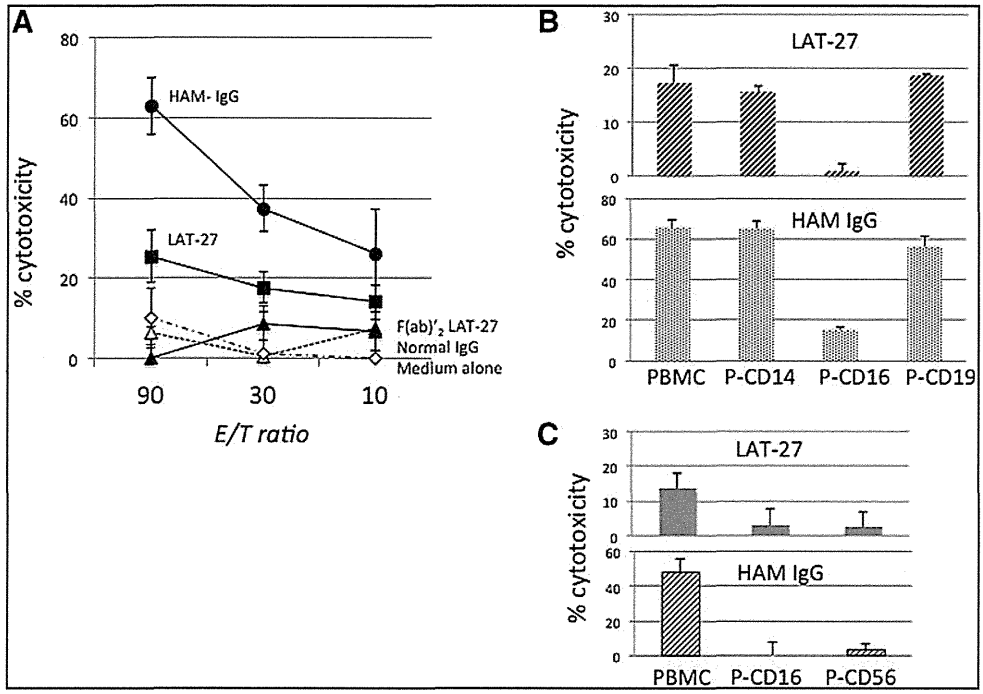

FIG. 7. The CD16$^+$ CD56$^+$ PBMCs mediate antibody-dependent cellular cytotoxicity (ADCC) in the presence of LAT-27 or HAM-IgG. **(A)** ^{51}Cr-labeled HTLV-1-infected cells were cocultured *in vitro* with autologous fresh PBMCs at various E/T ratios in the presence or absence of 10 μg/ml of LAT-27 or F(ab')$_2$ LAT-27, or 100 μg/ml of normal human or HAM-IgG for 24 h. Each coculture was performed in triplicate, and the amount of radioactivity in the culture supernatants was determined. Data shown are representative of three independent experiments. **(B, C)** Effector PBMCs before or after depletion of CD14$^+$, CD16$^+$, CD19$^+$, or CD56$^+$ cells were assayed for ADCC activity against autologous HTLV-1-infected cells in the presence of LAT-27 (10 μg/ml) or HAM-IgG (100 μg/ml) in triplicate wells in the 24 h ^{51}Cr-release assay. Data shown are representative of two independent experiments.

against activated autologous T cells.[44] Further studies are in progress to address this mechanism.

Based on the data presented herein, it is suggested that humanized LAT-27 mAb might have potential as a passive vaccine against HTLV-1 infection for HTLV-1-uninfected individuals at high risk of HTLV-1 infection, including babies born to HTLV-1 carriers and drug abusers who are also at high risk of HIV infection, and for HTLV-1 carriers whose anti-HTLV-1 neutralizing and ADCC-inducing antibody titers are low. One concern is the potential interference of LAT-27 activity by other nonneutralizing or non-ADCC-inducing antibodies that may interfere with the binding of LAT-27 to gp46. We have performed some experiments and obtained data showing that LAT-12, which blocked the binding of LAT-27 to HTLV-1-infected cells, did not interfere with either LAT-27-mediated syncytium blocking[29] and/or the eradication of HTLV-1-infected cells with autologous PBMCs (Supplementary Fig. S4). It seems likely that the binding affinities of neutralizing antibodies to gp46 expressed on actively living cells are higher than those of nonneutralizing antibodies. Thus, validation of humanized LAT-27 in animal models is currently one of our objectives.

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Y.Tak. and A.H. carried out the ADCC assays. R.T. and A.K. produced, purified, labeled monoclonal antibodies, confirmed their specificities, and made in-house EILSA for p24. M.S. participated in the determination of proviral loads and performed the statistical analysis. M.K. established HTLV-1-infected cells from patients and participated in the design of the study. A.A.A. participated in the design of the study and helped to draft the manuscript. Y.T. conceived the study, participated in its design and coordination, carried out the coculture assays, and drafted the manuscript. All authors read and approved the final manuscript.

Author Disclosure Statement

No competing financial interests exist.

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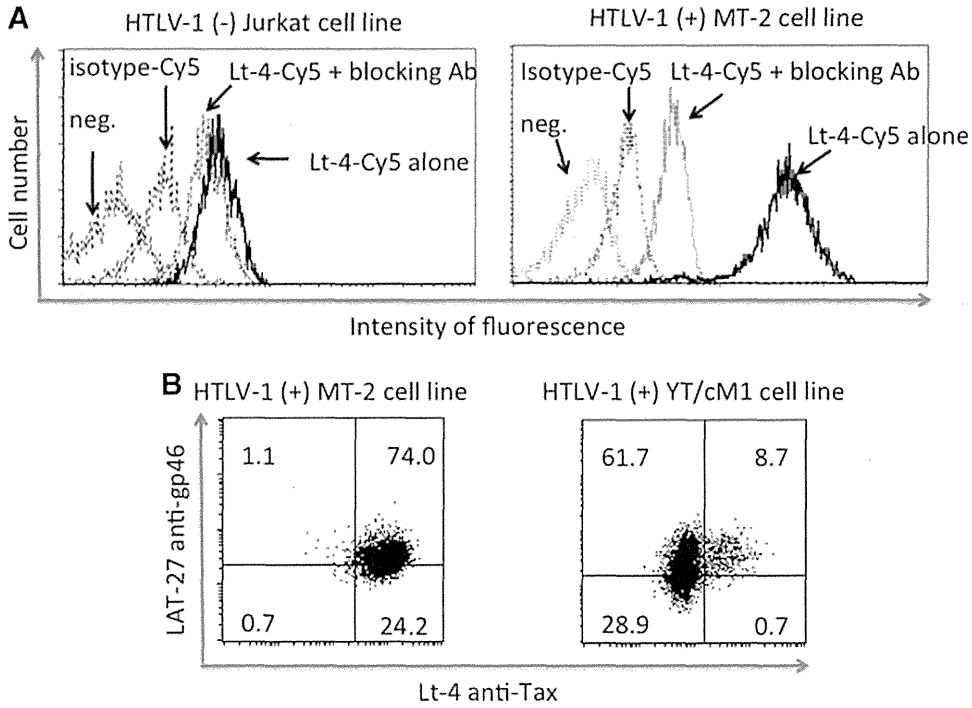
Address correspondence to:
Yuetsu Tanaka
Department of Immunology
Graduate School of Medicine
University of the Ryukyus
207 Uehara, Nishihara-cho
Okinawa 903-0215
Japan

E-mail: yuetsu@s4.dion.ne.jp

Supplementary Data

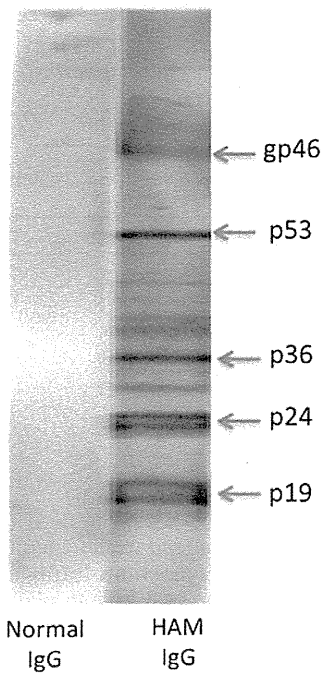


SUPPLEMENTARY FIG. S1. Flow cytometry of Tax and HTLV-I gp46 antigens. **(A)** Tax-specific and nonspecific staining by Cy5-labeled Lt-4. HTLV-I-negative Jurkat cells and HTLV-I-positive MT-2 cells were stained with either Cy5-labeled Lt-4 or Cy5-labeled mouse isotype control (IgG3) in the presence or absence of a 500 times excess of nonlabeled Lt-4 (blocking Ab). **(B)** Typical dual staining of MT-2 and another HTLV-I-immortalized T cell line (YT/cM1) with FITC-LAT-27 and Cy5-Lt-4. Negative controls for the two mAbs were obtained from cells stained in the presence of a 500 times excess of nonlabeled homologous blocking mAbs as explained above.

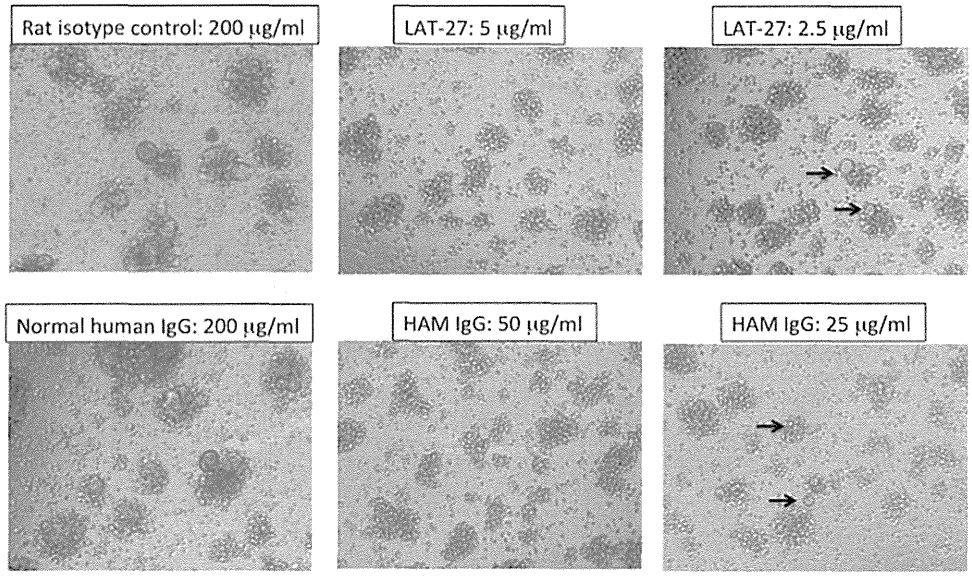
A HTLV-I-coated gelatin particle agglutination assay (SERODIA®HTLV-I)

Sample (1 mg/ml)	Titer
Normal-IgG	< 8 x
HAM-IgG	4,096 x

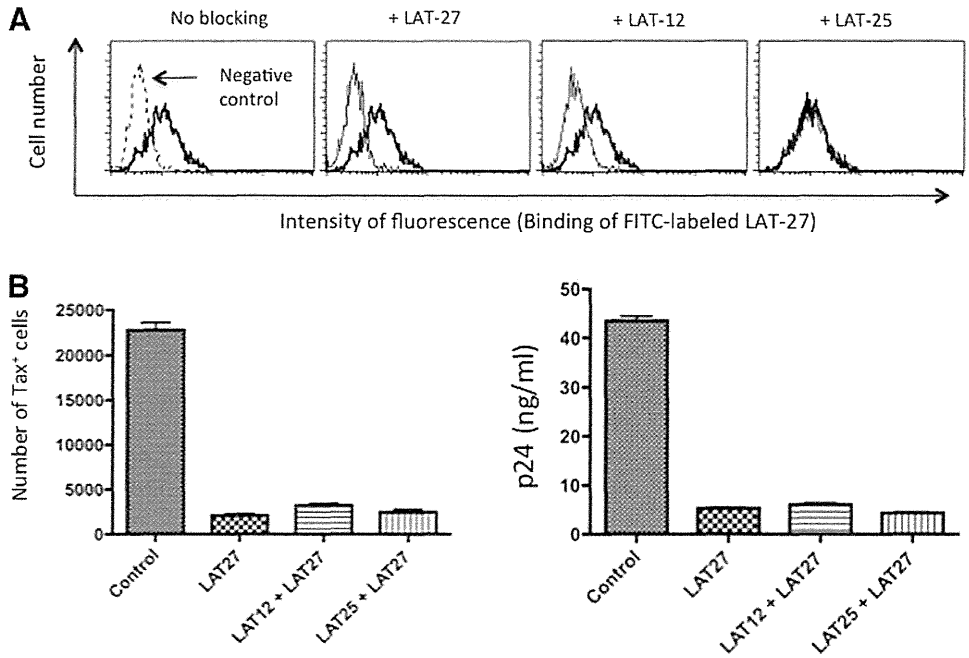
B Western blot analysis (PROBLOT HTLV-I)



SUPPLEMENTARY FIG. S2. Characterization of anti-HTLV-I antibody profile of HAM-IgG. **(A)** Purified HAM-IgG at 1 mg/ml was serially diluted and subjected to a commercial anti-HTLV-I agglutination assay (SERODIA®HTLV-I, Fujirebio Inc.). Titers were expressed as the reciprocal dilution that showed a positive reaction. **(B)** Using a commercial anti-HTLV-I western blot assay, IgG (10 μg/ml) from pooled plasma of normal donors and HAM patients was examined for HTLV-I antibodies.



SUPPLEMENTARY FIG. S3. Titration of HTLV-I-neutralizing antibody titers of LAT-27 and HAM-IgG. Two-fold diluted IgG samples were added to the coculture of ILT-M1 and Jurkat cells, and the minimum IgG concentration required for complete blockade of syncytium formation was determined. Note that the control rat isotype (rat IgG anti-HCV) and control IgG from pooled normal human plasma did not neutralize even a 200 μg/ml (final concentration). Arrows indicate small syncytia escaped from neutralization.



SUPPLEMENTARY FIG. S4. Lack of interference by nonneutralizing anti-gp46 mAb in LAT-27 mediated HTLV-1 suppression in the presence of autologous PBMCs. **(A)** Binding of FITC-labeled LAT-27 to ILT-M1 cells in the presence of a 10 times higher concentration of competing mAb was analyzed by flow cytometry (FCM). Dotted line, binding of FITC-isotype control; thick and thin lines, bindings of FITC-LAT-27 in the absence and presence of competitors, respectively. **(B)** As shown in Fig. 6, the IL-2-dependent HTLV-1-infected $CD4^+$ T cells were exposed to autologous PBMCs with 10 μg/ml of isotype control (control) or LAT-27 in the presence or absence of 100 μg/ml of LAT-12 or LAT-25 twice at 3 day intervals. Two days after the second exposure, the absolute Tax^+ cell number/culture and HTLV-1 p24 levels produced in the culture supernatants were quantitated by FCM and ELISA, respectively. In the absence of PBMCs, the numbers of Tax^+ cells were 47,200 + 5,200, which was not affected by the addition of only LAT-12, LAT-25, or LAT-27 (data not shown) ($n = 4$).

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SHORT REPORT Open Access

The neutralizing function of the anti-HTLV-1 antibody is essential in preventing in vivo transmission of HTLV-1 to human T cells in NOD-SCID/γcnull (NOG) mice

Mineki Saito[1,2*], Reiko Tanaka[1], Hideki Fujii[1], Akira Kodama[1], Yoshiaki Takahashi[1], Toshio Matsuzaki[3], Hiroshi Takashima[3] and Yuetsu Tanaka[1]

Abstract

Background: Human T-cell leukemia virus type 1 (HTLV-1) causes both neoplastic and inflammatory diseases, including adult T-cell leukemia and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). Because these life-threatening and disabling diseases are not yet curable, it is important to prevent new HTLV-1 infections.

Findings: In this study, we have established a simple humanized mouse model of HTLV-1 infection for evaluating prophylactic and therapeutic interventions. In this model, HTLV-1-negative normal human peripheral blood mononuclear cells (PBMCs) are transplanted directly into the spleens of severely immunodeficient NOD-SCID/γcnull (NOG) mice, together with mitomycin-treated HTLV-1-producing T cells. Using this model, we tested the efficacy of monoclonal antibodies (mAbs) specific to HTLV-1 as well as human IgG isolated from HAM/TSP patients (HAM-IgG) in preventing HTLV-1-infection. One hour before and 24 h after transplantation of the human cells, each antibody sample was inoculated intraperitoneally. On day 14, human PBMCs isolated from the mouse spleens were tested for HTLV-1 infection. Whereas fresh CD4-positive and CD8-positive T cells isolated from untreated mice or mice treated with isotype control mAb, HTLV-1 non-neutralizing mAbs to envelope gp46, gag p19, and normal human IgG were all infected with HTLV-1; the mice treated with either HTLV-1 neutralizing anti-gp46 mAb or HAM-IgG did not become infected.

Conclusions: Our data indicate that the neutralizing function of the antibody, but not the antigen specificity, is essential for preventing the *in vivo* transmission of HTLV-1. The present animal model will also be useful for the *in vivo* evaluation of the efficacy of candidate molecules to be used as prophylactic and therapeutic intervention against HTLV-1 infection.

Keywords: HTLV-1, NOG mice, Intrasplenic injection, Neutralizing antibody, Envelope gp46

Findings

Human T cell leukemia virus type-1 (HTLV-1) has been linked to the development of adult T-cell leukemia (ATL) and a chronic inflammatory disease called HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [1]. However, the mechanism of disease pathophysiology is still incompletely understood, and the treatments available are still unsatisfactory. Therefore, studies should be conducted to develop an effective method for preventing the occurrence of new infections, as well as to identify the mechanism of disease development and effective treatment following infection. This will require the development of a small animal model that can be exploited as a tool for the screening and evaluation of HTLV-1 infection. However, although HTLV-1 consistently infects rabbits [2,3], some non-human primates [4,5], and to a lesser extent, rats [6,7], the virus does not

* Correspondence: mineki@med.kawasaki-m.ac.jp
[1]Department of Immunology, Graduate School of Medicine, University of the Ryukyus, 207 Uehara, Okinawa 903-0215, Japan
[2]Department of Microbiology, Kawasaki Medical School, 577 Matsushima, Kurashiki, Okayama 701-0192, Japan
Full list of author information is available at the end of the article

efficiently infect murine cells. Previous studies have indicated that viral transmission in mice, using typical methods of infection, results in inconsistent infections and limited virus expression in tissues [8-10].

Here we established a novel mouse model to evaluate primary HTLV-1 infection of human lymphocytes *in vivo*. In this model, HTLV-1-negative healthy human peripheral blood mononuclear cells (PBMCs) (2×10^6/mouse) were transplanted directly into the spleens of severely immunodeficient NOD-SCID/γcnull (NOG) mice, together with cells from the mitomycin C (MMC)-treated HTLV-1-infected cell line ILT-M1 (1×10^6/mouse), which is an IL-2-dependent CD8+ T cell line derived from a HAM/TSP patient (kindly provided by Prof. Kannagi of Tokyo Medical and Dental University). Cell suspensions in a final volume of 50 μl were administered by intrasplenic injection (hereafter called hu-PBMC-NOG-spl mice). As previously reported [11], the severe immune deficiency of the NOG strain enables efficient engraftment of the human T cells, and a reduction in mouse death caused by severe graft-versus-host disease (GVHD), compared to those inoculated into the peritoneal cavity, which is the more common route of administration. In fact, all mice grew normally without piloerection or weight loss until 14 days after transplantation (i.e., the time of sacrifice).

First, we isolated the bulk spleen cells from hu-PBMC-NOG-spl mice sacrificed 14 days post inoculation/infection. Using flow cytometry (FCM), live cells were gated on their forward and side light scatter characteristics, and cell surface markers within the HLA-class I-positive population (i.e., human cells) were analyzed (Figure 1A). The numbers of recovered human cells (i.e. HLA-class I positive cells) from the mouse spleens were 1.48×10^7 (Donor #1), 1.29×10^7 (Donor #2) and 1.92×10^7 (Donor #3), respectively, which are much higher than the numbers of inoculated human cells, suggesting successful engraftment. The increased numbers of human T cells in the mouse spleens within two weeks after inoculation may have been caused by xenoreactive lymphocyte proliferation, since recent report by Søndergaard et al. suggested that injection of human PBMCs into NOG mice cause polyclonal expansion and activation of functional human T cells [12]. Meanwhile, human T cell expansion due to HTLV-1 is unlikely, since there is no clear difference in numbers of human T cells in the mouse spleens between mice treated with PBS (i.e., HTLV-1-infected) and mice treated with neutralizing antibodies (i.e., HTLV-1-uninfected) (data not shown). There tended to be higher frequencies of CD4-positive cells than CD8-positive cells (Figure 1B). Next, in order to confirm HTLV-1 infection, we isolated human CD4- and CD8-positive T cells by positive immunoselection from the bulk spleen cells, and then amplified a fragment of the HTLV-1 pX region by genomic PCR (Figure 1C). As shown in Figure 1C, similar to the naturally HTLV-1-infected PBMCs from healthy carriers and HAM/TSP patients, an HTLV-1 proviral DNA band was detected in all the isolated human CD4- and CD8-positive cell samples tested. We also performed RT-PCR in order to detect viral mRNA (tax and HBZ) in these human CD4- and CD8-positive T cells. As shown, all of the CD4 and CD8 cells tested expressed both tax and HBZ mRNA (Figure 1D). The poor visibility of tax mRNA bands of CD8 cells suggest that the possible contamination of residual ILT-M1 cells, which are positive for CD8 and strongly express tax mRNA, is unlikely. To further 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]. Next, 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 in Additional file 2: Figure S1, 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. The median proviral DNA copy numbers (proviral load: PVL) in 1×10^4 of both the human CD4 and human CD8 cells recovered from three hu-PBMC-NOG-spl mice, each inoculated with human PBMCs from different donors, were 9,533 and 4,546, respectively (i.e., 0.95 and 0.45 copies/cell, respectively), suggesting highly efficient cell-to-cell transmission of HTLV-1 from infected to uninfected human lymphocytes *in vivo*. Although a previous study also showed the successful engraftment of an HTLV-1-transformed cell line and uninfected PBMCs in NOG mice, the HTLV-1 PVL in spleen was very low and less than 1% of cells were infected with HTLV-1 [14]. In this previous study, 10^7 uninfected human PBMCs were injected intraperitoneally, and those PBMCs were infected with HTLV-1 by intraperitoneal inoculation of MMC-treated HTLV-1-infected MT-2 cells (10^3 or 10^4 cells/mouse) [14]. The different infection efficiencies between the previous and present studies clearly indicate that the efficient engraftment of the human T cells *in vivo* could be achieved by this route of inoculation. As shown in the present study, an intrasplenic transfer of human PBMCs can reduce the number of PBMCs required for the initial inoculation by approximately 1 log unit for the generation of more than 10^7 human T cells within two weeks, probably because human lymphocytes directly inoculated into the mouse spleen are efficiently activated, and thus HTLV-1 could efficiently infect human T cells *in vivo*. The microanatomic environment of the secondary lymphoid organs, such as the spleen, might also play an important role in the efficient cell-to-cell transmission of HTLV-1.

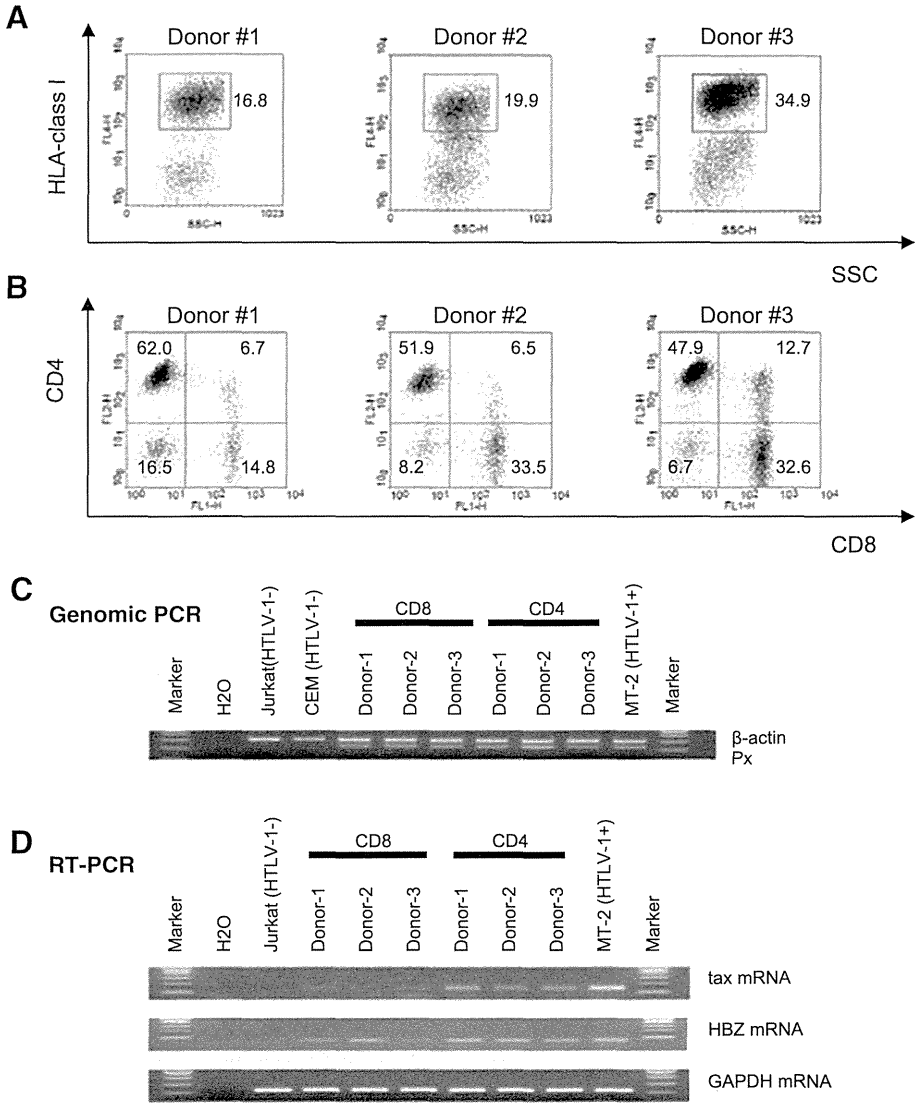

Figure 1 *In vivo* infection of HTLV-1 in engrafted human PBMCs in hu-PBMC-NOG-spl mice. A. Live cells were gated on their forward and side light scatter characteristics, and then cell surface markers within the HLA-class I-positive population were analyzed. **B**. There tended to be higher frequencies of CD4-positive cells than CD8-positive cells. The numbers represent the percentage of the cell population within the HLA-class I-positive gate. **C**. Genomic PCR to confirm HTLV-1 infection. Genomic DNA was extracted from human CD4 and CD8-positive T cells recovered from the spleens of hu-PBMC-NOG-spl mice sacrificed 14 days post infection, and then a fragment of the HTLV-1 pX region was amplified. β-actin was used as a control. The lower limit of detection was one copy of HTLV-1 tax per 10^4 PBMCs. **D**. RT-PCR to confirm HTLV-1 infection. RNA was extracted from human CD4 and CD8-positive T cells recovered from the spleens of hu-PBMC-NOG-spl mice sacrificed 14 days post infection. cDNA was synthesized and amplified from HTLV-1 tax and the HBZ region as described previously [15]. GAPDH was used as a control.

It is well known that viral gene transcription of HTLV-1 in vivo is suppressed in the PBMCs of most HTLV-1-infected individuals [16]. To test whether this phenomenon occurs even in hu-PBMC-NOG-spl mice, we examined HTLV-1 transactivator Tax protein expression in fresh and cultured human lymphocytes recovered from the spleens of infected hu-PBMC-NOG-spl mice by FCM. Similar to naturally HTLV-1-infected cells from healthy carriers and HAM/TSP patients [16], the fresh human lymphocytes recovered from the mouse spleens expressed very low levels of Tax protein (Figure 2A, upper panel). However, Tax expression was rapidly induced after short-term (16 h) cultivation ex vivo (Figure 2A, lower panel). Furthermore, these Tax-expressing CD4-positive cells were more frequently positive for chemokine (C-C motif) receptor 4 (CCR4) than Tax-negative CD4-positive cells (Figure 2B), as previously reported in natural HTLV-1 infections [17,18]. However, although most of the CD4-

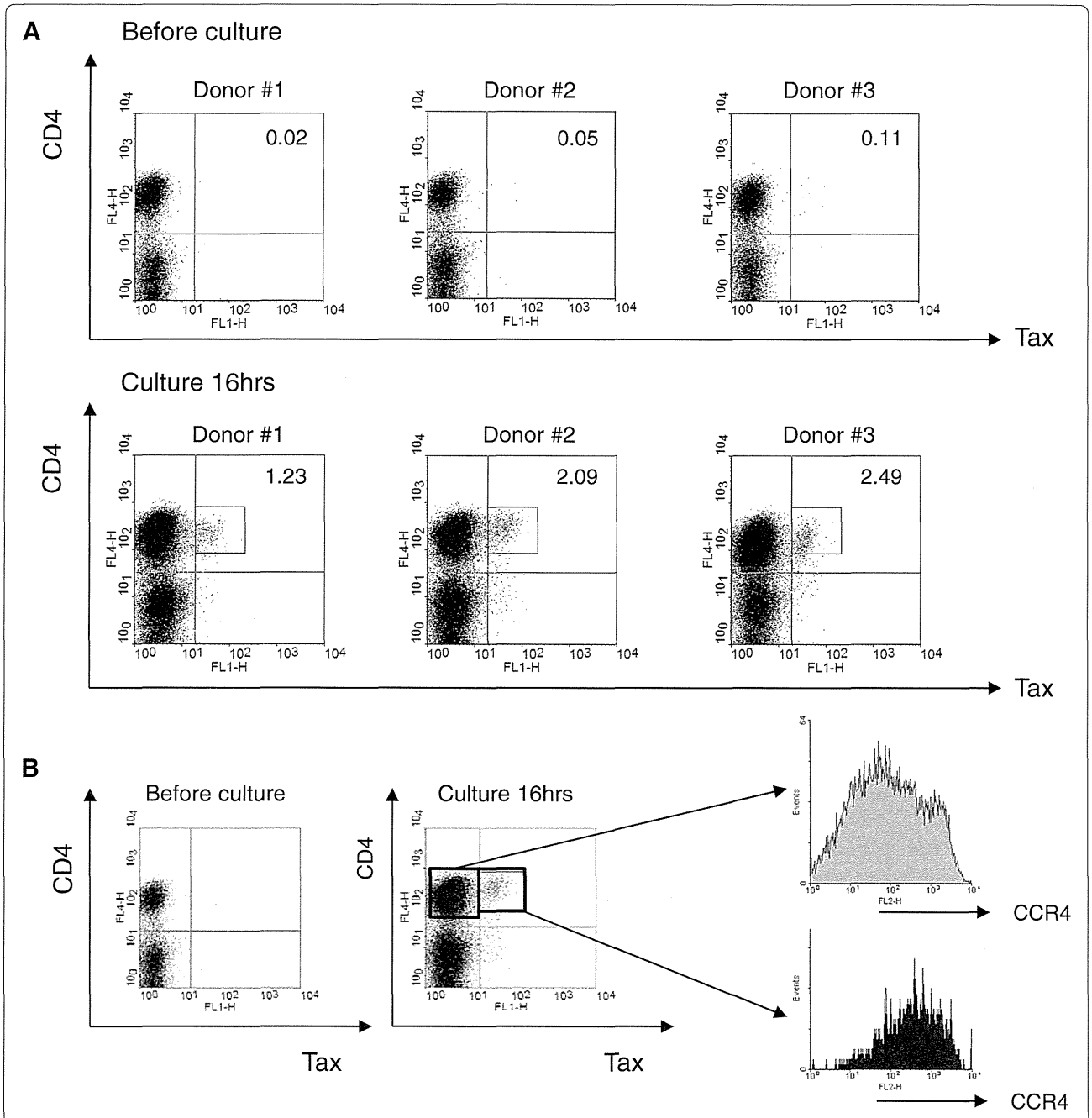

Figure 2 Characteristics of HTLV-1-infected human T cells recovered from hu-PBMC-NOG-spl mice. Tax protein expression in human lymphocytes recovered from the spleens of hu-PBMC-NOG-spl mice by flow cytometry. **A.** Human lymphocytes recovered from mouse spleens express very low levels of Tax protein (upper panel). After a short-term (16 h) cultivation ex vivo, Tax expression was rapidly induced (lower panel). The numbers represent the percentage of the Tax-positive cell population within the HLA-class I-positive gate. **B.** Tax-expressing cells are more frequently positive for CCR4 than Tax-negative cells.

positive T cells recovered from mouse spleens were infected with HTLV-1, the number of Tax positive cells after ex vivo culture appeared to be small. This observation might be attributed to the culture conditions of this experiment. Specifically, we cultured whole cells isolated from the recipient mouse spleens, indicating the mixed cultures of inoculated human PBMCs and mouse cells including stromal cells. Recently, Kinpara et al. reported that expression of HTLV-1 in HTLV-1-infected T cells is markedly suppressed at both the mRNA and protein levels upon co-culture of human cells and mouse stromal cells, in part via the type I interferon

(IFN) response [19]. It is therefore plausible that the observed small number of Tax-expressing cells after ex vivo culture is likely due to co-culture with mouse stromal cells derived from the spleen. Meanwhile, we observed that the percentage of Tax-expressing cells in the same culture conditions varies from one patient to another even in HAM/TSP patients with similar PVL (Saito et al., unpublished data). Furthermore, the severely immune-deficient NOG mice used in this study do not have any acquired immune response against inoculated HTLV-1 infected cells, such as HTLV-1-specific Abs, helper T cells, and cytotoxic T lymphocytes. These observations suggest that not only culture conditions but also cellular factors might be involved in the number of Tax-expressing cells. It also needs to be clarified whether the small number of Tax-expressing cells can be explained by multiple infection of single cell. Further investigations of such factors would be important for controlling HTLV-1 infection and disease development in vivo.

It is well established that the HTLV-1 virions are not very infectious, and thus cell-to-cell transmission is more efficient both *in vivo* and *in vitro* [20,21]. The surface glycoproteins of HTLV-1, which are recognized by neutralizing antibodies, play important roles in cell-to-cell transmission [22,23]. Indeed, previous reports have indicated that passive transfer of HTLV-1 Env-specific-neutralizing antibodies is effective in preventing *in vivo* infection in macaques [5,24] and rabbit [25,26] models. However, these studies evaluated the in vivo transmission of HTLV-1 to non-human cells, which are more resistant to HTLV-1 infection than human cells are. In this study, we tested the protective efficacy of various anti-HTLV-1 antibodies against HTLV-1 transmission into human lymphocytes *in vivo* in the hu-PBMC-NOG-spl mouse model. The mice immunized with the anti-HTLV-1 gp46 neutralizing mAb (clone LAT-27) were completely protected against HTLV-1 infection whereas other non-neutralizing antibodies such as anti-gp46 mAb (clone LAT-25), anti-Gag (clone GIN-7), anti-HCV (clone MO-8), and anti-OX40 mAb (clone B-7B5) did not protect against infection (Figure 3A). The HTLV-1 proviral DNA was not detected by quantitative real-time PCR in the human lymphocytes recovered from hu-PBMC-NOG-spl mice that received passive transfer of LAT-27, indicating that the neutralizing function is an essential factor in preventing in vivo HTLV-1 transmission. Furthermore, passive immunization with human polyclonal anti-HTLV-1 IgG from HAM/TSP patients (HAM-IgG) can also protect against HTLV-1 infection in vivo, whereas human immunoglobulin isolated from HTLV-1-negative donors (NC-IgG) did not (Figure 3A). Consistent with the results of the quantitative real-time PCR, FCM studies also showed that the human CD4-positive cells recovered from mouse spleens immunized with either LAT-27 or HAM-IgG, express only trace amounts of Tax protein after short-term (16 h) cultivation *ex vivo*, which may be the result of background false-positive staining. In contrast, a significant amount of Tax protein was expressed in human lymphocytes recovered from non-immunized mouse spleens (PBS-injected) or mouse spleens immunized with NC-IgG (Figure 3B). These results demonstrate the requirement for the neutralizing function of the anti-HTLV-1 antibody in preventing *in vivo* transmission. It is noteworthy that neutralizing anti-Env gp46 clone LAT-27 and HAM-IgG completely blocked the in vivo transmission of HTLV-1 in human lymphocytes, even in the conditions that permit the vigorous proliferation of human lymphocytes that enables HTLV-1 to rapidly spread by cell-to-cell contact. However, antibody injection only once after PBMC transplantation did not block the HTLV-1 infection in vivo, suggesting that the pre-existing neutralizing anti-Env Abs are critical for preventing HTLV-1 infection (Additional file 3: Figure S2). This result also suggests that *in vivo* transmission is established within 24 hours after transfer of HTLV-1-infected cells. Importantly, although neutralizing Abs used in this study displayed antibody-dependent cell-mediated cytotoxicity (ADCC) activity in vitro in our previous study [27], such neutralizing and ADCC activities of anti-Env Abs are not crucial for the elimination of HTLV-1-infected cells once HTLV-1 infection is established *in vivo*. Indeed, titers of existing neutralizing and ADCC Abs did not correlate with HTLV-1 PVL (i.e., numbers of HTLV-1-infected cells in vivo) (Saito et al., unpublished data). Moreover, HAM/TSP patients also showed high titers of such Abs, indicating that these Abs are not potent in preventing the onset of HAM/TSP in infected individuals. These data also indicate the importance of passive immunization before infection.

Recently, we reported that both LAT-27 and HAM-IgG, but not non-neutralizing LAT-25 and NC-IgG, are capable of depleting and/or eliminating HTLV-1-infected cells in the presence of autologous PBMCs in vitro. This occurs in part via ADCC, preventing the spontaneous immortalization of T cells [27]. Thus, the neutralizing activity is essential for preventing HTLV-1 infection as well as malignant transformation. In the present study, although non-neutralizing anti-Env gp46 (clone LAT-25) and anti-Gag p19 (clone GIN-7), as well as control antibodies (anti-HCV clone MO-8, anti-OX40 clone B-7B5), and normal human IgG (NC-IgG) did not completely block the infection, we observed that non-neutralizing LAT-25 mAb and anti-OX40 mAb decreased the number of HTLV-1 infected cells to some extent (Figure 3A). Since OX40 is a cell-surface molecule specifically expressed on HTLV-1-infected and activated T cells [28], and LAT-25 recognizes the HTLV-1 Env protein, these data may suggest a novel effect of IgG specifically reacting with a

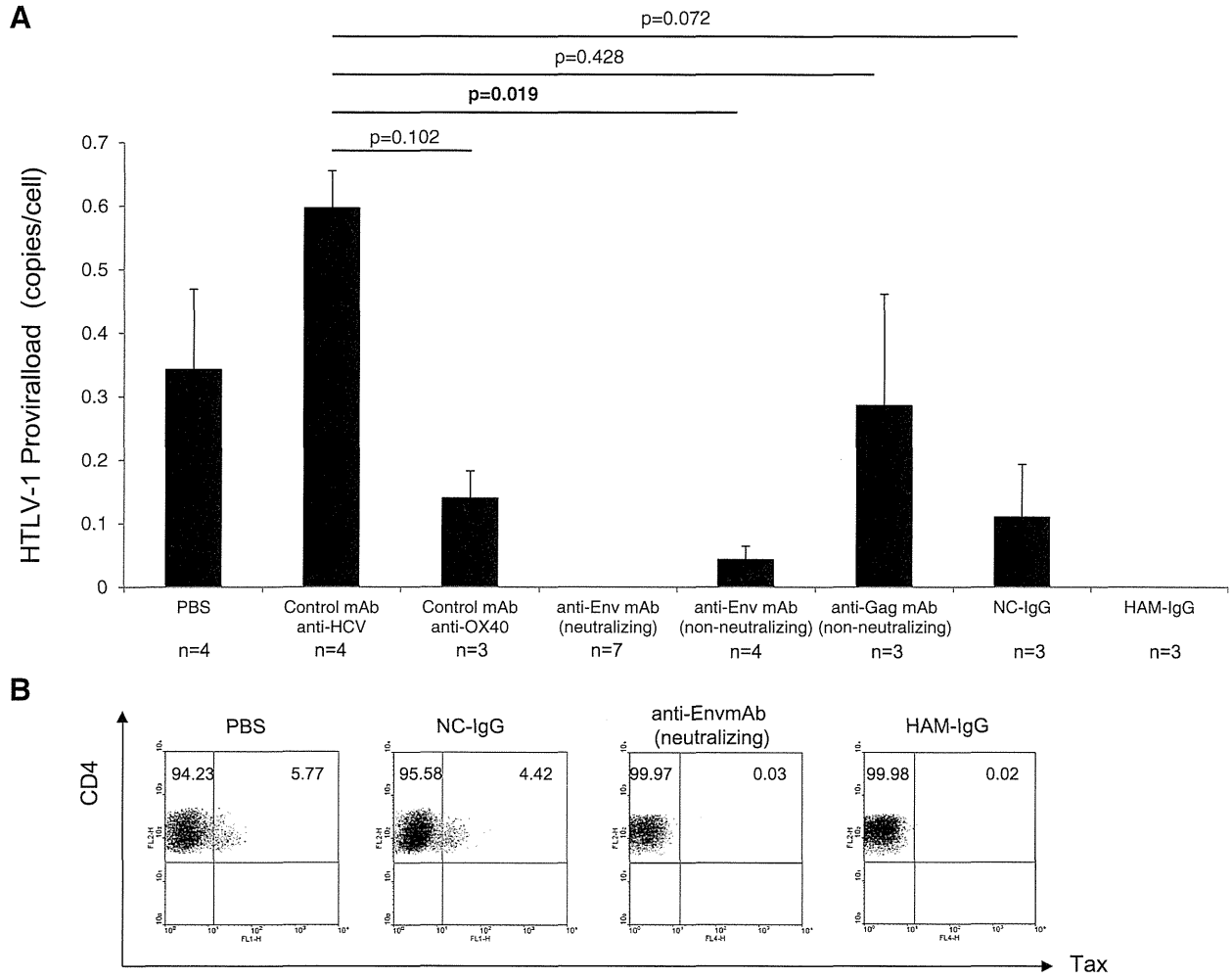

Figure 3 HTLV-1 infection in hu-PBMC-NOG-spl mice was completely inhibited by neutralizing, but not non-neutralizing, antibodies. *In vivo* transmission of HTLV-1 and protective efficacy of various monoclonal antibodies was evaluated using quantitative real-time PCR analysis of HTLV-1 proviral DNA. Genomic DNA was extracted from the human lymphocytes recovered from hu-PBMC-NOG-spl mice. **A**. All of the mice immunized with neutralizing mAbs against Env (clone LAT-27) were completely protected against HTLV-1 infection, whereas non-neutralizing mAbs against Env (clone LAT-25), anti-Gag (clone GIN-7), anti-HCV (clone MO-8), or anti-OX40 mAb (clone B-7B5) did not protect against infection. The mice immunized with human immunoglobulin isolated from HAM/TSP patients (HAM-IgG) were also protected against HTLV-1 infection, whereas human IgG isolated from normal uninfected controls (NC-IgG) did not protect against infection. Results are shown as mean ± SE. To test for significant differences among the different groups, one-way analysis of variance was performed, followed by Scheffe's multiple comparisons test. The lower limit of detection was one copy of HTLV-1 tax per 10^4 PBMCs. **B**. Flow cytometric studies indicated that the human lymphocytes recovered from mouse spleens immunized with anti-Env neutralizing mAbs or HAM-IgG express only a trace amount of Tax protein after short-term (16 h) cultivation *ex vivo*, which may be a background false-positive staining artifact. In contrast, a significant amount of Tax protein was expressed in human lymphocytes recovered from non-immunized mouse spleens (PBS-injected) or mouse spleens immunized with NC-IgG. The numbers represent the percentage of the cell population within the HLA-class I-positive/CD4-positive gate.

number of membrane receptors on HTLV-1-infected and/or activated T cells *in vivo*. As shown in Figure 3A, human IgGs isolated from uninfected people also suppressed the PVL, indicating that the administered non-specific IgGs also can help to eliminate the inoculated HTLV-1-infected cells (i.e., ILT-M1 cells). It is well established that the intravenous immunoglobulins (IVIg) therapy is effective in various diseases including autoimmune diseases and life-threatening infections. Although the precise mechanism of action of injected IVIg is not fully understood, several pathophysiological mechanisms such as suppression of idiotypic antibodies, saturation of Fc receptors on macrophages, modulation of complement activation, and suppression of various immunomodulators such as cytokines, chemokines, and metalloproteinases have been proposed [29]. It is therefore possible that the administered non-specific immunoglobulin in our mouse model also can help to eliminate the inoculated HTLV-1-infected

cells (i.e. ILT-M1 cells) via unknown mechanisms, resulting in a decreased efficiency of *in vivo* infection.

In conclusion, we have established a novel and simple small animal model to study primary HTLV-1 infection in vivo. Although our mouse model is not the animal models of HAM/TSP or ATL, the present study has demonstrated an important rational basis for passive immunization against HTLV-1 infection in humans. Using our mouse model, *in vivo* evaluation of the efficacy of drug candidates could also be investigated in future studies.

Additional files

Additional file 1: Table S1. Sequences flanking the integration site of HTLV-1 provirus in ILT-M1 cell and primer sequences used for integration site-specific PCR.

Additional file 2: Figure S1. 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.

Additional file 3: Figure S2. 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.

Abbreviations

HTLV-1: Human T-cell leukemia virus type-1; ATL: Adult T-cell leukemia; HAM/TSP: HTLV-1-associated myelopathy/tropical spastic paraparesis; ADCC: Antibody-dependent cellular cytotoxicity; PVL: Proviral load.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MS designed and performed the experiments, analyzed the data, and wrote the paper; RT, HF, AK and YT performed experiments, analyzed and interpreted data; TM and HT provided clinical samples and assembled clinical database; YT made contribution to the conception and design of the study, and wrote the paper. All authors read and approved the final manuscript.

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Author details

[1]Department of Immunology, Graduate School of Medicine, University of the Ryukyus, 207 Uehara, Okinawa 903-0215, Japan. [2]Department of Microbiology, Kawasaki Medical School, 577 Matsushima, Kurashiki, Okayama 701-0192, Japan. [3]Department of Neurology and Geriatrics, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan.

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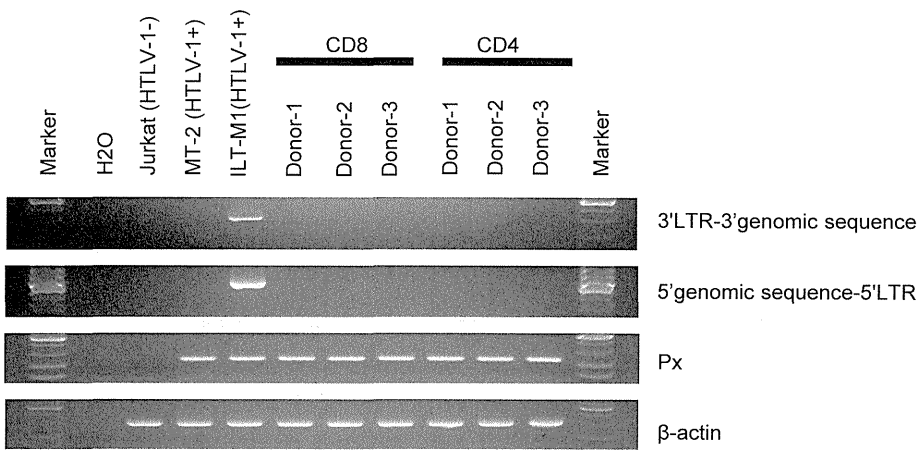
Supplemental Table 1

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

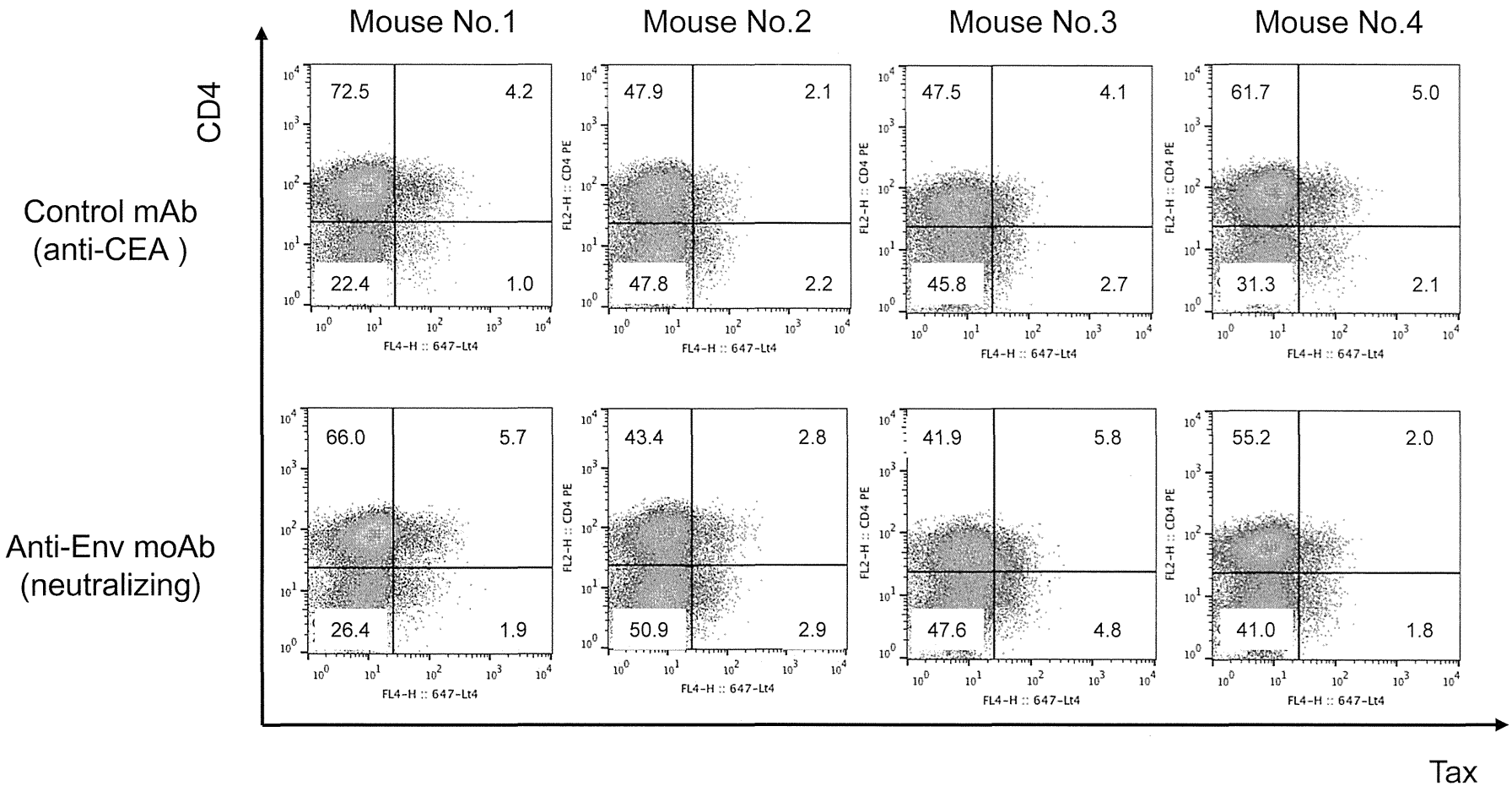
5'-genomic region	5'-LTR	3'-LTR	3'-genomic region
TGCTTTGTCATCTGTGCGTTCAGTTCA	**TGACAATGACCATGAGCCCCAAATATC**	**TCCAGGAGAGAAACTTAGTACACA**	AGTTCACAGAGTTTCACCTTTCTCTTCA
Forward Primer for the 5'-genomic region	Reverse Primer for the 5'-LTR	Forward Primer for the 3'-LTR	Reverse Primer for the 3'-genomic 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

Guangyong Ma[a], Jun-ichirou Yasunaga[a,1], Hirofumi Akari[b], and Masao Matsuoka[a,1]

[a]Laboratory of Virus Control, Institute for Virus Research, Kyoto University, Kyoto 606-8507, Japan; and [b]Center for Human Evolution Modeling Research, Primate Research Institute, Kyoto University, Inuyama, Aichi 484-8506, Japan

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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.

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[1]To whom correspondence may be addressed. Email: mmatsuok@virus.kyoto-u.ac.jp or jyasunag@virus.kyoto-u.ac.jp.

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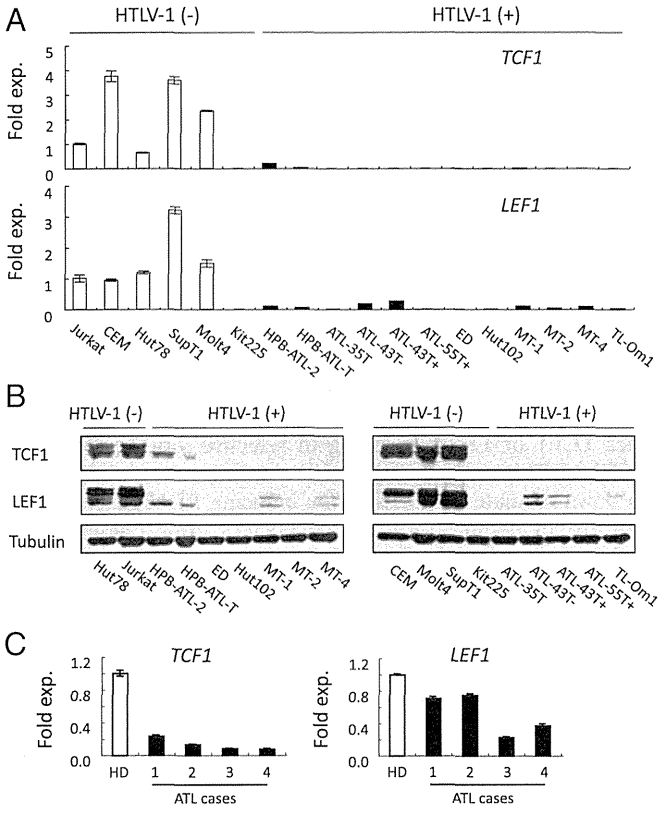

Fig. 1. TCF1 and LEF1 are expressed at low levels in HTLV-1–infected T cells. (*A*) TCF1 and LEF1 mRNA expression is invariably low in HTLV-1–infected cell lines. Total RNA was extracted for each cell line and subjected to quantitative real-time PCR (qPCR) analysis. Results are shown as relative mRNA 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

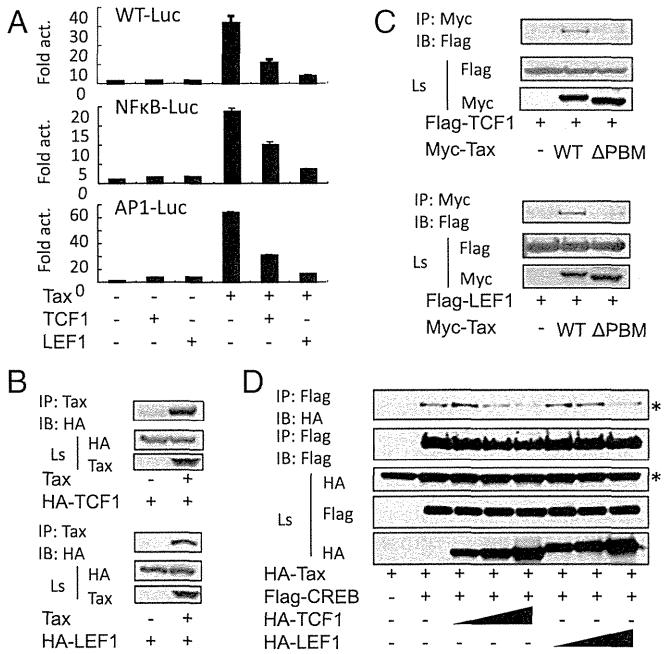

Fig. 2. TCF1 and LEF1 each interact with Tax and impair its transactivating ability. (*A*) TCF1 and LEF1 each repress Tax-mediated activation of WT-Luc (*Top*), NFκB-Luc (*Middle*), and AP1-Luc (*Bottom*). Reporter assays were performed in Jurkat cells. (*B*) Physical interactions between TCF1 and Tax (*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.

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