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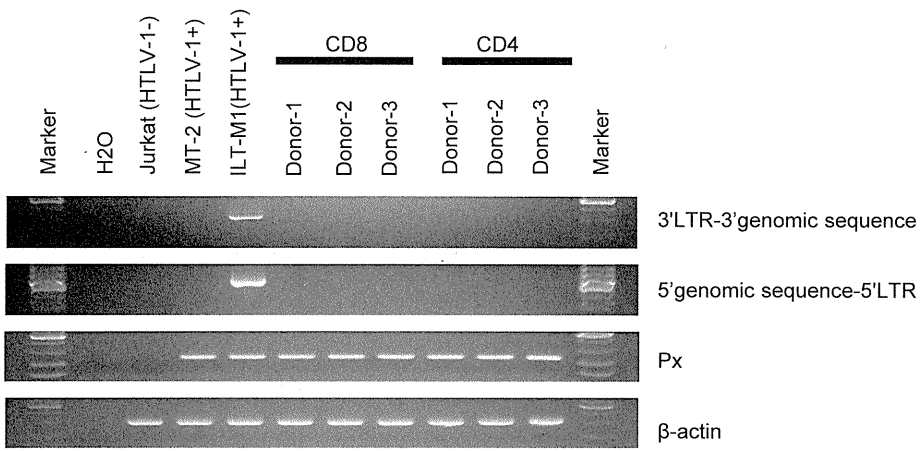
Supplemental Table 1

Sequences flanking the integration site of HTLV-1 provirus in ILT-M1 cell and primer sequences used for integration site-specific PCR.

5'-genomic region	5'-LTR	3'-LTR	3'-genomic region
TGCTTTGTCATCTGTGCGTTCAGTTCA	**TGACAATGACCATGAGCCCCAAATATC**	**TCCAGGAGAGAAACTTAGTACACA**	AGTTCACAGAGTTTCACCTTTCTCTTCA

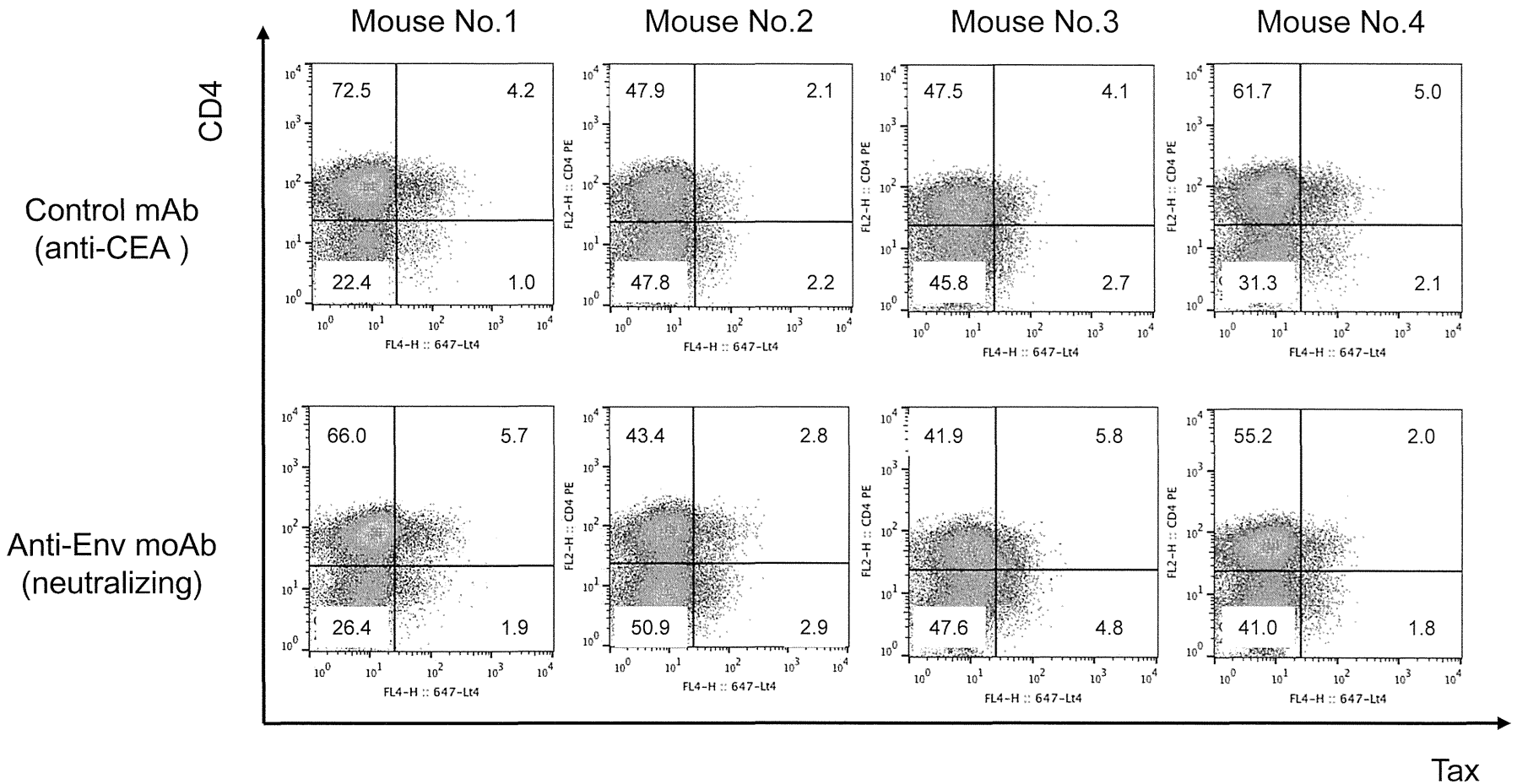
Forward Primer for the 5'-genomic region	Reverse Primer for the 5'-LTR	Forward Primer for the 3'-LTR	Reverse Primer for the 3'-genomic region
5'-TGCAGATTTCAAGCGCTTCTAGG-3'	5'-TTAGTCTGGGCCCTGACCTTTTCA-3'	5'-CAACTCTACGTCTTTGTTTCGT-3'	5'-GTAAATGAGAAATCCCGCTTCCA-3'

Flanking sequences of 5'- and 3'-LTR were determined by inverse-PCR.
HTLV-1 proviral sequences are shown in boldface.



To rule out the possible contamination of residual ILT-M1 cells, inverse PCR amplification was carried out to determine the sequences adjacent to HTLV-1 LTRs (both 3′- and 5′-LTR) using the DNA extracted from ILT-M1 cells, as previously described [13]. Then, integration site-specific PCR was carried out using primer pairs that encompass HTLV-1 LTRs (both 3′ and 5′ LTR) and flanking host sequences (Additional file 1: Table S1). As shown, no integration site specific bands were observed except for ILT-M1 cells, suggesting that the possible contamination of HTLV-1 genome derived from the residual ILT-M1 cells is unlikely.

Supplemental Figure 1



Flow cytometric studies showed that the human lymphocytes recovered from mouse spleens express the amount of Tax protein after short-term (16 h) cultivation ex vivo, indicating that the neutralizing anti-Env Ab (clone LAT-27) injection once after PBMC transplantation did not block the in vivo transmission of HTLV-1.

Supplemental Figure 2

TCF1 and LEF1 act as T-cell intrinsic HTLV-1 antagonists by targeting Tax

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Human T-cell leukemia virus type 1 (HTLV-1) is a delta-type retrovirus that induces malignant and inflammatory diseases during its long persistence in vivo. HTLV-1 can infect various kinds of cells; however, HTLV-1 provirus is predominantly found in peripheral CD4 T cells in vivo. Here we find that TCF1 and LEF1, two Wnt transcription factors that are specifically expressed in T cells, inhibit viral replication through antagonizing Tax functions. TCF1 and LEF1 can each interact with Tax and inhibit Tax-dependent viral expression and activation of NF-κB and AP-1. As a result, HTLV-1 replication is suppressed in the presence of either TCF1 or LEF1. On the other hand, T-cell activation suppresses the expression of both TCF1 and LEF1, and this suppression enables Tax to function as an activator. We analyzed the thymus of a simian T-cell leukemia virus type 1 (STLV-1) infected Japanese macaque, and found a negative correlation between proviral load and TCF1/LEF1 expression in various T-cell subsets, supporting the idea that TCF1 and LEF1 negatively regulate HTLV-1 replication and the proliferation of infected cells. Thus, this study identified TCF1 and LEF1 as Tax antagonistic factors in vivo, a fact which may critically influence the peripheral T-cell tropism of this virus.

HTLV-1 | Tax | TCF1 | LEF1

Human T-cell leukemia virus type 1 (HTLV-1) causes a malignancy named adult T-cell leukemia (ATL) and several inflammatory diseases including HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP) (1, 2). HTLV-1 encodes a critical transactivator, Tax, that induces the activation and subsequent clonal expansion of infected T cells in vivo (2, 3). Tax is transcribed from the viral promoter 5′ long terminal repeat (LTR), where it further enhances HTLV-1 viral transcription by recruiting cellular CREB protein to Tax-responsive elements (TRE). However, Tax expression is frequently silenced in ATL cells due to genetic and epigenetic changes in the viral 5′ LTR and the *tax* gene (4–7), a possible consequence of host immune surveillance (8). On the other hand, the viral 3′ LTR remains intact and is responsible for consistent expression of the HTLV-1 bZIP factor (HBZ), a negative strand encoded accessory gene, in all ATL cells (9).

T-cell factor 1 (TCF1) and lymphoid-enhancer binding factor 1 (LEF1) are transcription factors of the Wnt pathway that bind to β-catenin to coactivate the downstream cascade (10, 11). They are predominantly expressed in T-lineage cells, with immature thymocytes having the highest expression (12). Thymocyte development was impaired in TCF1 knockout mice (13). Although LEF1 knockout did not significantly affect T-cell development, deficiency in both TCF1 and LEF1 resulted in a complete block at the immature single positive stage, indicating a functional redundancy of TCF1/LEF1 and their indispensible role in driving T-cell development (14). In contrast, their functions in peripheral T cells remain poorly characterized although a quite different role has been suggested due to their reduced expression upon T-cell receptor (TCR) engagement in CD8 T cells (15).

HTLV-1 is peripheral mature T-cell tropic. However, the mechanism of this tropism remains to be elucidated. Here we find that TCF1 and LEF1 are T-cell intrinsic factors that suppress HTLV-1 replication via antagonizing Tax. They interact with Tax and suppress its transactivating abilities. As a result, viral transcription and replication are greatly suppressed by either TCF1 or LEF1, resulting in selective viral replication in TCF1/LEF1 low-expressing T cells. At the same time, Tax is able to down-regulate TCF1/LEF1 by inducing STAT5a expression. We further demonstrate that thymocytes from a simian T-cell leukemia virus type 1 (STLV-1) infected Japanese macaque have low viral abundance and low 5′ LTR activity, negatively correlating with their high expression of TCF1 and LEF1.

Results

TCF1/LEF1 Are Expressed at Low Levels in HTLV-1–Infected T Cells. Previously we reported that HBZ impaired the DNA-binding ability of TCF1/LEF1 and thereby suppressed the canonical Wnt pathway, shaping an HTLV-1 favorable host environment (16). Interestingly, upon further study, we found that TCF1 and LEF1 mRNA and protein levels were invariably low in HTLV-1–infected cell lines, in contrast to most HTLV-1–negative T-cell lines except Kit225 (Fig. 1 *A* and *B*). Fresh ATL cells exhibited reduced expression of TCF1 and LEF1 compared with CD4 T cells from a healthy donor (Fig. 1*C*). Moreover, by analyzing microarray data of HTLV-1–infected individuals including asymptomatic carriers (AC), HAM/TSP, and ATL patients (GSE19080 and GSE33615), we observed similar down-regulation of TCF1 and LEF1 (Fig. S1 *A* and *B*).

Significance

HTLV-1 is a peripheral T-cell tropic virus and induces proliferation of CD4+ T cells, resulting in T-cell malignancy and inflammatory diseases. Recent studies demonstrated that several restriction factors inhibiting HIV are also inhibitory to HTLV-1. We identified two T-cell–specific proteins, TCF1 and LEF1, as HTLV-1 restriction factors that determine the peripheral T-cell tropism of this virus by targeting Tax. They are highly expressed in immature thymocytes and thereby become a natural intrinsic barrier for HTLV-1 replication in the thymus. However, their expression can be down-regulated by Tax, as well as by activation and differentiation of T cells. These findings provide a mechanistic understanding of how HTLV-1 induces T-cell malignancies in the periphery but never in the thymus.

Author contributions: G.M., J.-i.Y., and M.M. designed research; G.M. and J.-i.Y. performed research; H.A. contributed new reagents/analytic tools; G.M., J.-i.Y., and M.M. analyzed data; G.M., J.-i.Y., and M.M. wrote the paper.

The authors declare no conflict of interest.

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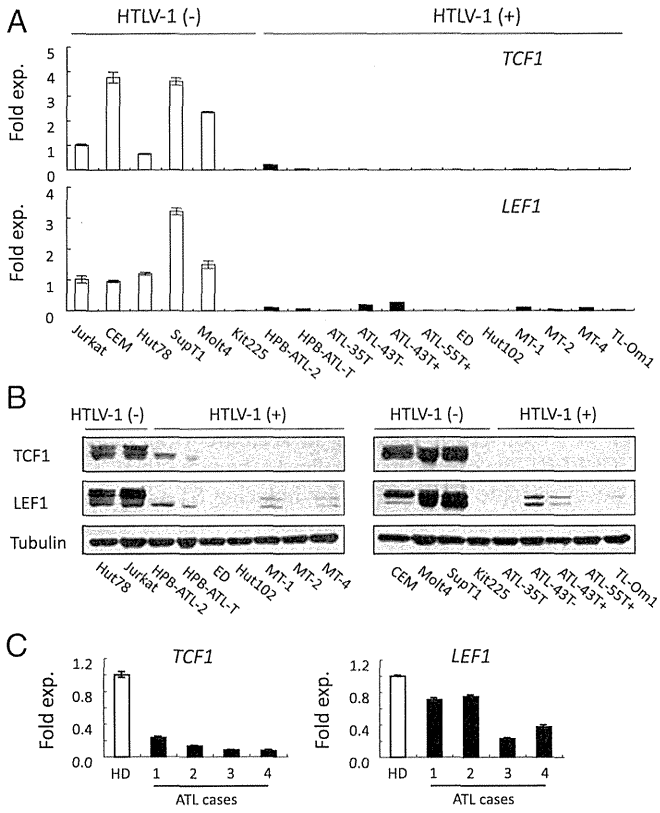

Fig. 1. TCF1 and LEF1 are expressed at low levels in HTLV-1–infected T cells. (*A*) TCF1 and LEF1 mRNA expression is invariably low in HTLV-1–infected cell lines. Total RNA was extracted for each cell line and subjected to quantitative real-time PCR (qPCR) analysis. Results are shown as relative mRNA expression of TCF1 or LEF1 normalized to that of 18S rRNA. (*B*) TCF1 and LEF1 protein expression of cell lines used in *A*. α-tubulin expression was used as a control. (*C*) TCF1 and LEF1 mRNA expression is lower in fresh ATL cases. Peripheral CD4 T cells from a healthy donor (HD) and four ATL patients were subjected to RNA extraction and following qPCR analysis. Results are shown as relative mRNA expression of TCF1 or LEF1 normalized to that of 18S rRNA. "Fold exp." indicates fold expression of normalized mRNA level of TCF1 or LEF1.

TCF1 and LEF1 Interact with Tax and Impair its Transactivating Ability. TCF family members have been recently reported to inhibit HIV type 1 (HIV-1) basal transcription (17). Therefore, we analyzed effects of TCF1 and LEF1 on transcription from the HTLV-1 LTR. As observed in HIV-1, we found that Tax-mediated activation of WT-Luc, which contains five tandem repeats of the TRE from HTLV-1 5′ LTR, was inhibited by TCF1 or LEF1 (Fig. 2*A*). Moreover, activation of the NFκB and AP1 pathways by Tax was also suppressed by TCF1 or LEF1 (Fig. 2*A*). Neither the activator of the Wnt pathway β-catenin nor the inhibitor Axin2 had such effects (Fig. S2*A*), indicating that the effects of TCF1 and LEF1 were mediated in a Wnt-independent manner. Furthermore, neither TCF1 nor LEF1 could inhibit the activation of these reporters by other transcription factors (Fig. S2*B*), suggesting that TCF1 and LEF1 specifically impair Tax function. We performed coimmunoprecipitation (co-IP) and found that TCF1 and LEF1 could each associate physically with Tax in vivo (Fig. 2*B*). Using a series of deletion mutants of Tax, we found that TCF1 and LEF1 predominantly bound to the C-terminal region of Tax (Fig. S2*C*). The PDZ-binding motif (PBM) is known to be localized in the C-terminal end of Tax (3). We found that removal of the PBM greatly impaired Tax binding to TCF1 or LEF1 (Fig. 2*C*), indicating that the PBM of Tax is critical for its binding with TCF1/LEF1. However, Tax bound to distinct regions of TCF1 and LEF1. The central regulation domain of TCF1 was indispensable for binding to Tax whereas all three domains were required for LEF1 to bind to Tax properly (Fig. S3*A*). Reporter assays with WT-Luc also functionally verified this result (Fig. S3*B*).

Nevertheless, due to their broad-spectrum antagonism of Tax, we suspected TCF1 and LEF1 might competitively bind to Tax over other host factors that are hijacked by Tax for transactivation of the viral LTR. CREB is recruited by Tax for its activation of the HTLV-1 5′ LTR (3). We found that TCF1 or LEF1 dose-dependently displaced CREB from Tax (Fig. 2*D*), which suggests that TCF1 and LEF1 each hinder the interaction between Tax and CREB. Thus, these data demonstrate that TCF1 and LEF1 are Tax antagonists that likely execute their inhibition via direct interaction with Tax.

TCF1 and LEF1 Inhibit HTLV-1 Replication by Antagonizing Tax. Next we examined the biological effects of this antagonism on Tax. HTLV-1 replication depends on Tax-driven transcription from the 5′ LTR. To address whether TCF1 and LEF1 are detrimental to HTLV-1 replication, we used an infectious clone of HTLV-1, pX1MT-M (18). HTLV-1 virus production measured by p19 ELISA was inhibited by TCF1 or LEF1 in a dose-dependent manner (Fig. 3*A*). Furthermore, expression of viral proteins that rely on Tax, such as gp46, p19, p24, and even Tax itself, was suppressed by TCF1 or LEF1 (Fig. 3*A*). We also found that endogenous TCF1 or LEF1 is also able to suppress HTLV-1 replication (Fig. S4).

On the other hand, HBZ transcription, which is initiated from viral 3′ LTR and slightly enhanced by Tax (19), was not suppressed but rather enhanced by TCF1 or LEF1 (Fig. 3*B*), in sharp contrast to Tax (Fig. 3*B*). To see whether this was associated with differential regulation of the HTLV-1 5′ and 3′ LTRs

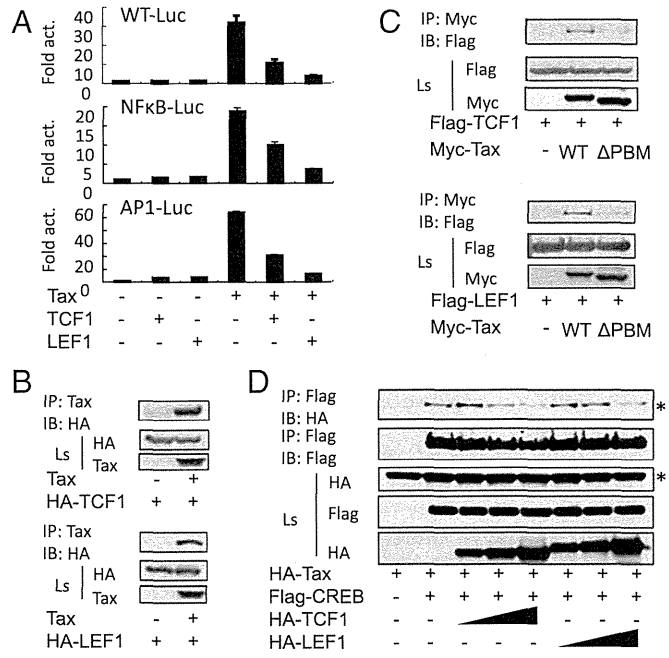

Fig. 2. TCF1 and LEF1 each interact with Tax and impair its transactivating ability. (*A*) TCF1 and LEF1 each repress Tax-mediated activation of WT-Luc (*Top*), NFκB-Luc (*Middle*), and AP1-Luc (*Bottom*). Reporter assays were performed in Jurkat cells. (*B*) Physical interactions between TCF1 and Tax (*Upper*), and LEF1 and Tax (*Lower*). (*C*) A ΔPBM mutant of Tax has impaired binding to TCF1 (*Upper*) and LEF1 (*Lower*) compared with WT Tax. (*D*) Physical interactions between Tax and CREB are inhibited by TCF1 or LEF1 in a dose-dependent manner. Tax-specific bands are denoted with an asterisk. All immunoprecipitations were performed in 293FT cells. "Ls" indicates the whole cell lysate.

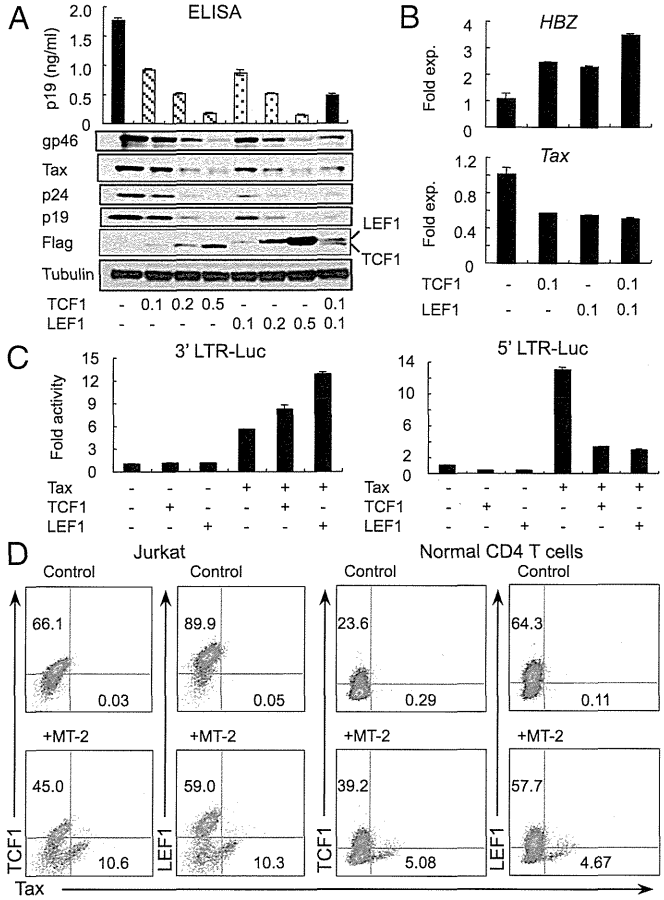

Fig. 3. TCF1 and LEF1 each inhibit HTLV-1 replication by antagonizing Tax. (*A*) TCF1 and LEF1 each inhibits HTLV-1 production (*Upper*) and protein expression (*Lower*). pX1MT-M (0.5 μg) was transfected with or without TCF1 or LEF1 into 293FT cells. 48 h later, supernatants were collected for p19 ELISA 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

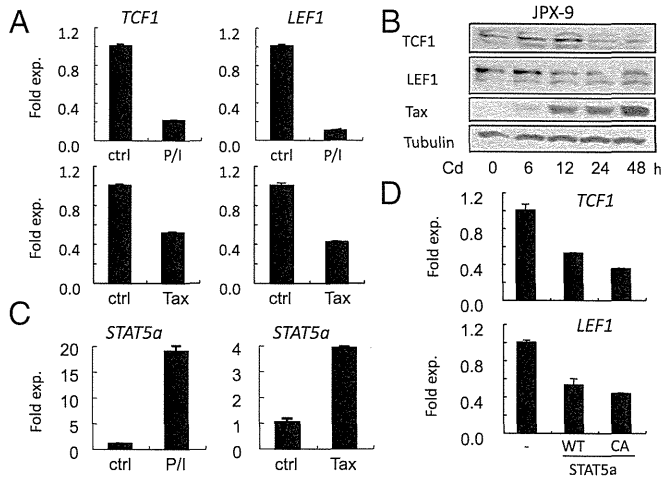

Fig. 4. Tax down-regulates the expression of TCF1 and LEF1 via STAT5a. (*A*) P/I stimulation (*Upper*) or Tax overexpression (*Lower*) inhibits TCF1/LEF1 transcription in Jurkat. For P/I stimulation, cells were treated with 50 ng/mL of PMA and 500 ng/mL of ionomycin (P/I) for 5 h and then subjected to 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).

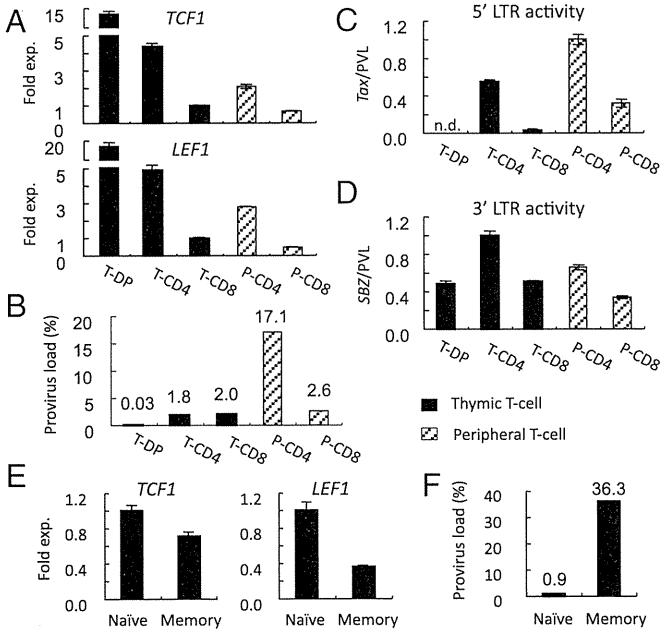

Fig. 5. TCF1 and LEF1 expression correlate negatively with STLV-1 proviral load in vivo. (*A*) TCF1 (*Upper*) and LEF1 (*Lower*) transcription in sorted CD4/CD8 double positive (T-DP), CD4 single positive (SP) thymocytes (T-CD4), CD8 SP thymocytes (T-CD8) and peripheral CD4 (P-CD4) and CD8 (P-CD8) SP 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

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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).

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

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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-

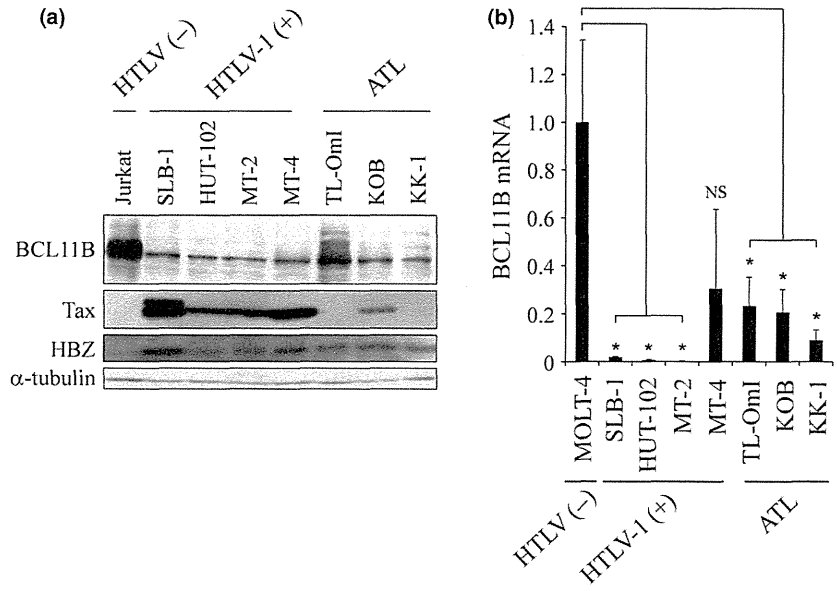

Fig. 1. The downregulation of BCL11B expression in HTLV-1-infected T-cell lines. (a) Cell lysates were prepared from the indicated human T-cell lines, and the expression levels of BCL11B, Tax, HBZ and α-tubulin in the cell lysates were measured by western blot analysis. (b) Total RNA was prepared from 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.

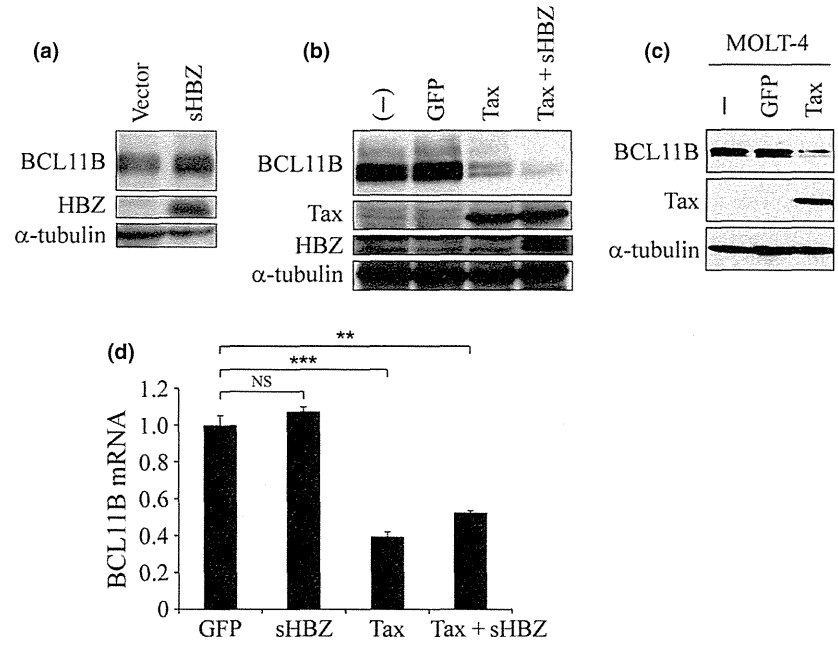

Fig. 2. Tax represses BCL11B expression in T-cells. (a–c) Cell lysates were prepared from Jurkat (a, b) or MOLT-4 cells (c) infected with the indicated lentiviruses, and the expression of BCL11B, Tax, HBZ and α-tubulin in the cell lysates was examined by western blot analysis. A lentivirus expressing 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

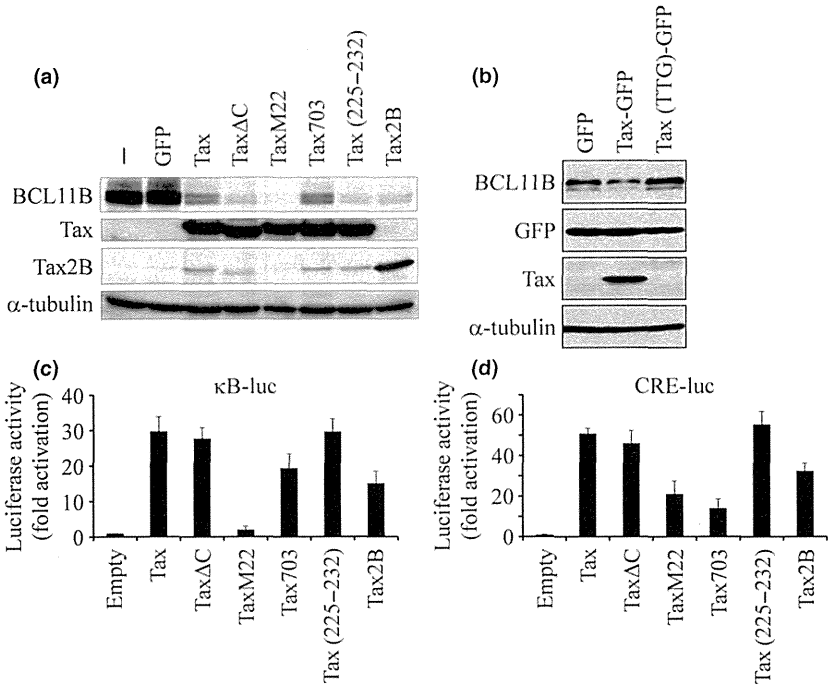

Fig. 3. Tax mutants also reduced the expression of BCL11B in T-cells. (a, b) Cell lysates were prepared from Jurkat cells infected with the indicated lentiviruses, and the expression levels of BCL11B, Tax, Tax2B, GFP and α-tubulin in the cell lysates were measured by western blot analysis. A lentivirus 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.

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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

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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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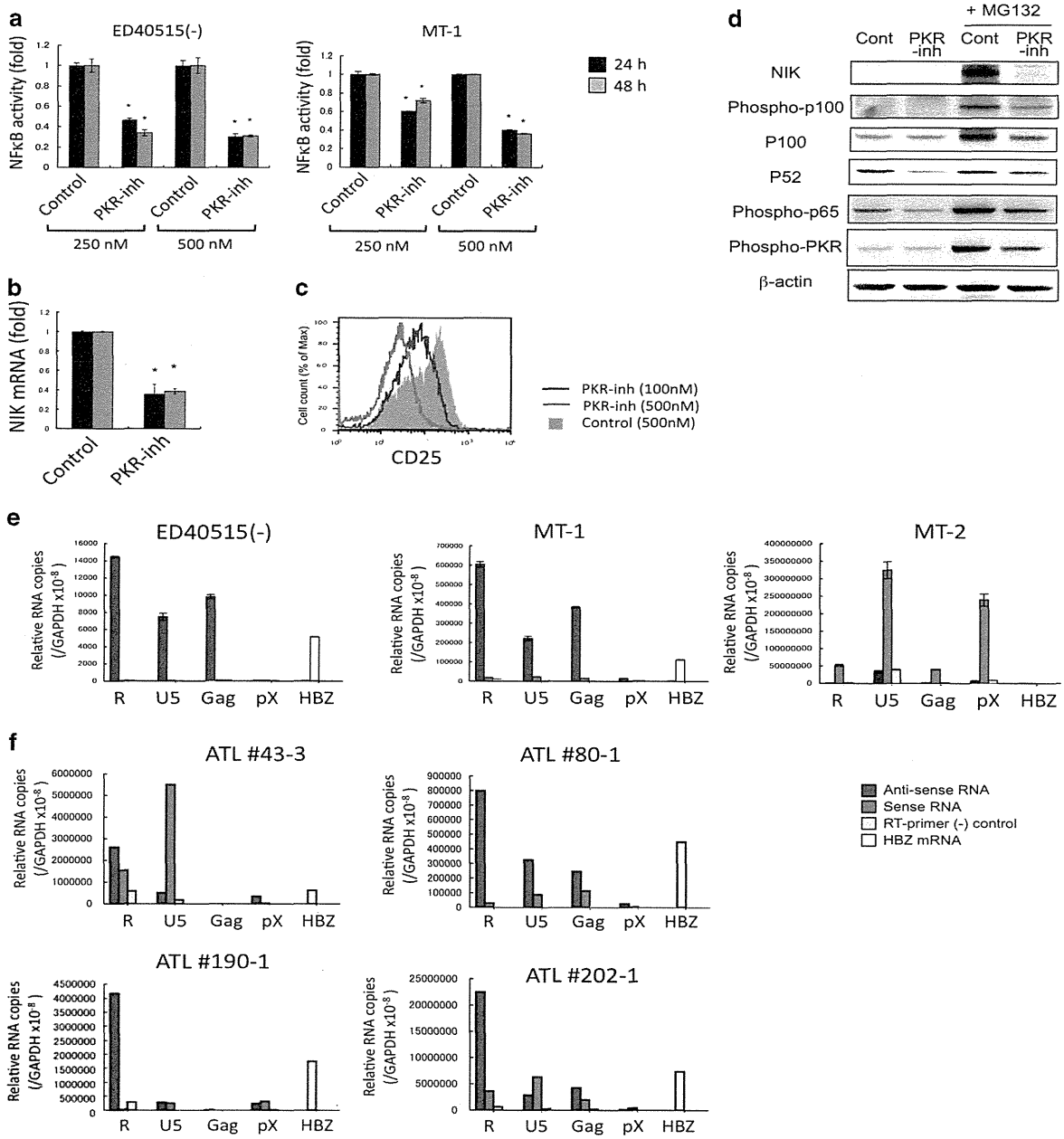
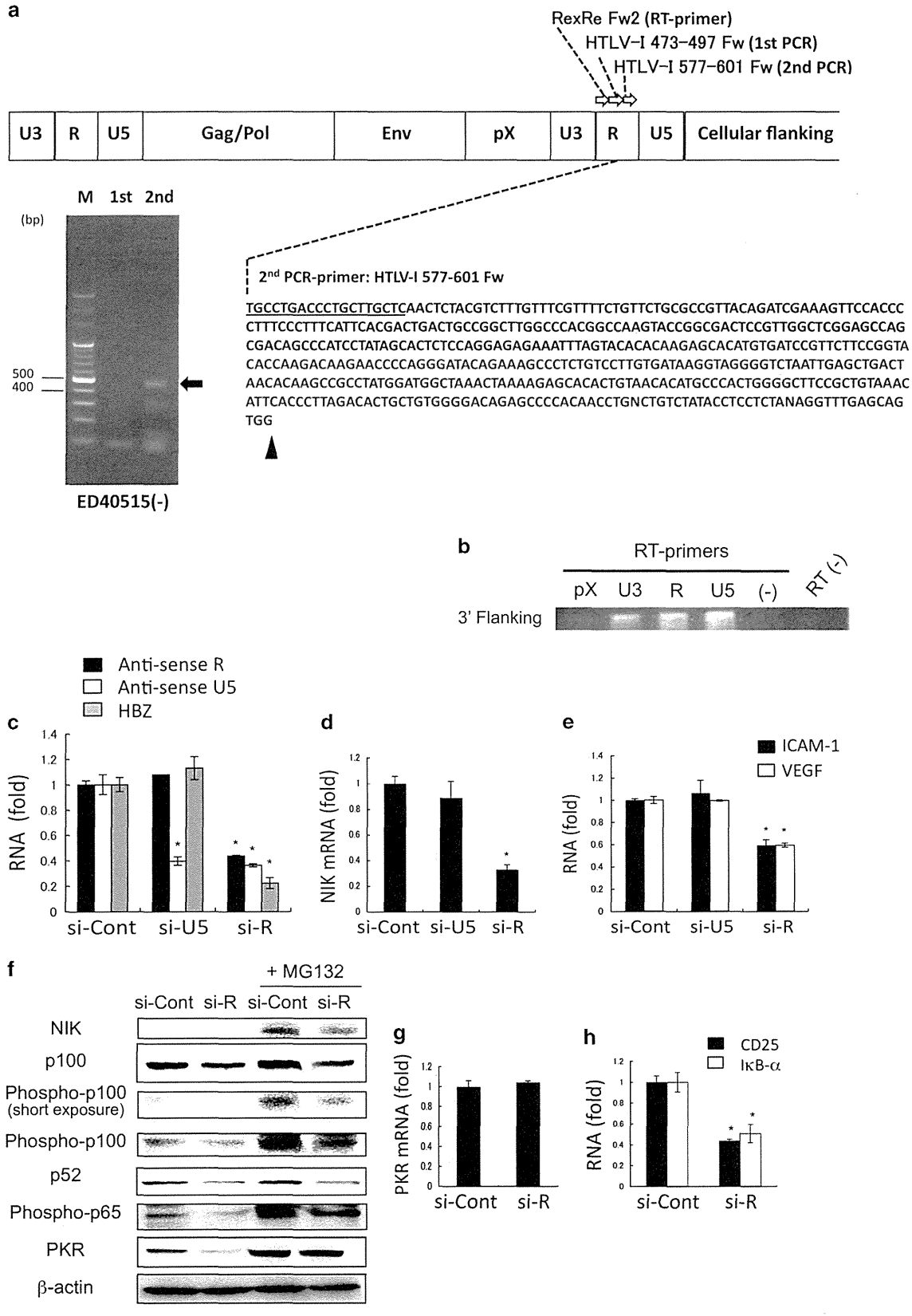


Figure 1. Suppression of NFκB activity by a PKR-inhibitor and the presence of antisense RNA at the HTLV-1 LTR in Tax-negative ATL cells. (**a**) ED40515(−) (left) and MT-1 (right) cells containing reporter genes for NFκB and the thymidine kinase (TK) promoter were incubated with a chemical PKR inhibitor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