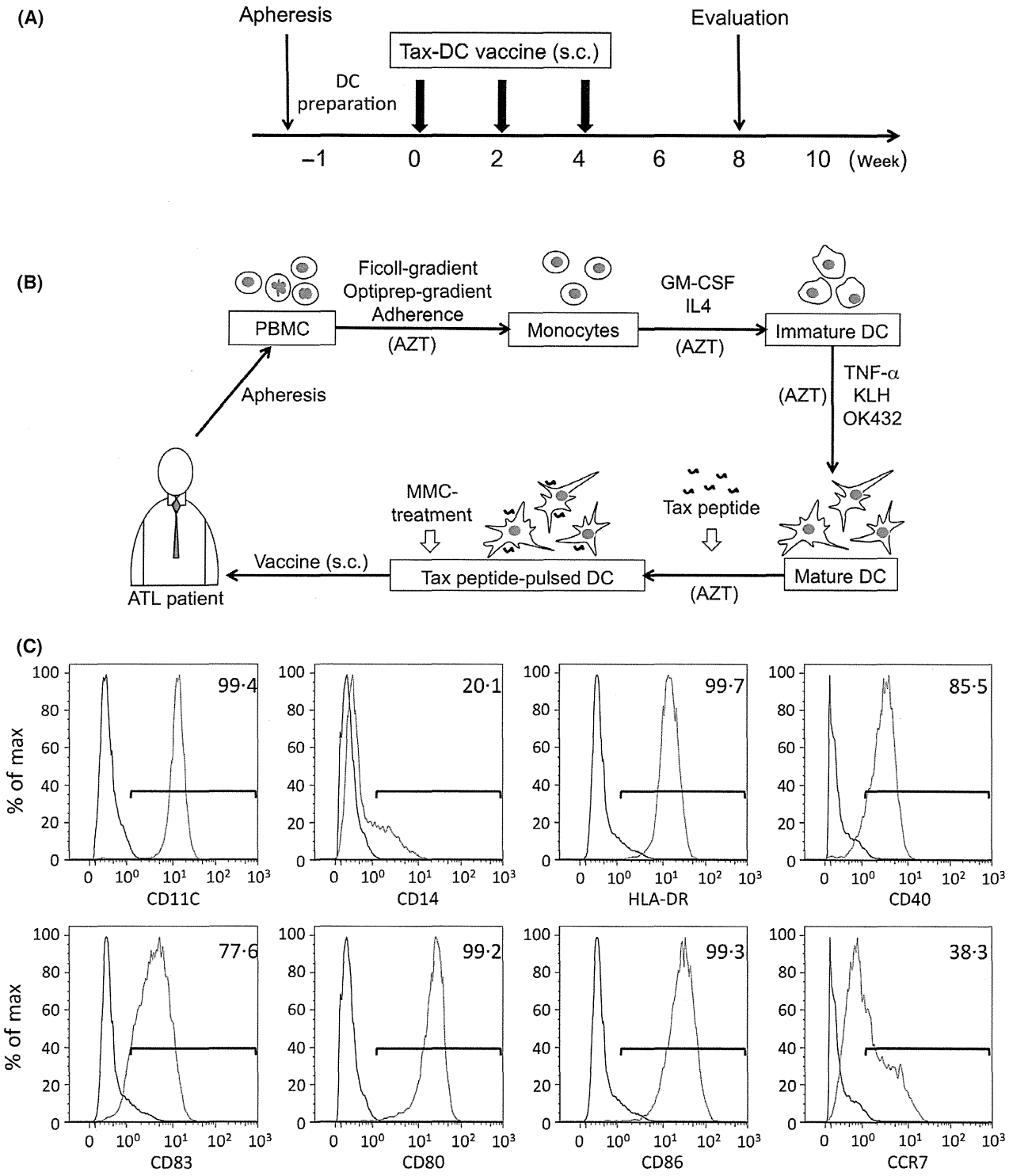

Fig 1. Outline of the Tax-DC vaccine therapy. (A) Schedule for the Tax-DC vaccine therapy. (B) Preparation of the monocyte-derived dendritic cells (DCs). Monocytes were enriched via serial density gradient centrifugation, and the adherent cells were cultured in the presence of granulocyte-macrophage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

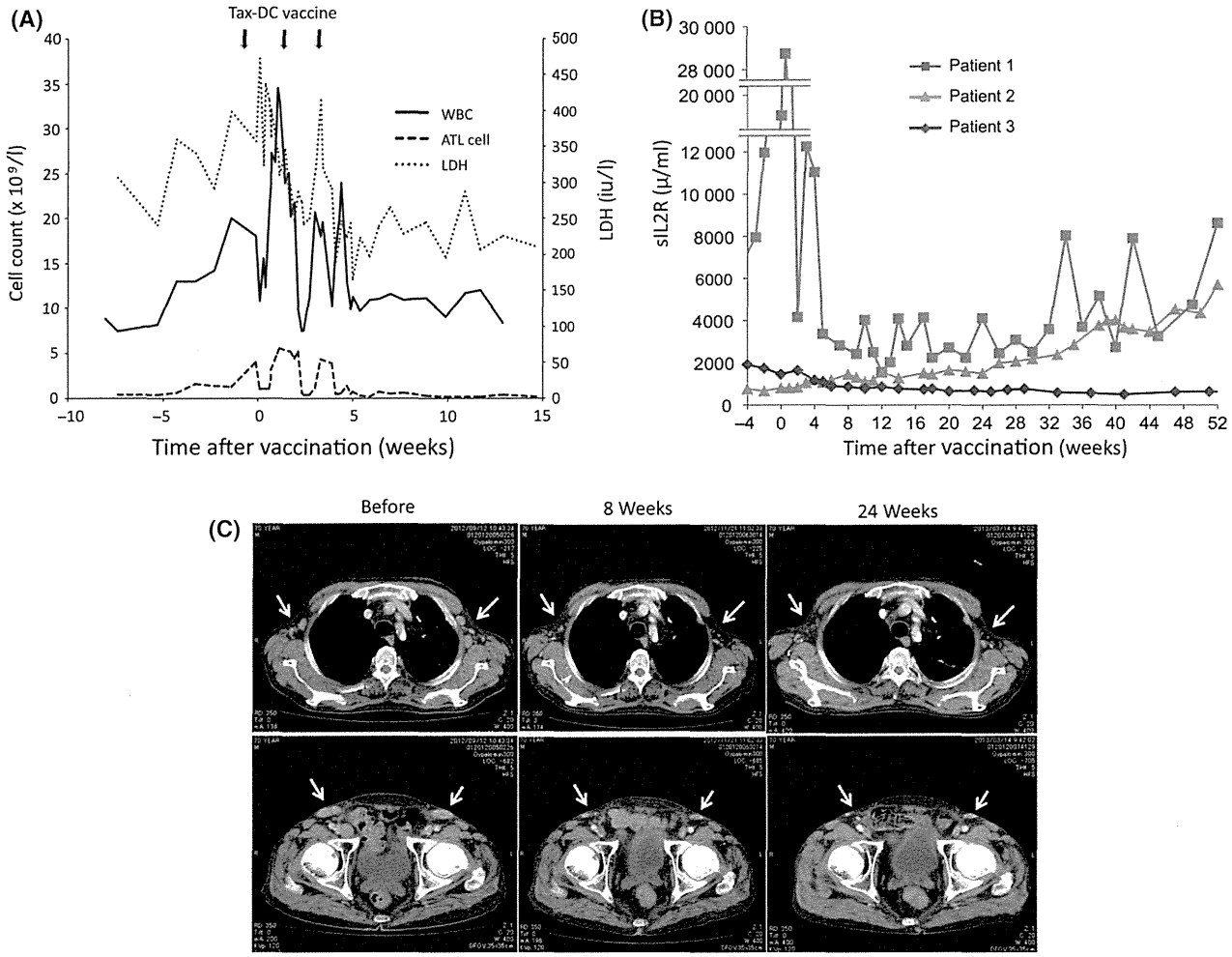

Fig 2. Clinical courses of the patients after the Tax-DC vaccine therapy. (A) Changes in the peripheral white blood cell count (WBC, solid line), ATL cell count (dashed line) and lactate dehydrogenase (LDH) level (fine dotted line) during the initial 15 weeks in Patient 1. The arrows indicate the days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