

antibodies. To optimize the syncytium inhibition assay, we screened various coculture combinations of HTLV-1-producing cells with a variety of HTLV-1-negative target cells, and selected the HTLV-1-producing T cell line ILT-M1 and the HTLV-1-negative T cell line Jurkat. Overnight coculture of the ILT-M1 and Jurkat cells resulted in the generation of numerous large syncytia (Fig. 1). Using this assay system, we titrated the syncytia-blocking activity of monoclonal LAT-27 and polyclonal IgG purified from pooled plasma from HAM patients (HAM-IgG). HAM-IgG was used as a positive anti-HTLV-1 antibody control because it contained high titers of antibodies against HTLV-1 antigens (Supplementary Fig. S2). The minimum concentrations required for the “complete” inhibition of syncytia formation by LAT-27 and HAM-IgG antibodies were calculated to be 5 $\mu\text{g/ml}$ and 50 $\mu\text{g/ml}$, respectively (Supplementary Fig. S3). To adjust for decay in antibody activities during cultivation at 37°C, we used LAT-27 and HAM-IgG at concentrations of 10 and 100 $\mu\text{g/ml}$, respectively, in all subsequent experiments.

To confirm the gp46 specificity of LAT-27 and HAM-IgG in this syncytium inhibition assay, an affinity-column-purified gp46 antigen³⁶ was added to an aliquot of either LAT-27 or HAM-IgG solution prior to cocultivation. Controls consisted of incubating an aliquot of the cocultures in media alone (shaded bars denoted by 0) or media containing 10 $\mu\text{g/ml}$ of gp46 (dark bars also denoted by 0). As shown in Fig. 2, HAM-IgG incubated in media alone clearly inhibited syncytia formation in a dose-dependent manner (at 12.5 ~ 100 $\mu\text{g/ml}$). However, preincubation of the HAM-IgG at 12.5 ~ 100 $\mu\text{g/ml}$ with 10 $\mu\text{g/ml}$ of affinity-purified gp46 resulted in significant reversal of inhibition, suggesting that gp46 was the main target for the neutralization activity present in the human anti-HTLV-1 antibodies. Similar results were obtained when LAT-27 instead of HAM-IgG was preincubated with gp46 (data not shown).

LAT-27 as reported previously²⁹ and HAM-IgG completely inhibited HTLV-1-mediated T cell immortalization of

normal activated T cells *in vitro* at concentrations of 10 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$, respectively (Fig. 3).

Effect of antibodies on spontaneous HTLV-1 antigen expression in vitro

To evaluate the role of anti-gp46 neutralizing antibodies against T cells carrying endogenous HTLV-1 from naturally infected donors, we cultured freshly isolated PBMCs from HAM/TSP patients in the presence or absence of various antibodies for 24 h. To exclude any potential effects of CD8⁺ CTL that are present within the PBMCs from the HAM/TSP patients,¹⁰ PBMCs were depleted of CD8⁺ T cells prior to the assay. For quantitation of the frequencies of HTLV-1 antigen-expressing cells, we stained an aliquot of the cells for the expression of intracellular Tax antigen utilizing our standardized anti-Tax mAb, which has generally been used to detect HTLV-1-infected cells.^{10,38} At a concentration of 10 $\mu\text{g/ml}$, LAT-27 reduced the frequency of Tax⁺ cells (Fig. 4A). This reduction was antigen and epitope specific since neither the isotype control rat IgG2b mAb (anti-HCV envelope) nor the anti-gp46 nonneutralizing mAb (LAT-25) and the other anti-gp46 nonneutralizing mAbs (clones LAT-12 and MET-3) that compete with LAT-27 in an antibody binding assay showed any detectable inhibitory effect (data not shown). The reduction in the frequency of Tax⁺ cells by LAT-27 was partially reversed by a mixture of anti-CD16 and anti-CD32 mAbs when added at the initiation of the assay, suggesting an involvement of Fc receptors in this reduction assay.

As shown in Fig. 4B, after prolonged culture (2 weeks) the suppressive effect of LAT-27 became more evident since there remained few if any Tax⁺ cells in the LAT-27-treated cultures of PBMCs from each of the HAM patients tested. A similar suppressive effect was observed for HAM-IgG but not normal human IgG (Fig. 4B). It should be noted that in the present culture conditions, similar to what has been generally observed for the PBMC cultures from HTLV-1-infected

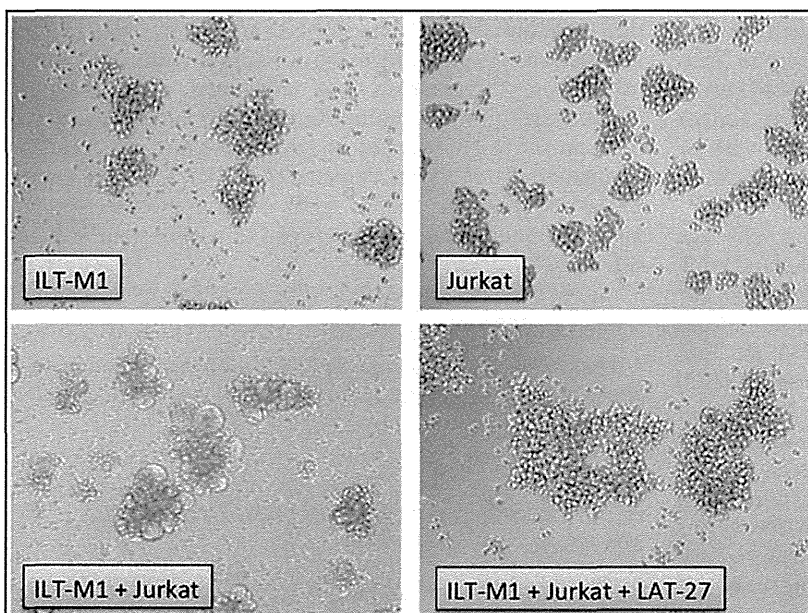


FIG. 1. Human T cell leukemia virus type-1 (HTLV-1)-mediated syncytia formation. HTLV-1⁺ ILT-M1 and HTLV-1⁻ Jurkat cells were either cultured alone or cocultured at a cell-to-cell ratio of 1:1 in the presence or absence of 10 $\mu\text{g/ml}$ LAT-27 for 18 h. Syncytia were microscopically observed using an inverted microscope at a magnification of 100 \times . Representative data from three independent experiments are shown.

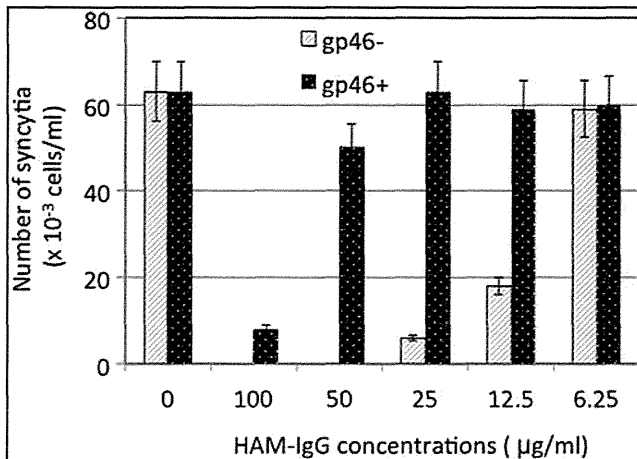


FIG. 2. Anti-gp46 antibodies are major HTLV-1 neutralizing antibodies in HAM-IgG. HAM-IgG at graded concentrations (0~100 µg/ml) was preincubated with either affinity-purified gp46 antigen (black bars) at 10 µg/ml for 10 min or incubated with medium alone (gray shaded bars, labeled as “gp46-”) and tested for syncytia inhibition activity. The numbers of syncytia were manually counted using a “Burker-Turk” hemocytometer. Representative data from three independent experiments are shown.

donors, the frequency of Tax⁺ cells gradually decreased during 2 weeks in culture even in IL-2 medium alone and thus it was not likely due to an effect of the addition of the control rat isotype IgG or normal IgG. Spontaneous immortalization of T cells by HTLV-1 was observed in the PBMC cultures from two-thirds of the HAM patients treated with medium

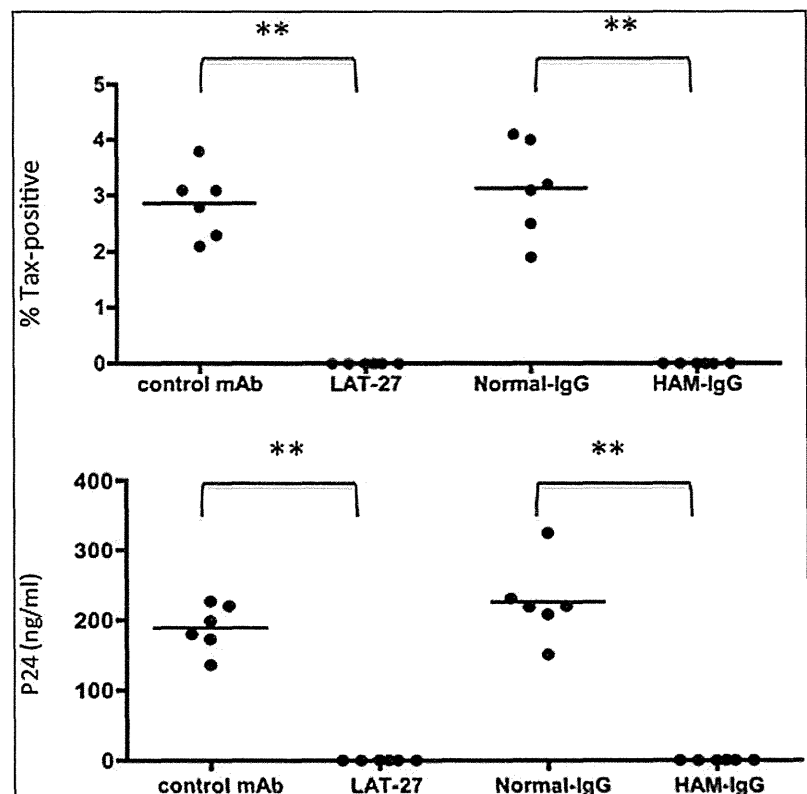
alone, isotype control, or normal IgG, but not in those treated with LAT-27 or HAM-IgG, as judged 6 weeks after culture (data not shown).

Importantly, neither LAT-27 nor HAM-IgG (data not shown) showed any detectable blocking effects on HTLV-1 Tax expression (Fig. 5) and p24 antigen production (data not shown) in long-term cultured HTLV-1-infected cell lines. It is known that the levels of Tax-positive cells vary depending on the cell lines being utilized due to DNA methylation, hypoacetylation of histones, or epigenetic changes of provirus.³⁹ Thus, we reasoned that the addition of the neutralizing antibodies blocks *de novo* expansion of HTLV-1 infection and/or eliminates the HTLV-1 gp46 expressing T cells via an FcR-dependent pathway in combination with effector cells contained within the PBMCs.

FcR-dependent elimination of HTLV-1-infected cells by antibodies

Due to the limitations on the availability of PBMC samples from HAM/TSP patients, we established a number of IL-2-dependent HTLV-1-infected CD4⁺ T cell lines from PBMCs of normal donors to determine whether ADCC was involved in the suppression of HTLV-1-infected cells. These HTLV-1⁺CD4⁺ T cell lines were cocultured with autologous fresh PBMCs in the presence or absence of various antibodies including F(ab')₂ of LAT-27, which showed HTLV-1 neutralization at a minimum concentration of 2.5 µg/ml (data not shown). HAM-IgG was included as an ADCC-positive control. After 3 days in culture, these cells were stained for cell surface CD4 and intracellular Tax antigen, and analyzed on a gated population of cells that displayed high forward and side scatters, which included a majority of the HTLV-1-infected

FIG. 3. LAT-27 and HAM-IgG completely block HTLV-1-mediated T cell immortalization *in vitro*. Activated peripheral blood mononuclear cells (PBMCs) from normal donors were seeded into six wells of 96-well U-bottom plates (1 × 10⁵ cells/0.1 ml/well) and cocultured with an equal number of mitomycin C-treated ILT-M1 cells in the presence or absence of 10 µg/ml of LAT-27 or rat isotype control, 100 µg/ml of normal human IgG, or HAM-IgG. Half of the medium was replaced every 3~5 days with new similar fresh media, and if necessary, cultures were split into 1:2. Each data point reflects the frequency of Tax⁺ cells or the levels of p24 in the culture supernatants of each well 6 weeks after culture. Data shown are representative of three independent experiments. The differences between the controls and the experimental data were highly significant, denoted as ***p* < 0.01. The negative control used for LAT-27 was an isotype control (rat IgG2b anti-HCV).



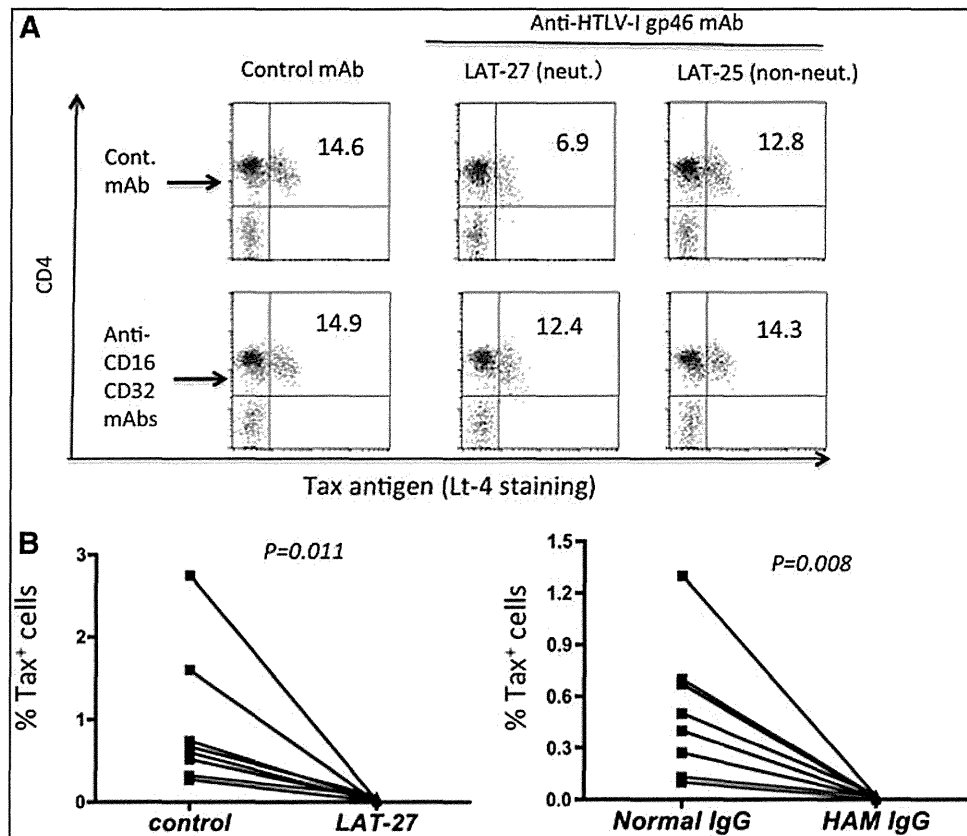


FIG. 4. Reduction of Tax-expressing cells in *in vitro* cultures of PBMCs from HAM patients in the presence of HTLV-1 neutralizing monoclonal antibody (mAb). (A) PBMCs from HAM patients were depleted of CD8⁺ T cells and cultured *in vitro* for 24 h at 1×10^6 cells/ml in interleukin (IL)-2-containing medium in the presence or absence of 10 μ g/ml antibodies indicated in the figure. The cells were then stained for cell surface CD4 and intracellular Tax antigen as described in the Materials and Methods section. The numbers in each dot-plot show the percentage of CD4⁺ Tax⁺ cells. The mixture of antibodies against human Fc receptors (FcR) (anti-CD16 and CD32) was added to block FcR function. Data shown are representative of three independent experiments using PBMCs from different donors. (B) PBMCs from HAM patients ($n=8$) were depleted of CD8⁺ T cells and cultured *in vitro* in IL-2-containing medium in the presence of (1) LAT-27 or an isotype control mAb at 10 μ g/ml or (2) HAM-IgG or normal human IgG at 100 μ g/ml for 2 weeks. The cells were stained for Tax antigen and the total percentage of Tax⁺ cells was calculated. The control used for LAT-27 was an isotype control (rat IgG2b anti-HCV mAb). The negative control mAb for anti-CD16 and CD32 was mouse IgG1 against HIV-1 (clone 2C2).

CD4⁺ T cells but not normal PBMCs. However, no detectable reduction of Tax⁺ cells was observed in the cultures treated with either LAT-27 or HAM-IgG cocultured in the presence of PBMCs (data not shown). Thus, these cells were washed and cocultured again for an additional 3 days with the same antibodies and fresh PBMCs.

As shown in Fig. 6A, although fresh PBMCs alone reduced the frequency of Tax⁺ cells to some extent, a marked reduction was seen in the presence of LAT-27 and HAM-IgG. In a similar fashion, the production of HTLV-1 p24 in the culture supernatants was markedly reduced by LAT-27 and HAM-IgG in the presence of autologous PBMCs. As shown in Fig. 6B, when these cultures were exposed one more time to the same antibodies and fresh PBMCs, LAT-27 IgG and HAM-IgG, but not F(ab')₂ of LAT-27 or normal IgG, further reduced the frequency of Tax⁺ cells. These data suggest that the addition of LAT-27 as well as HAM-IgG eliminates the HTLV-1 gp46 antigen-expressing cells via an FcR-dependent manner while blocking the spread of HTLV-1 to new target cells including fresh PBMCs in the same cell cultures *in vitro*. The involvement of complement-dependent

cytotoxicity was ruled out because the fetal calf serum used in the present study was heat inactivated prior to use.

ADCC against HTLV-1-infected cells by LAT-27

To examine whether LAT-27 could mediate ADCC in the present culture conditions, IL-2-dependent HTLV-1-infected T cells established from normal donors were labeled with ⁵¹Cr and cocultured with fresh autologous PBMCs in the presence or absence of antibodies. Significant ADCC activity was induced by HAM-IgG, but not LAT-27, by 6 h (data not shown). However, after 24 h at a high effector-to-target cell ratio, LAT-27, but not the F(ab')₂ fragment of LAT-27, showed significant cytotoxicity ($p < 0.01$) (Fig. 7A). When the effector PBMCs were depleted of either CD16⁺ or CD56⁺ cells, but not CD14⁺ or CD19⁺ cells, the ADCC activity mediated by either LAT-27 or HAM-IgG was significantly reduced ($p < 0.01$) (Fig. 7B and C). These data suggest that the CD16⁺ CD56⁺ subpopulation of PBMCs [representing natural killer (NK) cells] were most likely the main effector cells involved in the cell lysis. These results

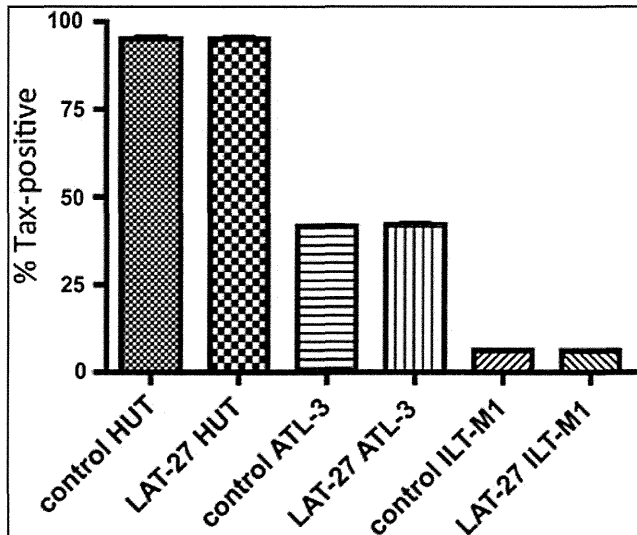


FIG. 5. LAT-27 alone does not affect long-term cultured HTLV-1-infected T cells. A standard HTLV-1-infected cell line HUT-102 (HUT), an IL-2-dependent CD4⁺ T cell line (ATL-3, generated from an ATL patient), and an IL-2-dependent CD8⁺ T cell line (ILT-M1) were cultured in the presence of 10 μ g/ml of either LAT-27 or isotype control (control) for 4 days, and the frequencies of Tax⁺ cells were determined by flow cytometry ($n=3$).

demonstrate that the monoclonal LAT-27, similar to the polyclonal HAM-IgG, is able to induce ADCC against HTLV-1-infected cells by autologous NK cells while protecting the spread of new infection with HTLV-1.

Discussion

The present study demonstrates that the monoclonal anti-HTLV-1 gp46 antibody clone LAT-27 generated by our laboratory mediates both HTLV-1 neutralization and HTLV-1-specific ADCC, and such ADCC activity might be capable of eliminating HTLV-1-infected T cells *in vitro* in the presence of autologous fresh PBMCs. Although fresh PBMCs alone showed a partial but significant inhibitory activity against HTLV-1-infected cells during prolonged *in vitro* cultivation, the data obtained here suggest that the HTLV-1-specific ADCC activity is the direct mechanism for this eradication. Similar suppressive activities were demonstrated for human IgG from HAM patients. This mechanism may explain the previous findings reported by Tochikura *et al.*²⁸ on the HTLV-1 suppressing activity of human anti-HTLV-1 antibodies. Furthermore, this mechanism may also explain in part why HTLV-1 antigen-expressing cells are not found *in vivo* in anti-HTLV-1 antibody-positive individuals. Although it is not known where and when HTLV-1 is produced *in vivo* in the infected individual, the continued presence of CD8⁺ T cells and antibodies specific for HTLV-1 indicates that HTLV-1 should be expressed periodically. Based on the results presented in this article, it might be possible that HTLV-1 expression occurs upon T cell stimulation in the periphery, but as soon as the cells express HTLV-1 gp46 antigen they might be instantly killed by the combination of anti-HTLV-1 ADCC-inducing antibodies and activated NK cells.

We submit that the addition of fresh PBMCs to the autologous HTLV-1-producing T cell cultures may result in it becoming readily infected and immortalized by HTLV-1. Thus, it is clear that the presence of neutralizing antibody is essential for the prevention of new infection of PBMCs and since ADCC effector mechanisms are functional during this time period, their contribution to the control of infection deserves merit. Interestingly, the ADCC induced by LAT-27 progressed slowly and the elimination of Tax⁺ cells became evident only after two consecutive exposures every 3 days in the present cell culture conditions. Since there was heterogeneity of the intensity of gp46 expression among cells in a single HTLV-1-infected cell line (data not shown), the findings suggest that the lysis of such gp46^{low} cells by ADCC requires a prolonged incubation period. Alternatively, since the repeated exposure against PBMCs resulted in an accumulation of live PBMCs, it is possible that a large number of effector fresh PBMCs might be required for the complete eradication by LAT-27, possibly due to the relatively low affinity of LAT-27 for human FcR.

Cell depletion experiments in the present study showed that the effector cells involved in the HTLV-1-specific ADCC in fresh PBMCs were either CD16⁺ or CD56⁺ cells, representing the cytolytic human NK cell subset, although it remains to be confirmed with purified NK cells. Because there are abundant circulating NK cells in the periphery in healthy donors, these findings strongly suggest that the HTLV-1-specific ADCC responses in the presence of neutralizing antibodies might have a role in controlling HTLV-1 *in vivo* in concert with HTLV-1-specific CTL responses in healthy HTLV-1 carriers. This view is supported by the findings that the ADCC effector function of PBMCs is lower in both HAM/TSP and ATL patients than healthy HTLV-1 carriers or normal donors,^{17,40} suggesting that defects in functional ADCC activities may contribute to the onset of HTLV-1-related diseases.

The level of ADCC of HTLV-1⁺ cells by LAT-27 was weaker than that induced by human polyclonal anti-HTLV-1 IgG. This might be due to the fact that LAT-27 is of rat origin and recognizes a single epitope on the gp46 (amino acids 191–196)²⁹ in contrast to the fact that HAM-IgG is of human origin and consists of high titers of polyclonal antibodies against multiple epitopes on gp46. In addition, it has been shown that mouse and rat IgG exhibit different ADCC activities with human NK cells depending on their subclasses, and that rat IgG2b (the subclass of LAT-27), but not IgG2a, triggers effective ADCC with human NK cells.⁴¹ Along these lines, it is possible that a humanized form of LAT-27 utilizing the human IgG1- or IgG3-Fc portion as a backbone would be far more effective than even the rat IgG2b of LAT-27.

This hypothesis has been confirmed by preliminary experiments using humanized LAT-27 consisting of human IgG1, which was generated in collaboration with Dr. Shimizu of IBL Inc. (Tanaka *et al.*, unpublished observations). In addition, epitope specificity and/or the affinity of anti-gp46 antibodies may also be involved in determining the ADCC-inducing activities. For example, LAT-25, which belongs to the rat IgG2b subclass and recognizes a C-terminal region of the gp46, did not eradicate HTLV-1⁺ cells (Fig. 7). Similarly, Kuroki *et al.* showed that a human mAb recognizing gp46 amino acids 191–196 (similar to the epitope recognized by LAT-27) could induce ADCC, but another human mAb

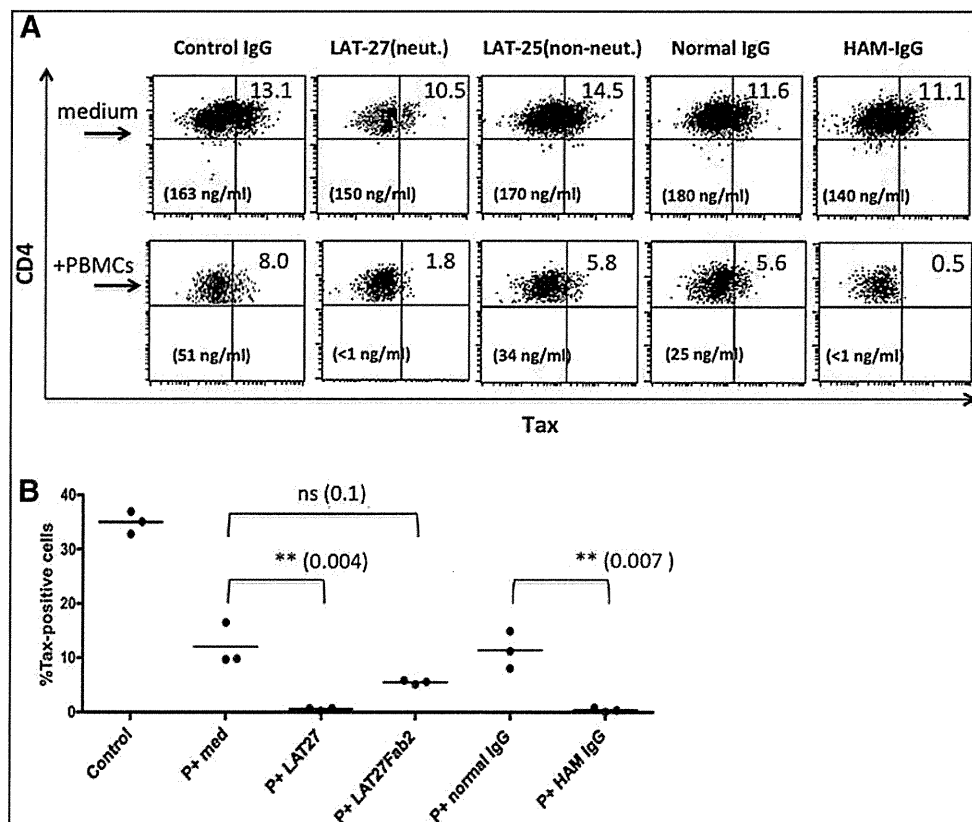


FIG. 6. Elimination of Tax⁺ cells and reduction of HTLV-1 p24 production in IL-2-dependent HTLV-1-infected T cells cocultured with autologous PBMCs in the presence of LAT-27 or HAM-IgG. **(A)** IL-2-dependent CD4⁺ HTLV-1-infected T cells established from the PBMCs of normal donors were repeatedly exposed to autologous PBMCs (+PBMCs) in the presence of 10 μ g/ml of LAT-27 or isotype control, or 100 μ g/ml of HAM-IgG, or normal human IgG twice at 3 day intervals. Two days after the second exposure, the high forward and side scatter gated populations of cells that contained a majority of the HTLV-1⁺ cells but not PBMCs were analyzed for the frequencies of CD4⁺ Tax⁺ cells. Percentages of CD4⁺ Tax⁺ cells are shown in the upper right quadrant. The numbers in parentheses show the levels of HTLV-1 p24 produced in the culture supernatants. Data shown are representative of three independent experiments using PBMCs from different donors. **(B)** As shown in **(A)**, IL-2-dependent CD4⁺ HTLV-1-infected T cells were cultured *in vitro* either alone (control) or exposed to autologous PBMCs (P+) in the presence of 10 μ g/ml of LAT-27 or F(ab')₂ LAT-27, or 100 μ g/ml of normal human or HAM-IgG in triplicate wells with three supplementations provided at 3 day intervals. Two days after the third exposure, the cells were examined for the frequencies of CD4⁺ Tax⁺ cells. Data shown are representative of three independent experiments using HTLV-1-infected cells and PBMCs from different donors.

recognizing the gp46 amino acids 187–193 could not, even though the two mAbs bind similarly to the cell surface of HTLV-1-infected cells and belong to the ADCC-inducing human IgG1.²²

It remains to be determined whether there are clonal populations of human IgGs that can mediate both the neutralization and ADCC against HTLV-1. So far, it has been shown that the two activities could be operating separately by different epitope-specific human mAbs against gp46.²² Recently, Kuo *et al.*²⁴ showed that both neutralizing and non-neutralizing mouse anti-gp46 mAbs can activate neutrophils and mediate its burst activity in the presence of an HTLV-1-infected MT-2 cell line, and concluded that HTLV-1-specific ADCC capacity is not coupled to the neutralizing capacity of the antibody. Thus, these articles highlight the finding of LAT-27 as a special antibody. Analyses of the conformational and antigenic structure of gp46 expressed on the cell surface will be necessary to address this issue further.

Another possible target for ADCC on HTLV-1-expressing cells is the envelope gp21; however, it has been unclear

whether human anti-gp21 antibodies function in ADCC. In addition, the recent finding that the glycosylation of Fc-IgG plays an important role in anti-HIV-1 ADCC effector mechanisms⁴² suggests that this issue needs to also be considered in the evaluation of anti-HTLV-1 gp46 antibodies and for vaccine formulations in general. Nevertheless, it is clear that the simultaneous operation of neutralization and ADCC by single or polyclonal antibodies is essential to recognize and eliminate HTLV-1⁺ cells since not only T cells but also the NK cells are permissive to HTLV-1 infection.⁴³

The present study also showed that fresh PBMCs had a partial and significant but not complete suppressive activity against autologous HTLV-1-infected cells in the absence of anti-HTLV-1 antibodies. Our preliminary experiments indicate that monocytes might be involved in this partial suppression because PBMCs depleted of CD14⁺ cells, but not of NK cells, were no longer suppressive in the absence of LAT-27 (data not shown). Since HTLV-1-infected T cells are continuously activated due to the Tax antigen, one possible mechanism is a monocyte-dependent cell death (MDCD)

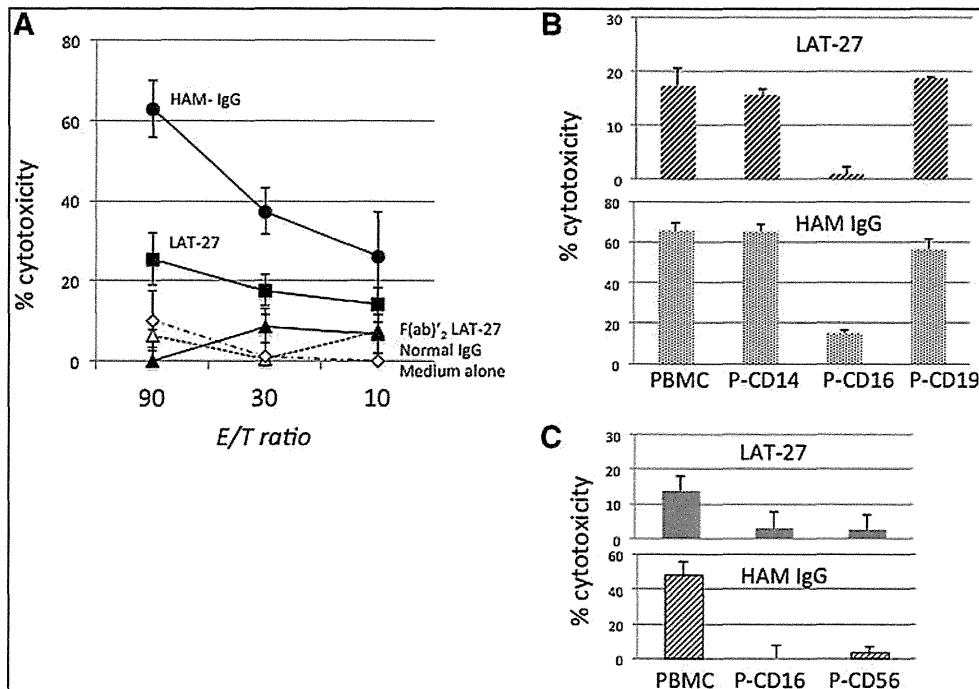


FIG. 7. The CD16⁺ CD56⁺ PBMCs mediate antibody-dependent cellular cytotoxicity (ADCC) in the presence of LAT-27 or HAM-IgG. (A) ⁵¹Cr-labeled HTLV-1-infected cells were cocultured *in vitro* with autologous fresh PBMCs at various E/T ratios in the presence or absence of 10 μg/ml of LAT-27 or F(ab)₂ LAT-27, 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 ⁵¹Cr-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 blocking²⁹ 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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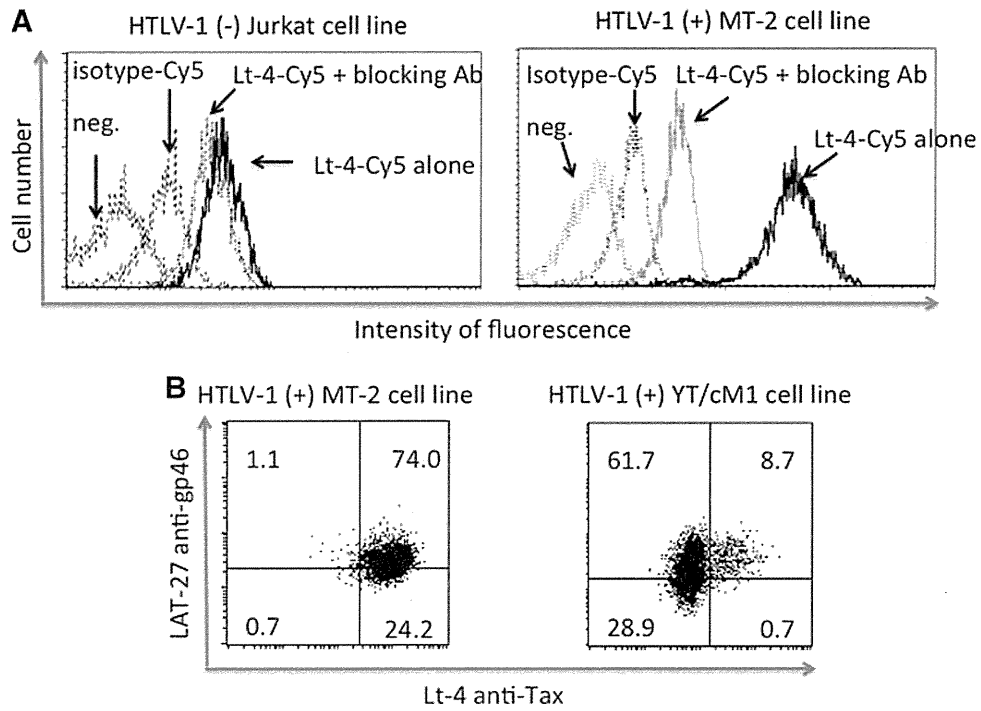
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Supplementary Data

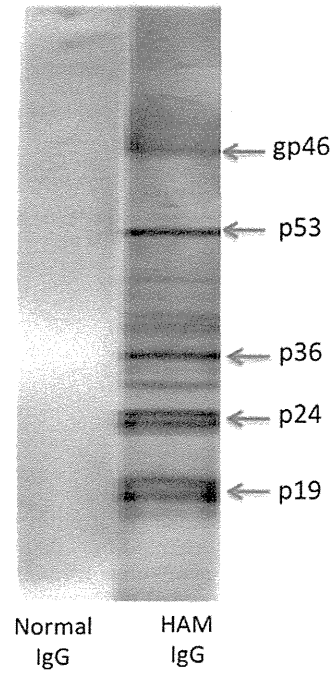


SUPPLEMENTARY FIG. S1. Flow cytometry of Tax and HTLV-I gp46 antigens. **(A)** Tax-specific and nonspecific staining by Cy5-labeled Lt-4. HTLV-I-negative Jurkat cells and HTLV-I-positive MT-2 cells were stained with either Cy5-labeled Lt-4 or Cy5-labeled mouse isotype control (IgG3) in the presence or 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.

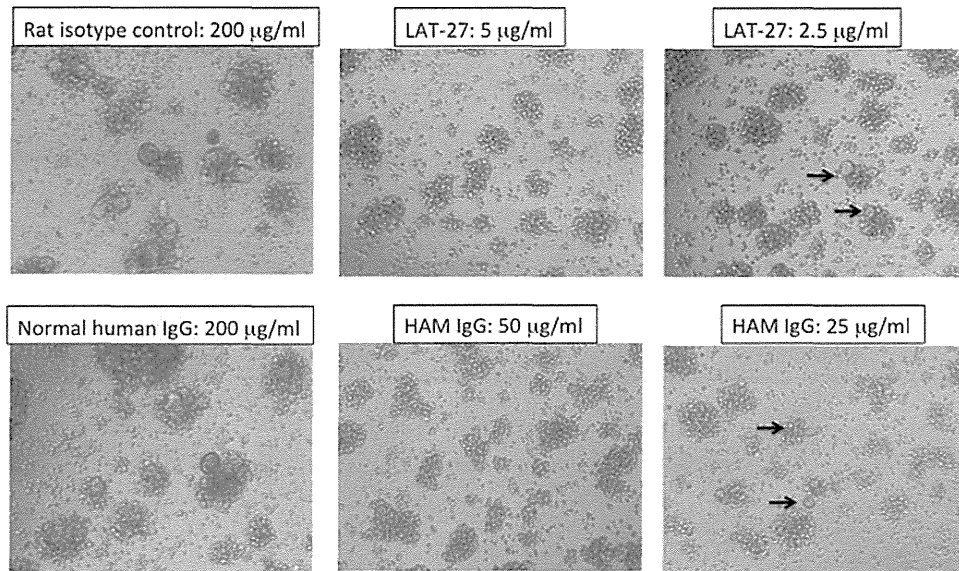
A HTLV-I-coated gelatin particle
agglutination assay
(SERODIA®HTLV-I)

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

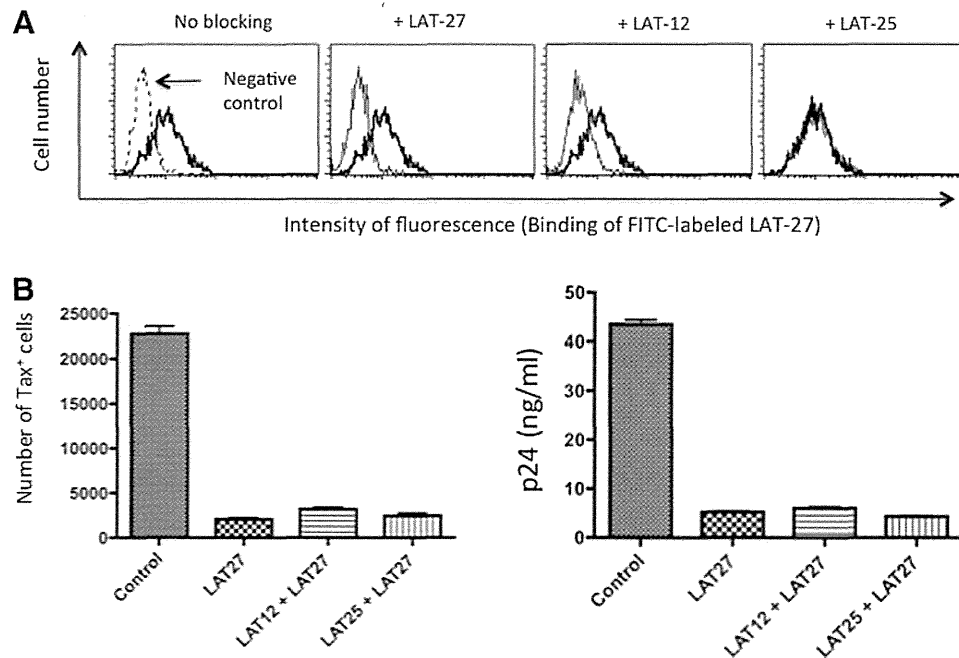
B Western blot analysis
(PROBLOT HTLV-I)



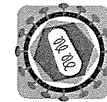
SUPPLEMENTARY FIG. S2. Characterization of anti-HTLV-I antibody profile of HAM-IgG. **(A)** Purified HAM-IgG at 1 mg/ml was serially diluted and subjected to a commercial anti-HTLV-I agglutination assay (SERODIA®HTLV-I, Fujirebio Inc.). Titers were expressed as the reciprocal dilution that showed a positive 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 $\mu\text{g/ml}$ of isotype control (control) or LAT-27 in the presence or absence of 100 $\mu\text{g/ml}$ 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) ($n=4$).



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/ γ cnul (NOG) mice

Mineki Saito^{1,2*}, Reiko Tanaka¹, Hideki Fujii¹, Akira Kodama¹, Yoshiaki Takahashi¹, Toshio Matsuzaki³, Hiroshi Takashima³ and Yuetsu Tanaka¹

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/ γ cnul (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

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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/ γ cnnull (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 μ l 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.

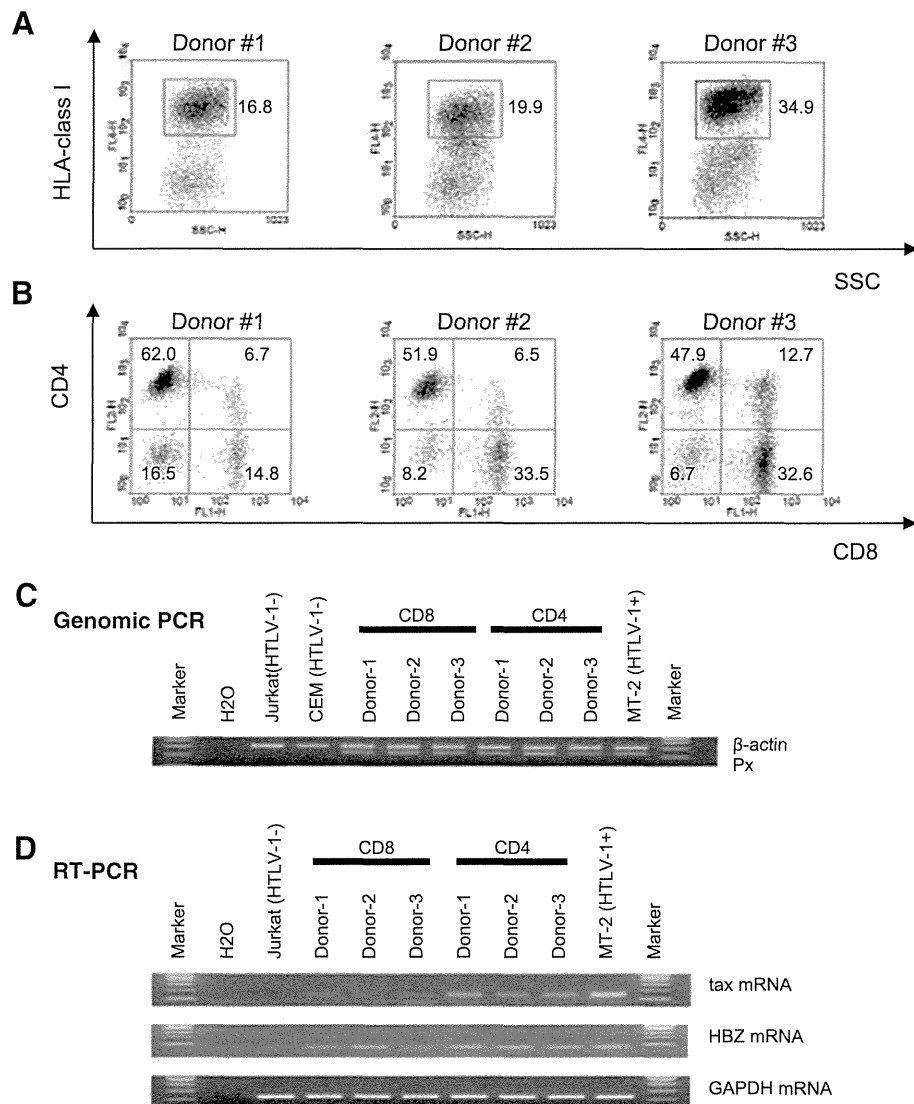
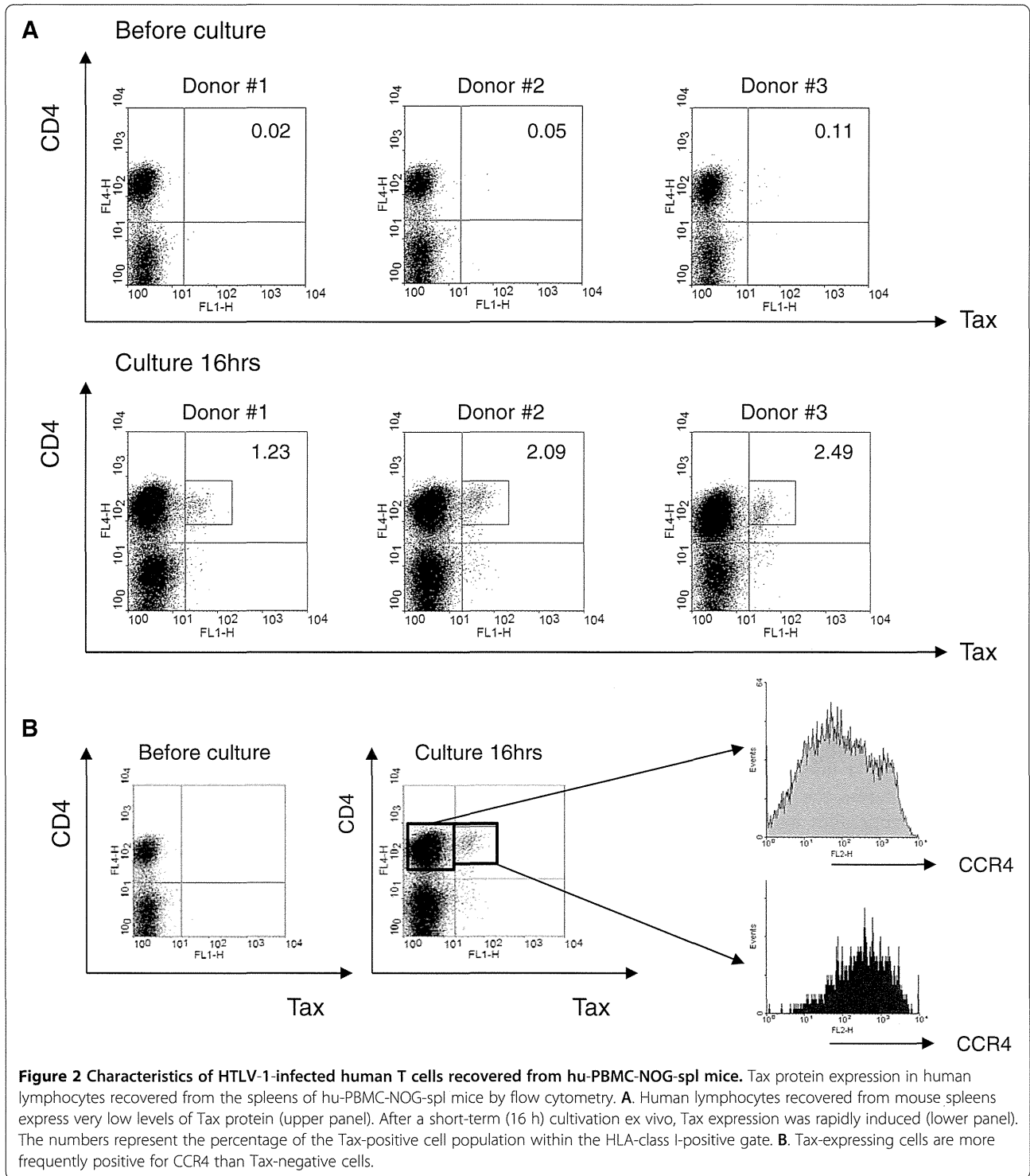


Figure 1 In vivo infection of HTLV-1 in engrafted human PBMCs in hu-PBMC-NOG-spl mice. **A**. Live cells were gated on their forward and side light scatter characteristics, and then cell surface markers within the HLA-class I-positive population were analyzed. **B**. There tended to be higher frequencies 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-



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

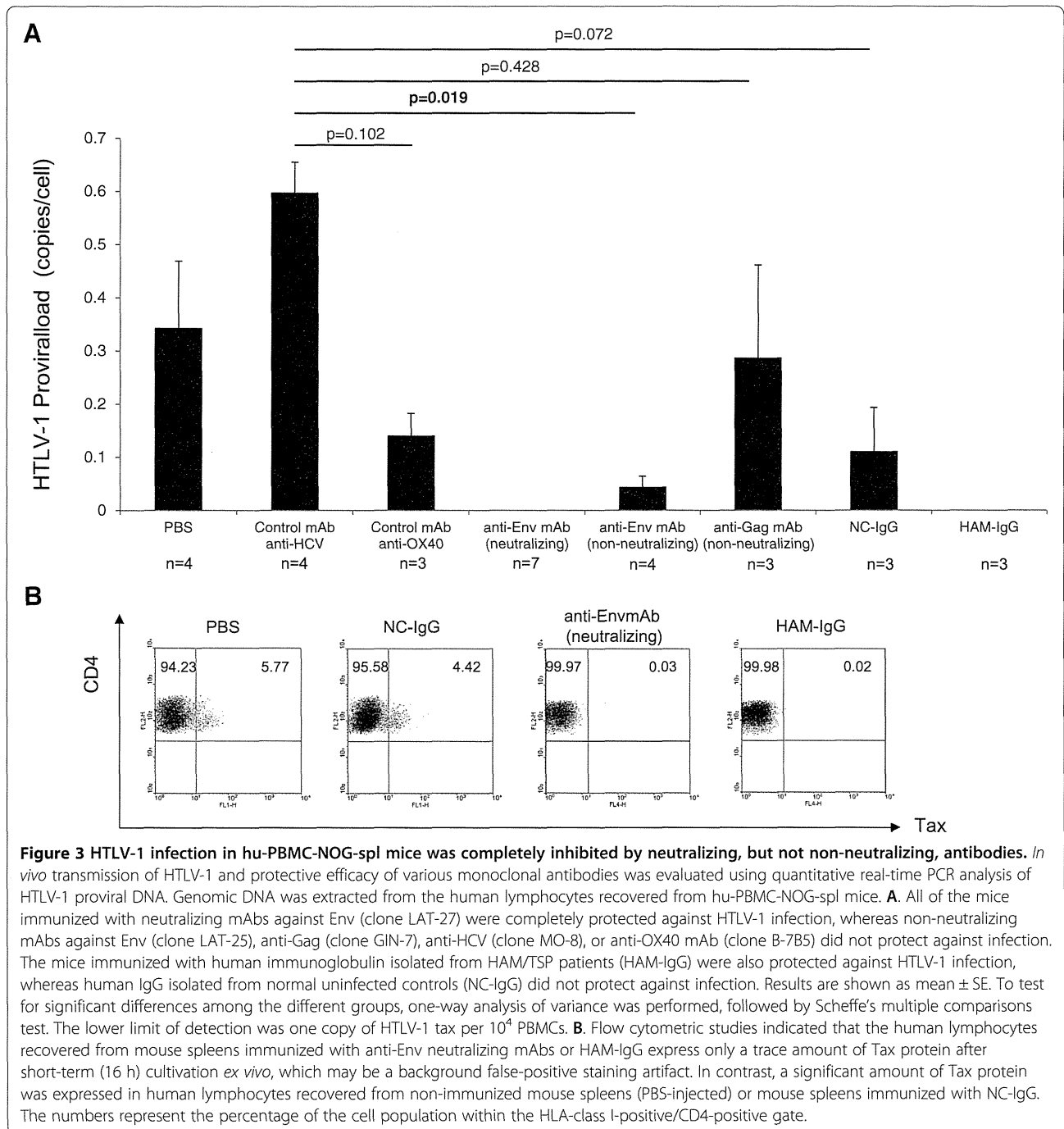
mixed cultures of inoculated human PBMCs and mouse cells including stromal cells. Recently, Kinpara et al. reported that expression of HTLV-1 in HTLV-1-infected T cells is markedly suppressed at both the mRNA and protein levels upon co-culture of human cells and mouse stromal cells, in part via the type 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



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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