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

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Abstract

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- κ B 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.

Significance Statement (120-word-maximum)

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 novel 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 downregulated 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.

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Introduction

Human T-cell leukemia virus type 1 (HTLV-1) causes a malignancy named adult T-cell leukemia (ATL) and several inflammatory diseases including HTLV-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 co-activate 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 didn't 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 indispensable 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 novel 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 downregulate 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.

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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 downregulation of TCF1 and LEF1 (Fig. S1*A* and *B*).

TCF1 and LEF1 interact with Tax and impair its transactivating ability. TCF family members have been recently reported to inhibit human immunodeficiency virus 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 NF κ B 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 co-immunoprecipitation (co-IP) and found that TCF1 and LEF1 could each associate physically with Tax *in vivo* (Fig. 2B). Using a series of deletion mutants of Tax, we found that TCF1 and LEF1 predominantly bound to the C-terminal region of Tax (Fig. S2C). 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. 2C), 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. S3A). Reporter assays with WT-Luc also functionally verified this result (Fig. S3B).

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. 2D), 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. 3A). 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. 3A). 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. 3B), in sharp contrast to Tax (Fig. 3B). To see whether this was associated with differential regulation of the HTLV-1 5' and 3' LTRs 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. 3C). This explains why HBZ transcription increased in the presence of TCF1/LEF1 (Fig. 3B). Consistent with the WT-Luc result (Fig. 2A), TCF1 or LEF1 significantly suppressed Tax-induced 5' LTR activation (Fig. 3C). 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. 3D), suggesting that TCF1/LEF1 restricts HTLV-1 *de novo* viral expression and its replication.

Tax downregulates TCF1 and LEF1 via STAT5a. Antigen encounter or T-cell activation were reported to trigger TCF1/LEF1 downregulation (15). We confirmed that phorbol myristate acetate (PMA)/ionomycin (P/I) stimulation downregulate TCF1 and LEF1 in Jurkat and primary CD4 T cells (Fig. 4A and Fig. S5A). 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. S5B), and suppressed the expression of TCF1 and LEF1 in Jurkat cells (Fig. 4A). Furthermore, cadmium-induced Tax expression in JPX-9, a modified Jurkat line that expresses Tax under a metallothionein promoter (20), also downregulated TCF1 and LEF1 (Fig. 4B). However, Tax (Fig. S5C) did not inhibit transcription from the TCF1 and LEF1 promoters. To see whether the NF κ B, NFAT or AP1 pathways, the three major TCR downstream pathways, are involved in TCF1/LEF1 downregulation (21), we activated them by electroporation of the corresponding transcription factors into Jurkat (Fig. S5D). However, neither single nor combined activation of these pathways clearly suppressed TCF1 or LEF1 expression (Fig. S5E). 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). Since 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. 4C). 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. 4D).

Higher expression of TCF1 and LEF1 is associated with low STLTV-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 STLTV-1 encoded Tax and STLTV-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. 5A) whereas their PVL was the lowest (Fig. 5B). This 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 2-fold higher in TCF1/LEF1 expression (Fig. 5A) 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. 5B). Similar measurements were made in thymic (T-CD8) and peripheral CD8 T cells (P-CD8) (Fig. 5A 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. 5B). Along with the fact that thymic CD8 and CD4 T cells had similar PVLs, this 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. 3B). The 5' LTR was clearly more active in peripheral CD4 or

CD8 T cells than their thymic counterparts (Fig. 5C). 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. 5D). 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. 5E and F), which is in agreement with the fact that HTLV-1 infected cells have mostly a memory phenotype (28).

Discussion

During co-evolution 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 since 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 towards 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 since 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 to 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 downregulation of TCF1/LEF1 always occurs in activated or differentiated peripheral T cells (38). HTLV-1 may exploit this downregulation to achieve its expansion, since downregulation of TCF1/LEF1 allows Tax to execute its functions. A previous report also indicated that pre-activated primary T cells are easier to transform by HTLV-1 (39). Downregulation of TCF1/LEF1 upon T-cell activation/differentiation would allow Tax expression and subsequent HTLV-1 expansion.

Downregulation of TCF1/LEF1 also occurs as T cells develop or differentiate, from DP to SP in the thymus (Fig. 5A), or from naïve to memory in the periphery (Fig. 5E). 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 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 STL-1. However, in order 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, post-infection 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 STL-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. 3C). 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- κ B pathway has been considered a critical function of Tax for cellular transformation (2, 3), the recent study have reported that hyper-activation of NF- κ B 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- κ B 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 while 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 novel 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 years 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% 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 previously (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 Dr. Fujisawa. pX1MT-M was a generous gift from Dr. Derse. NF κ B-Luc and AP1-Luc were purchased from Stratagene. 3' LTR-Luc was described previously (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 Roche. 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 previously (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 while 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 3000 rpm 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 supporting information for details (Fig. S6).

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

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References

1. Gallo RC (2005) History of the discoveries of the first human retroviruses: HTLV-1 and HTLV-2. *Oncogene* 24(39):5926-5930.
2. Matsuoka M & Jeang KT (2007) Human T-cell leukaemia virus type 1 (HTLV-1) infectivity and cellular transformation. *Nat Rev Cancer* 7(4):270-280.
3. Grassmann R, Aboud M, & Jeang KT (2005) Molecular mechanisms of cellular transformation by HTLV-1 Tax. *Oncogene* 24(39):5976-5985.
4. Tamiya S, *et al.* (1996) Two types of defective human T-lymphotropic virus type I provirus in adult T-cell leukemia. *Blood* 88(8):3065-3073.
5. Takeda S, *et al.* (2004) Genetic and epigenetic inactivation of tax gene in adult T-cell leukemia cells. *Int J Cancer* 109(4):559-567.
6. Furukawa Y, Kubota R, Tara M, Izumo S, & Osame M (2001) Existence of escape mutant in HTLV-I tax during the development of adult T-cell leukemia. *Blood* 97(4):987-993.
7. Koiwa T, *et al.* (2002) 5'-long terminal repeat-selective CpG methylation of latent human T-cell leukemia virus type 1 provirus in vitro and in vivo. *Journal of virology* 76(18):9389-9397.
8. Bangham CR (2009) CTL quality and the control of human retroviral infections. *Eur J Immunol* 39(7):1700-1712.
9. Satou Y, Yasunaga J, Yoshida M, & Matsuoka M (2006) HTLV-I basic leucine zipper factor gene mRNA supports proliferation of adult T cell leukemia cells. *Proceedings of the National Academy of Sciences of the United States of America* 103(3):720-725.
10. Staal FJ & Clevers HC (2005) WNT signalling and haematopoiesis: a WNT-WNT situation. *Nature reviews. Immunology* 5(1):21-30.
11. Staal FJ, Luis TC, & Tiemessen MM (2008) WNT signalling in the immune system: WNT is spreading its wings. *Nature reviews. Immunology* 8(8):581-593.
12. Oosterwegel M, *et al.* (1993) Differential expression of the HMG box factors TCF-1 and LEF-1 during murine embryogenesis. *Development* 118(2):439-448.
13. Verbeek S, *et al.* (1995) An HMG-box-containing T-cell factor required for thymocyte differentiation. *Nature* 374(6517):70-74.

14. Okamura RM, *et al.* (1998) Redundant regulation of T cell differentiation and TCRalpha gene expression by the transcription factors LEF-1 and TCF-1. *Immunity* 8(1):11-20.
15. Willinger T, *et al.* (2006) Human naive CD8 T cells down-regulate expression of the WNT pathway transcription factors lymphoid enhancer binding factor 1 and transcription factor 7 (T cell factor-1) following antigen encounter in vitro and in vivo. *J Immunol* 176(3):1439-1446.
16. Ma G, Yasunaga J, Fan J, Yanagawa S, & Matsuoka M (2013) HTLV-1 bZIP factor dysregulates the Wnt pathways to support proliferation and migration of adult T-cell leukemia cells. *Oncogene* 32(36):4222-4230.
17. Narasipura SD, *et al.* (2012) Role of beta-catenin and TCF/LEF family members in transcriptional activity of HIV in astrocytes. *Journal of virology* 86(4):1911-1921.
18. Mitchell MS, *et al.* (2007) Phenotypic and genotypic comparisons of human T-cell leukemia virus type 1 reverse transcriptases from infected T-cell lines and patient samples. *Journal of virology* 81(9):4422-4428.
19. Yoshida M, Satou Y, Yasunaga J, Fujisawa J, & Matsuoka M (2008) Transcriptional control of spliced and unspliced human T-cell leukemia virus type 1 bZIP factor (HBZ) gene. *Journal of virology* 82(19):9359-9368.
20. Azimi N, *et al.* (1998) Human T cell lymphotropic virus type I Tax protein trans-activates interleukin 15 gene transcription through an NF-kappaB site. *Proceedings of the National Academy of Sciences of the United States of America* 95(5):2452-2457.
21. Macian F (2005) NFAT proteins: key regulators of T-cell development and function. *Nature reviews. Immunology* 5(6):472-484.
22. Welte T, *et al.* (1999) STAT5 interaction with the T cell receptor complex and stimulation of T cell proliferation. *Science* 283(5399):222-225.
23. Migone TS, *et al.* (1995) Constitutively activated Jak-STAT pathway in T cells transformed with HTLV-I. *Science* 269(5220):79-81.
24. Lin JX & Leonard WJ (2000) The role of Stat5a and Stat5b in signaling by IL-2 family cytokines. *Oncogene* 19(21):2566-2576.
25. Nakamura N, *et al.* (1999) Human T-cell leukemia virus type 1 Tax protein

- induces the expression of STAT1 and STAT5 genes in T-cells. *Oncogene* 18(17):2667-2675.
26. Miura M, *et al.* (2013) Characterization of simian T-cell leukemia virus type 1 in naturally infected Japanese macaques as a model of HTLV-1 infection. *Retrovirology* 10:118.
 27. Kannian P, *et al.* (2012) Distinct transformation tropism exhibited by human T lymphotropic virus type 1 (HTLV-1) and HTLV-2 is the result of postinfection T cell clonal expansion. *Journal of virology* 86(7):3757-3766.
 28. Yasunaga J, *et al.* (2001) Impaired production of naive T lymphocytes in human T-cell leukemia virus type I-infected individuals: its implications in the immunodeficient state. *Blood* 97(10):3177-3183.
 29. Wolf D & Goff SP (2008) Host restriction factors blocking retroviral replication. *Annual review of genetics* 42:143-163.
 30. Bieniasz PD (2004) Intrinsic immunity: a front-line defense against viral attack. *Nature immunology* 5(11):1109-1115.
 31. Blanco-Melo D, Venkatesh S, & Bieniasz PD (2012) Intrinsic cellular defenses against human immunodeficiency viruses. *Immunity* 37(3):399-411.
 32. Derse D, Hill SA, Princler G, Lloyd P, & Heidecker G (2007) Resistance of human T cell leukemia virus type 1 to APOBEC3G restriction is mediated by elements in nucleocapsid. *Proceedings of the National Academy of Sciences of the United States of America* 104(8):2915-2920.
 33. Sze A, *et al.* (2013) Host restriction factor SAMHD1 limits human T cell leukemia virus type 1 infection of monocytes via STING-mediated apoptosis. *Cell host & microbe* 14(4):422-434.
 34. Manel N, Battini JL, Taylor N, & Sitbon M (2005) HTLV-1 tropism and envelope receptor. *Oncogene* 24(39):6016-6025.
 35. Bertotto A, *et al.* (1990) Human breast milk T lymphocytes display the phenotype and functional characteristics of memory T cells. *Eur J Immunol* 20(8):1877-1880.
 36. Hasegawa H, *et al.* (2006) Thymus-derived leukemia-lymphoma in mice transgenic for the Tax gene of human T-lymphotropic virus type I. *Nature medicine* 12(4):466-472.

37. Weber BN, *et al.* (2011) A critical role for TCF-1 in T-lineage specification and differentiation. *Nature* 476(7358):63-68.
38. Xue HH & Zhao DM (2012) Regulation of mature T cell responses by the Wnt signaling pathway. *Ann N Y Acad Sci* 1247:16-33.
39. Merl S, *et al.* (1984) Efficient transformation of previously activated and dividing T lymphocytes by human T cell leukemia-lymphoma virus. *Blood* 64(5):967-974.
40. Villaudy J, *et al.* (2011) HTLV-1 propels thymic human T cell development in "human immune system" Rag2(-)/(-) gamma c(-)/(-) mice. *PLoS pathogens* 7(9):e1002231.
41. Belrose G, *et al.* (2011) Effects of valproate on Tax and HBZ expression in HTLV-1 and HAM/TSP T lymphocytes. *Blood* 118(9):2483-2491.
42. Zhi H, *et al.* (2011) NF-kappaB hyper-activation by HTLV-1 tax induces cellular senescence, but can be alleviated by the viral anti-sense protein HBZ. *PLoS pathogens* 7(4):e1002025.
43. Zhao T, *et al.* (2009) Human T-cell leukemia virus type 1 bZIP factor selectively suppresses the classical pathway of NF-kappaB. *Blood* 113(12):2755-2764.
44. Matsuoka M & Yasunaga J (2013) Human T-cell leukemia virus type 1: replication, proliferation and propagation by Tax and HTLV-1 bZIP factor. *Current opinion in virology* 3(6):684-691.
45. Peloponese JM, Jr., Yasunaga J, Kinjo T, Watashi K, & Jeang KT (2009) Peptidylproline cis-trans-isomerase Pin1 interacts with human T-cell leukemia virus type 1 tax and modulates its activation of NF-kappaB. *Journal of virology* 83(7):3238-3248.

Figure Legends

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.

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), NF κ B-Luc (middle) and AP1-Luc (bottom). Reporter assays were performed in Jurkat cells. (B) Physical interactions between TCF1 and Tax (top), and LEF1 and Tax (bottom). (C) A Δ PBM mutant of Tax has impaired binding to TCF1 (top) and LEF1 (bottom) compared to 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 *. 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 (top) and protein expression (bottom). pX1MT-M (0.5 μ g) was transfected with or without TCF1 or LEF1 into 293FT cells. 48 hours later, supernatants were collected for p19 ELISA and cells were lysed for western blot. (B) TCF1 and LEF1 each inhibit Tax transcription (bottom) but not HBZ transcription (top). pX1MT-M (0.5 μ g) was transfected with or without TCF1 or LEF1 into 293FT cells. 44 hours 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 (top) or cocultivated with lethally irradiated (150 Gy) MT-2 cells (bottom) at a 2:1 ratio. 48 hours 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.

Fig. 4 Tax downregulates the expression of TCF1 and LEF1 via STAT5a

(A) P/I stimulation (top) or Tax overexpression (bottom) 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 hours and then subjected to RNA extraction and qPCR analysis. Overexpression of Tax was achieved by electroporation and 24 hours later, RNA was extracted for qPCR. (B) Tax induction in JPX-9 downregulates the expression of TCF1 and LEF1. JPX-9 was cultured in RPMI supplemented with 20 μ M 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 downregulates TCF1 and LEF1. Jurkat was transfected with wild type (WT) or constitutively active (CA) STAT5a by electroporation. 24 hours later, RNA was extracted for qPCR. Fold exp. indicates fold expression.

Fig. 5 TCF1 and LEF1 expression correlate negatively with STLV-1 proviral load *in vivo*

(*A*) TCF1 (top) and LEF1 (bottom) 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⁺ (naïve) 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.

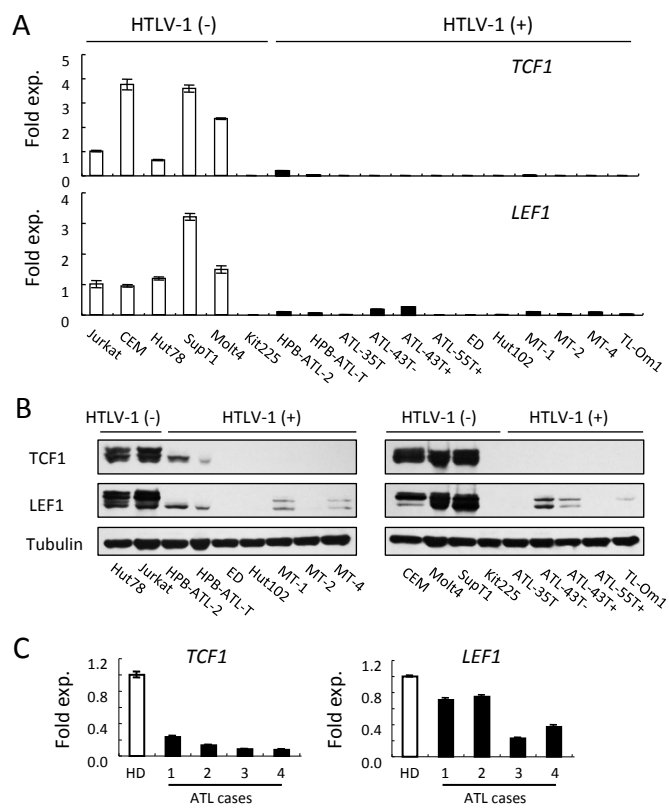


Figure 1

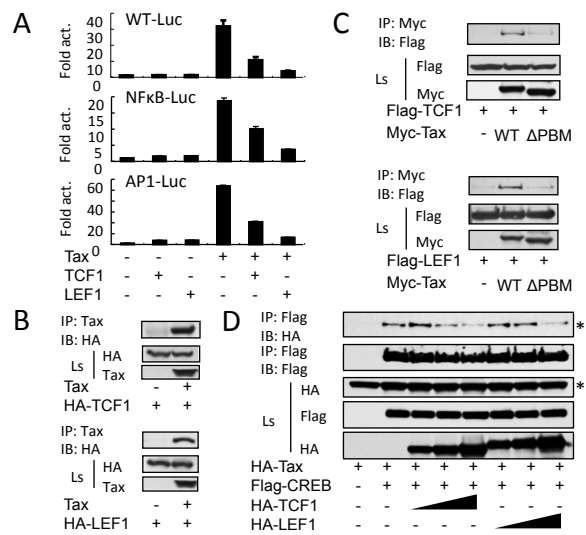


Figure 2

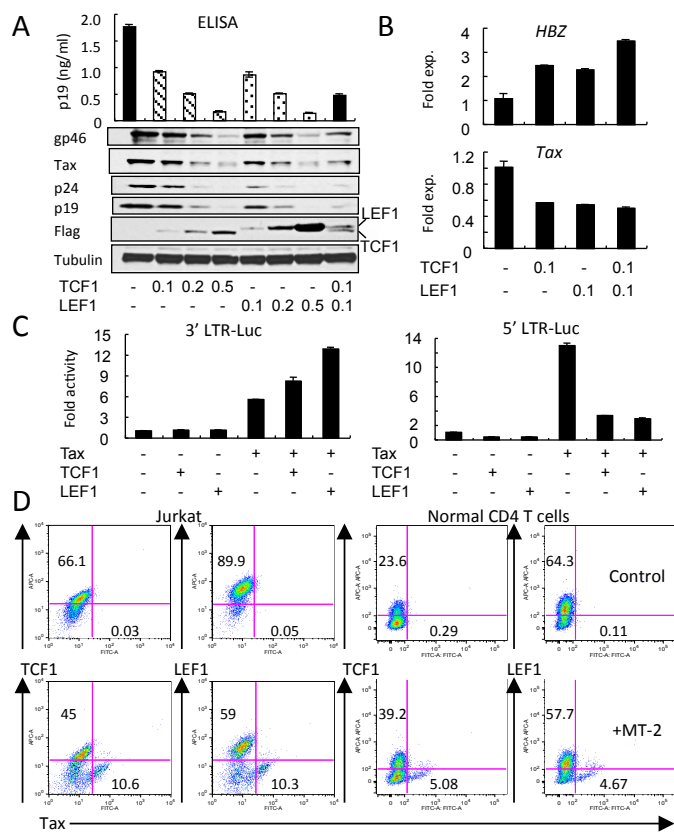


Figure 3

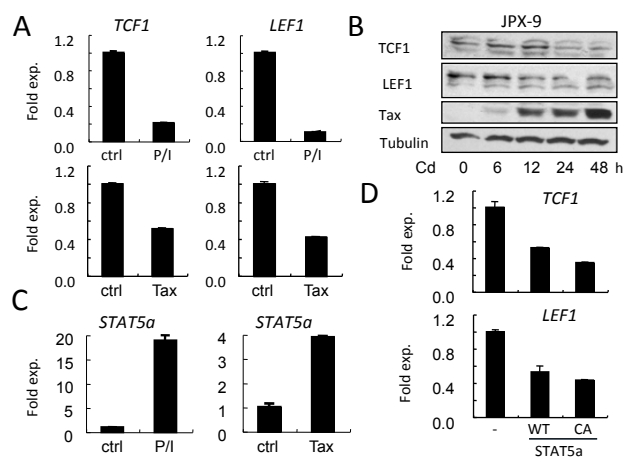


Figure 4

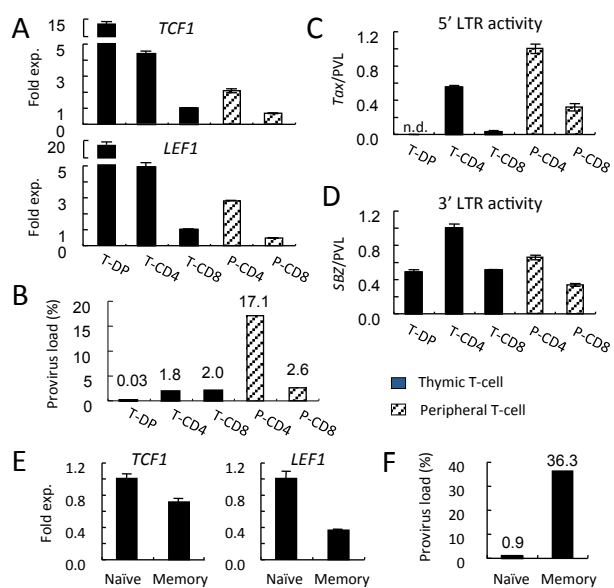


Figure 5