Although it has been suggested that HTLV-1 can stay dormant in infected cells and become resistant to immune effector mechanisms by ratcheting down its antigen production,[12] the continued presence of strong $CD8^+$ cytotoxic T lymphocyte (CTL) responses[13] and readily detectable levels of antibodies specific for HTLV-1 antigens in HTLV-1-infected people[14] indicates that persistent production of HTLV-1 must occur *in vivo* to maintain such effector mechanisms. Escape from immune effector mechanisms by spontaneous mutation of key residues is unlikely, due to the high degree of genomic stability that is characteristic of the HTLV-1 genome.[15] It has been suggested that HTLV-1-infected cells expressing HTLV-1 antigens occur at a low enough frequency that they are constantly being eliminated by HTLV-1-specific CTL *in vivo*[10] without leading to immune exhaustion. Besides CTL and virus neutralizing antibodies, there has been renewed interest in the potential role of antibody-dependent cellular cytotoxicity (ADCC) as an effector mechanism against a number of viral infections. This view has been highlighted by the recent demonstration of the potential role of ADCC in the only known partially successful human RV144 trial of a vaccine against human immunodeficiency virus type-1 (HIV-1).[16] The ADCC activity against HTLV-1 was first reported by Miyakoshi *et al.* in 1984[17] followed by a number of other reports.[18–21]

So far, several lines of evidence show that the HTLV-1 envelope gp46 antigen serves as a major target of ADCC.[22–24] Antibodies against gp46 antigen are commonly detected in the sera of HTLV-1-infected individuals.[25–27] However, the precise role of ADCC effector mechanism(s) in controlling HTLV-1 infection has been lacking. A possible involvement of anti-HTLV-1 antibodies in the suppression of spontaneous HTLV-1 antigen expression by HTLV-1-infected cells was first reported by Tochikura *et al.*[28] These investigators showed that serum IgG from HTLV-1-infected donors interfered with HTLV-1 antigen expression by *in vitro*-cultured PBMCs from both ATL patients and healthy HTLV-1 carriers. However, the precise mechanism by which this was mediated remained unclear.

In efforts to define the role of antibodies with neutralizing and ADCC-inducing activities in the control of HTLV-1 infection, we capitalized on the use of our previously defined rat anti-gp46 neutralizing monoclonal antibody (mAb) (LAT-27)[29] and pooled human anti-HTLV-1 IgG purified from HAM/TSP patients (HAM-IgG). Studies were conducted to evaluate the potential of these antibodies to block HTLV-1 infection and eliminate HTLV-1-infected cells from autologous T cell cultures that had previously been infected with HTLV-1 *in vivo* or *in vitro*. Results of these studies show that monoclonal LAT-27 and the polyclonal HAM-IgG are not only capable of mediating neutralization and ADCC, but are also highly effective in the elimination of HTLV-1-infected cells in the presence of fresh autologous PBMCs while preventing *de novo* infection with HTLV-1.

Materials and Methods

Reagents

The medium used throughout was RPMI 1640 medium (Sigma-Aldrich. Inc., St. Louis, MO) supplemented with 10% fetal calf serum (FCS), 100 U/ml of penicillin, and 100 μg/ml of streptomycin (hereinafter called RPMI medium). Anti-human CD3 (clone OKT-3) and anti-CD28 (clone 28.2) mAbs were purchased from the American Type Culture Collection (Rockville, MD) and Biolegend (San Diego, CA), respectively.

The rat and mouse mAbs utilized in the studies reported herein were produced and characterized by our laboratory previously.[29–34] These antibodies were rat IgG2b anti-gp46 (clones LAT-27 and LAT-25), rat IgG2a anti-gp46 (clone LAT-12), rat IgG2b anti-HCV (clone Mo-8), rat IgG2a anti HTLV-1 p24 (clone WAG-24), mouse IgG1 anti-HTLV-1 gp46 (clone MET-3), mouse IgG3 anti-HTLV-1 Tax (clone Lt-4), mouse IgG1 anti-p24 (clone NOR-1), and mouse IgG1 anti-HIV-1 p24 (clone 2C2). These in-house mAbs were purified from the ascites fluids of groups of CB.17-SCID mice carrying the appropriate hybridoma cell line. The ascites fluid was subjected to ammonium sulfate precipitation followed by gel filtration using Superdex G-200 (GE Healthcare, Tokyo, Japan). Aliquots of these mAbs were labeled with either fluorescein isothiocyanate (FITC), Alexafluor 488, Alexafluor 647, HRP (Dojindo, Kumamoto, Japan), or Cy-5 (GE Healthcare) according to the manufacturer's instructions. The FITC- or phycoerythrin (PE)-labeled mouse mAbs against human CD3, CD4, CD8, CD14, CD16, CD19, or CD56 and unlabeled mouse anti-CD16 and anti-CD32 mAbs were purchased from Abcam.

For cell depletion, magnetic beads labeled with anti-CD4, CD8, CD14, CD16, CD19, and antimouse IgG (Dynal) and those labeled with anti-CD56 mAb (LifeTec) were used according to the manufacturer's recommendations. Mitomycin-C (MMC) was commercially purchased from Kyowa Kirin (Tokyo, Japan) and used at 50 μg/ml in RPMI medium. A purified $F(ab')_2$ fragment of LAT-27 IgG generated by enzymatic digestion of LAT-27 IgG was purchased from IBL Inc. (Gunma, Japan). Human IgG was purified from pooled plasma from three normal donors (normal IgG) and three HAM patients (HAM-IgG) using protein-G affinity purification kits (GE Healthcare).

The protocols for the use of human PBMCs and animals were approved by the Human IRB and the Institutional Animal Care and Use Committee (IACUC) on clinical and animal research of the University of the Ryukyus prior to initiation of the present study.

Cell cultures

PBMCs were isolated from heparinized blood by standard density gradient centrifugation using Lympholyte (Cedarlane, Burlington, Canada). Some PBMCs were cryopreserved using a cell freezing media (Cell reservoir, Nakarai Tesque Inc., Kyoto, Japan). The method to activate PBMCs with anti-CD3 and CD28 mAbs has been described previously.[34] The HTLV-1-producing T cell lines utilized included MT-2, HUT102, IL-2-dependent $CD4^-CD8^+$ ILT-M1 cells derived from an HAM/TSP patient, $CD4^+CD8^-$ ILT-H2 cells, ATL-3 cells derived from ATL patients, and a number of other T cell lines derived from normal PBMCs following *in vitro* immortalization by cocultivation with MMC-treated ILT-M1 cells. These cell lines were maintained in culture using RPMI medium containing 20 U/ml IL-2.

The syncytium inhibition assay was performed using an assay that involved the coculture of ILT-M1 and Jurkat cells.[35] A suspension of ILT-M1 cells in a volume of 25 μl containing 5×10^4 cells in 20 U/ml IL-2 media was mixed with 50 μl of serially diluted antibody to be tested in a flat-bottom 96-well microtiter plate for 5 min followed by the addition of 5×10^4 Jurkat cells in a volume of 25 μl of medium. After coculture for 18$\sim$24 h at 37°C in a 5% CO_2 humidified incubator, syncytium formation was microscopically observed using an inverted microscope and the minimum concentration of antibody that showed complete blocking of syncytium formation was determined. In some experiments, gp46 antigen that had been affinity purified from the culture supernatants of MT-2 cells using our anti-gp46 mAb (MET-3) antibody-coupled Sepharose 4B column (GE Healthcare) was used as a target antigen to serve as a specificity control to block the syncytia neutralization of antibodies.[36]

The HTLV-1-immortalization inhibition assay was performed according to the method described previously with a slight modification.[29] Briefly, PBMCs from HTLV-1-negative healthy donors were activated with immobilized OKT-3 together with soluble anti-CD28 mAb overnight, and these cells (5×10^4 cells) were cocultured with an equal number of MMC-treated ILT-M1 cells in wells of round-bottom 96-well microtiter plates (BD) in 0.2 ml media containing 20 U/ml IL-2 at 37°C in a humidified 5% CO_2 incubator in the presence or absence of the test antibodies. The medium was replaced with fresh IL-2-containing media with or without antibody every 3–5 days. Aliquots of the co-cultured cells were monitored every week for intracellular expression of Tax antigen, and the culture supernatants were monitored for the production of p24.

The assay for inhibition of spontaneous HTLV-1 antigen expression in PBMCs from HAM/TSP patients was performed as follows. PBMCs from HAM/TSP patients after depletion of CD8$^+$ cells were cultured *in vitro* at 1×10^6 cells/ml in 20 U/ml IL-2-containing RPMI medium at 37°C in a 24-well plate (BD) in the presence of various anti-HTLV-1 mAbs, HAM-IgG, or controls. After 24 h, cells were harvested and an aliquot stained with anti-CD3, CD4, or CD8 mAb, followed by fixation and subsequent intracellular Tax staining. The frequency and absolute cell numbers of Tax-positive cells were analyzed by flow cytometry (FCM) using the Flowcount (Coulter). The remaining cells were further cultured for 2$\sim$6 weeks with a change of media with or without antibody every 3$\sim$4 days. If necessary, cultures were split into 1:2 or 1:4.

The elimination of HTLV-1 antigen-expressing cells was tested as follows. The IL-2-dependent HTLV-1-infected T cell lines established from PBMCs of normal donors (2×10^5 cells/ml) were cocultured with autologous fresh PBMCs (2×10^6 cells/ml) in 20 U/ml IL-2-containing RPMI medium in triplicate in a round-bottom 96-well microtiter plate (BD) in the presence or absence of various antibodies. After initial coculture for 3 days, these cultures were split, and one was cultured in the presence and the other in the absence of fresh PBMCs and antibodies for 3 days. If necessary, these cells were further treated with antibodies and fresh PBMCs every 3 days. These cell cultures were periodically monitored for changes in the levels of Tax-expressing cells and levels of p24 production.

Flow cytometry (FCM) and enzyme-linked immunosorbent assay (ELISA)

For the detection of HTLV-1 antigen-expressing cells, sample cells were analyzed using polychromatic FCM. Briefly, live cells were Fc receptor-blocked with 2 mg/ml pooled normal human IgG in FACS buffer [phosphate-buffered saline (PBS) containing 0.2% bovine serum albumin (BSA) and 0.1% sodium azide] for 10 min on ice, and prestained with fluorescent dye-labeled mAbs for 30 min. After washing with FACS buffer, the cells were fixed in 4% paraformaldehyde (PFA) in PBS for 5 min at room temperature followed by permeablization and washing in 0.5% saponin + 1% BSA (Sigma) containing FACS buffer. The cells were incubated with 0.1 μg/ml of Cy5-labeled anti-Tax antibody (clone Lt-4) for 30 min. Negative control cells were stained with Cy5-Lt-4 in the presence of 50 μg/ml of unlabeled Lt-4. These cells were analyzed using a FACSCalibur (BD) and the data obtained were analyzed using the Cell Quest software (BD). Typical staining of HTLV-1-infected T cell lines with Lt-4 and LAT-27 is also shown in Supplementary Fig. S1 (Supplementary Data are available online at www.liebertpub.com/aid).

Production of HTLV-1 was determined by the measurement of the HTLV-1 core p24 antigen levels in the culture supernatants using our in-house formulated and standardized ELISA kit using a pair of anti-HTLV-1 p24 mAbs. The sensitivity of this assay was determined to be 0.5 ng/ml of p24 (data not shown).

ADCC assay

HTLV-1-immortalized target cells from healthy donors were labeled with ^{51}Cr for 60 min as described previously[37] and mixed with varying ratios of fresh PBMCs (varying effector-to-target cell ratios) in the presence or absence of various antibodies for the indicated period of time in 20 U/ml IL-2-containing medium. Appropriate controls were included with each assay including target cells cultured in media alone (spontaneous release) and in 0.5 N HCl (100% release). After brief centrifugation, supernatants were harvested and ^{51}Cr activity in each sample was determined using a gamma counter. The net percentage ^{51}Cr release was calculated using standard methods as follows (cpm in experiment – cpm in medium)/(cpm in 0.5 N HCl – cpm in medium) $\times$ 100. In some experiments, PBMCs were depleted of CD4$^+$, CD8$^+$, CD14$^+$, CD16$^+$, CD19$^+$, or CD56$^+$ cells using appropriately conjugated immunomagnetic beads and tested for their effector activity.

Statistical analysis

Data were tested for statistical significance by the Student's *t* test using Prism software (GraphPad Software).

Results

HTLV-1 neutralizing activities of LAT-27 and human anti-HTLV-1-IgG in vitro

The syncytium inhibition assay has been generally used to evaluate HTLV-1 neutralization titers of anti-HTLV-1

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 μg/ml and 50 μg/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 μg/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 antigen[36] 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 μg/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 μg/ml). However, preincubation of the HAM-IgG at 12.5 ~ 100 μg/ml with 10 μg/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 previously[29] and HAM-IgG completely inhibited HTLV-1-mediated T cell immortalization of normal activated T cells *in vitro* at concentrations of 10 μg/ml and 100 μg/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 μg/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

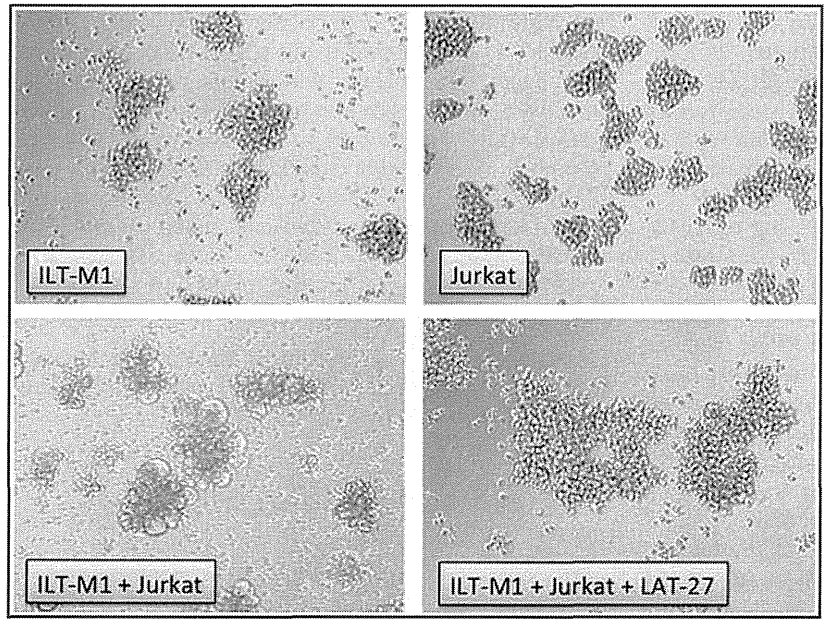

FIG. 1. Human T cell leukemia virus type-1 (HTLV-1)-mediated syncytia formation. $HTLV\text{-}1^+$ ILT-M1 and $HTLV\text{-}1^-$ Jurkat cells were either cultured alone or cocultured at a cell-to-cell ratio of 1:1 in the presence or absence of 10 μg/ml LAT-27 for 18 h. Syncytia were microscopically observed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

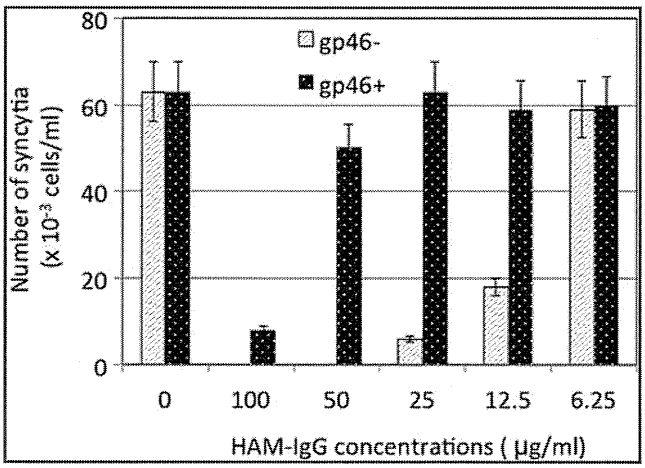

FIG. 2. Anti-gp46 antibodies are major HTLV-1 neutralizing antibodies in HAM-IgG. HAM-IgG at graded concentrations ($0 \sim 100\,\mu$g/ml) was preincubated with either affinity-purified gp46 antigen (black bars) at $10\,\mu$g/ml for 10 min or incubated with medium alone (gray shaded bars, labeled as "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 $2.5\,\mu$g/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

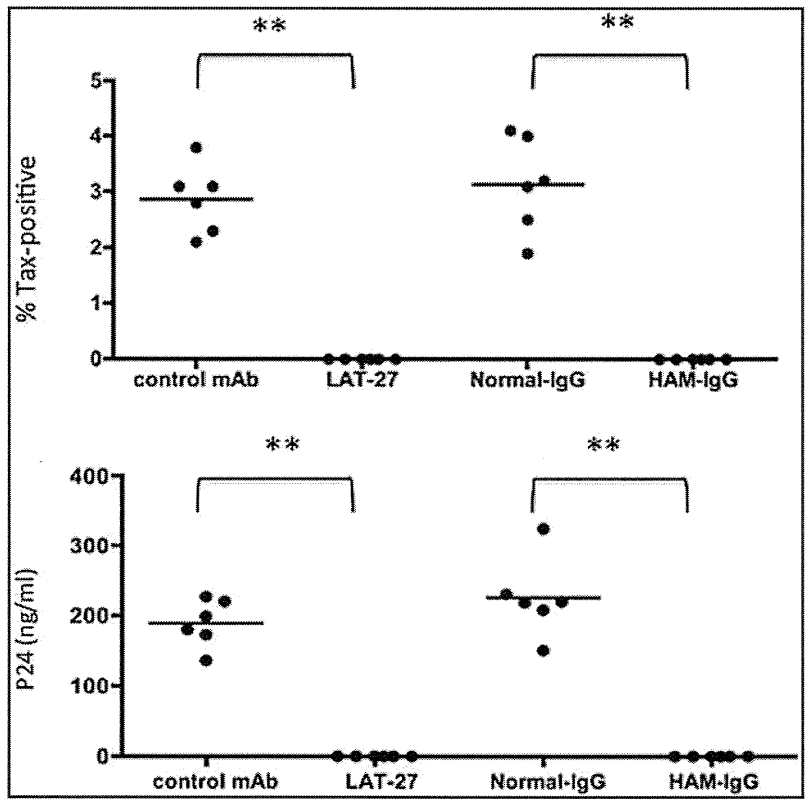

FIG. 3. LAT-27 and HAM-IgG completely block HTLV-1-mediated T cell immortalization *in vitro*. Activated peripheral blood mononuclear cells (PBMCs) from normal donors were seeded into six wells of 96-well U-bottom plates (1×10^5 cells/0.1 ml/well) and cocultured with an equal number of mitomycin C-treated ILT-M1 cells in the presence or absence of $10\,\mu$g/ml of LAT-27 or rat isotype control, $100\,\mu$g/ml of normal human IgG, or HAM-IgG. Half of the medium was replaced every $3 \sim 5$ 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).

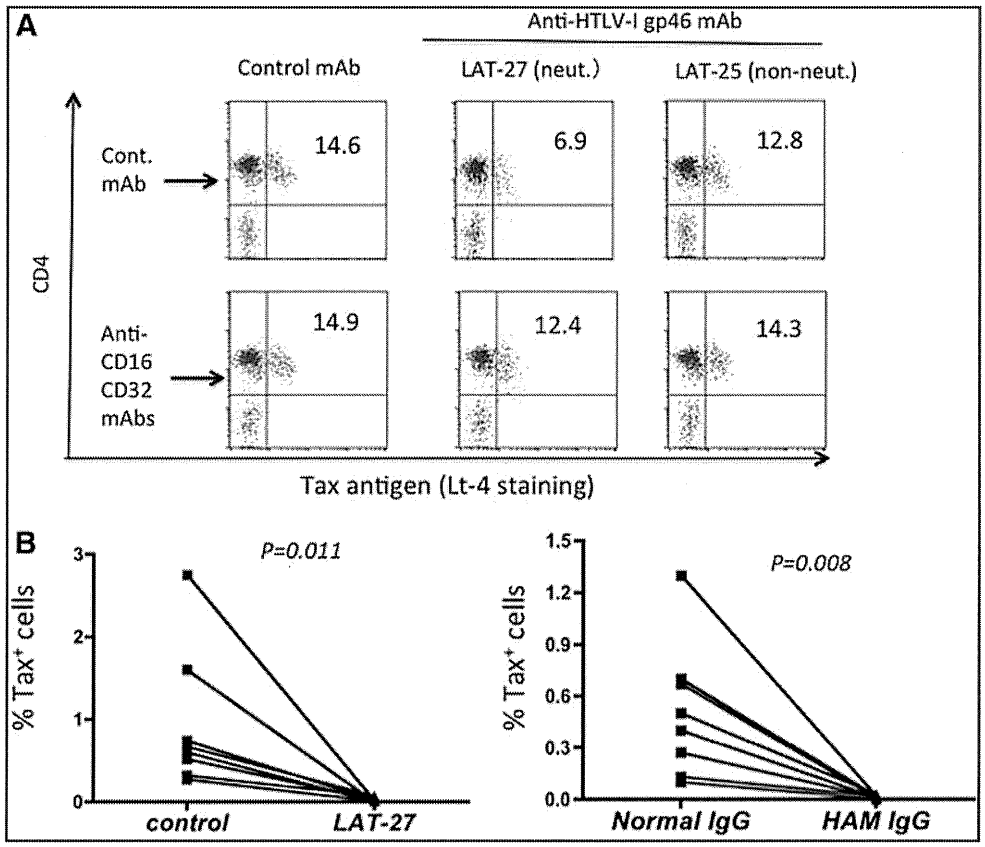

FIG. 4. Reduction of Tax-expressing cells in *in vitro* cultures of PBMCs from HAM patients in the presence of HTLV-1 neutralizing monoclonal antibody (mAb). **(A)** PBMCs from HAM patients were depleted of $CD8^+$ T cells and cultured *in vitro* for 24 h at 1×10^6 cells/ml in interleukin (IL)-2-containing medium in the presence or absence of 10 μg/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 μg/ml or (2) HAM-IgG or normal human IG at 100 μg/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 net 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 ^{51}Cr 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

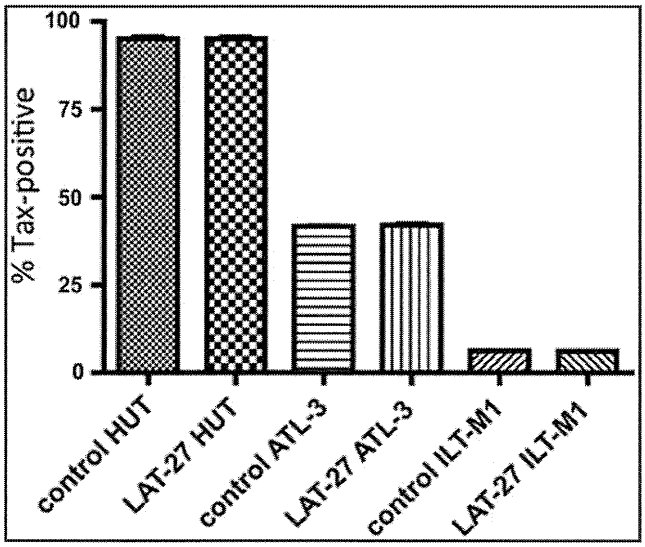

FIG. 5. LAT-27 alone does not affect long-term cultured HTLV-1-infected T cells. A standard HTLV-1-infected cell line HUT-102 (HUT), an IL-2-dependent CD4$^+$ T cell line (ATL-3, generated from an ATL patient), and an IL-2-dependent CD8$^+$ T cell line (ILT-M1) were cultured in the presence of 10 μg/ml 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-sepecific 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

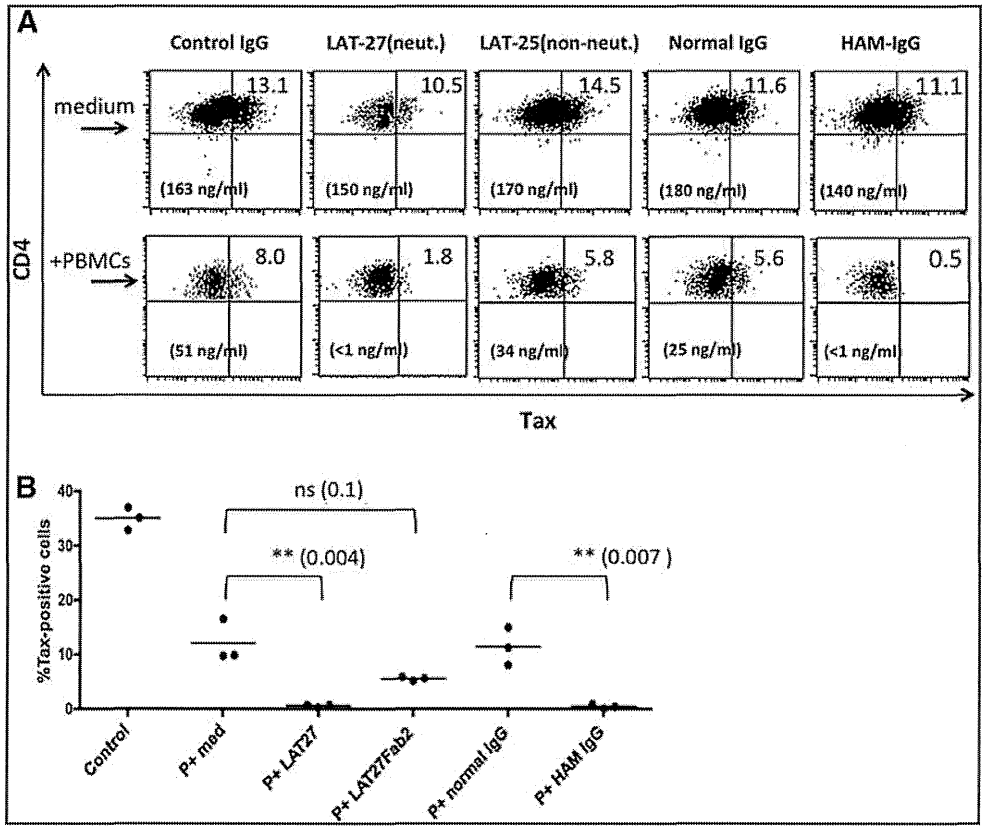

FIG. 6. Elimination of Tax^+ cells and reduction of HTLV-1 p24 production in IL-2-dependent HTLV-1-infected T cells cocultured with autologous PBMCs in the presence of LAT-27 or HAM-IgG. **(A)** IL-2-depnedent $CD4^+$ HTLV-1-infected T cells established from the PBMCs of normal donors were repeatedly exposed to autologous PBMCs (+PBMCs) in the presence of 10 μg/ml of LAT-27 or isotype control, or 100 μg/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 μg/ml of LAT-27 or $F(ab')_2$ LAT-27, or 100 μg/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 mechanisms[42] 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)

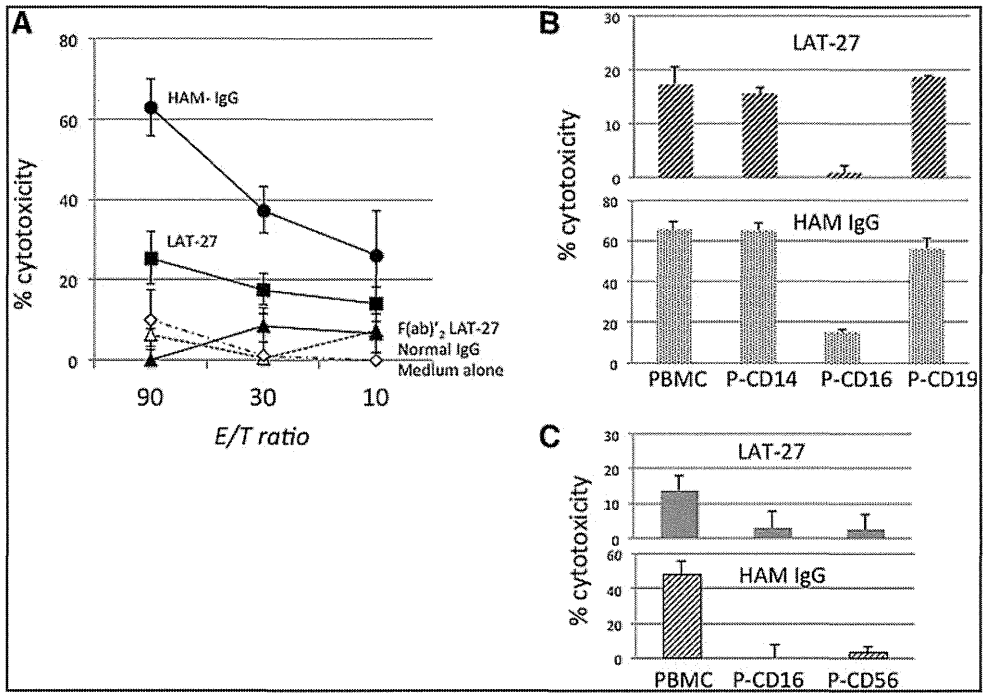

FIG. 7. The CD16$^+$ CD56$^+$ PBMCs mediate antibody-dependent cellular cytotoxicity (ADCC) in the presence of LAT-27 or HAM-IgG. **(A)** ^{51}Cr-labeled HTLV-1-infected cells were cocultured *in vitro* with autologous fresh PBMCs at various E/T ratios in the presence or absence of 10 μg/ml of LAT-27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

Acknowledgments

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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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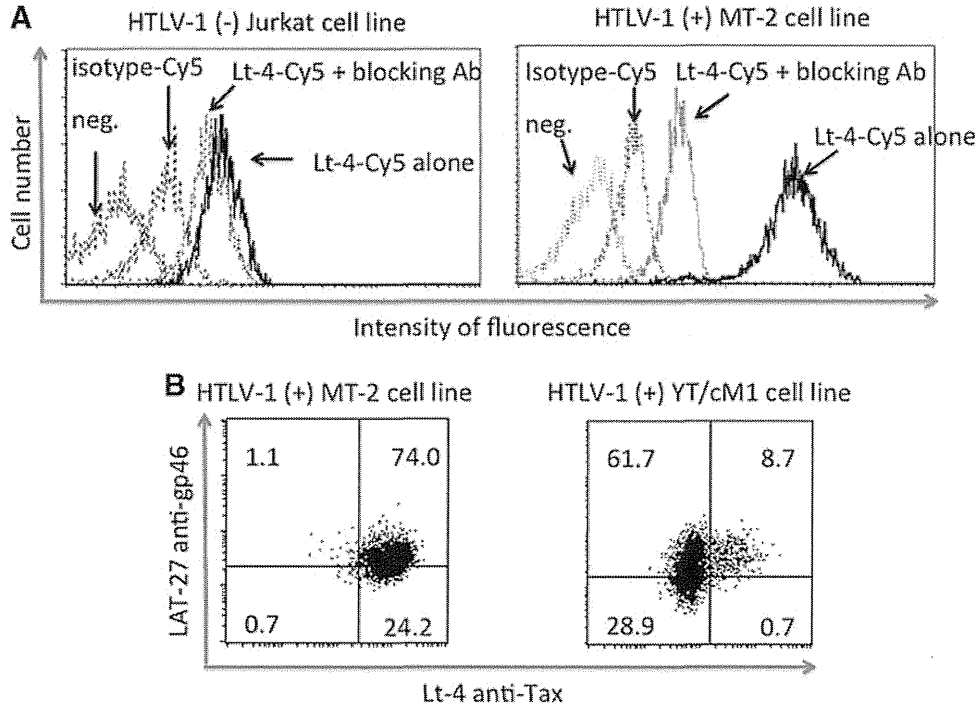
Supplementary Data

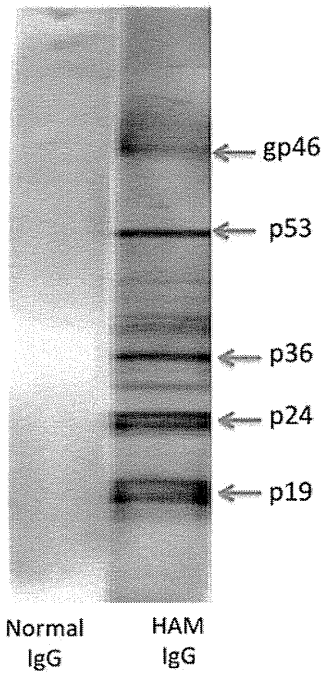


SUPPLEMENTARY FIG. S1. Flow cytometry of Tax and HTLV-I gp46 antigens. **(A)** Tax-specific and nonspecific staining by Cy5-labeled Lt-4. HTLV-I-negative Jurkat cells and HTLV-I-positive MT-2 cells were stained with either Cy5-labeled Lt-4 or Cy5-labeled mouse isotype control (IgG3) in the presence or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

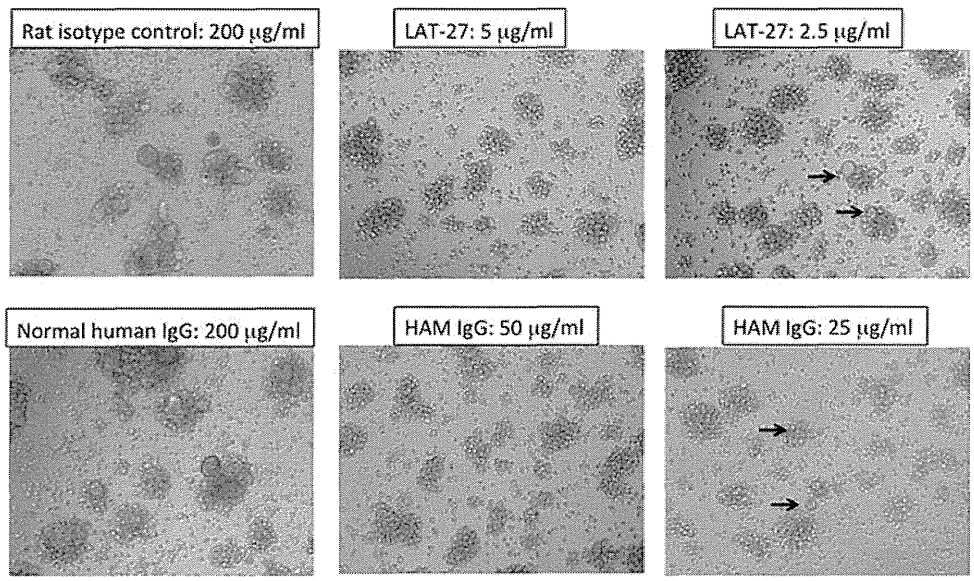
A HTLV-I-coated gelatin particle agglutination assay (SERODIA®HTLV-I)

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

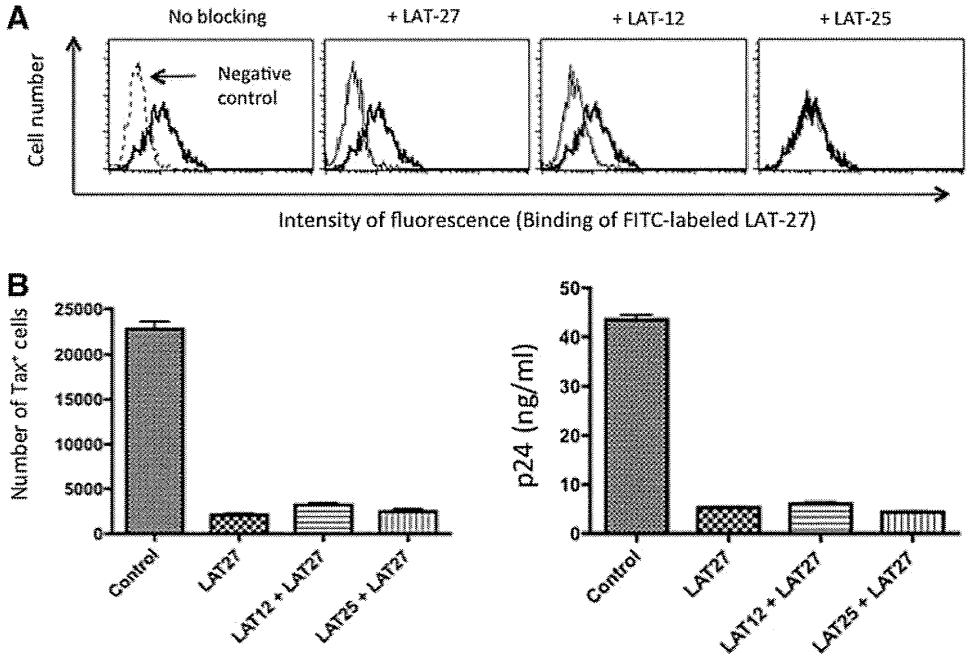
B Western blot analysis (PROBLOT HTLV-I)



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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COMMENTARY

Pathogenic conversion of forkhead box protein 3-positive T cells into T helper 17 cells: Is this also the case for multiple sclerosis?

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Abstract

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system that affects the brain and spinal cord. T helper 17 (Th17) cells have emerged as a key player in the pathogenesis of MS and other autoimmune disorders previously attributed to Th1 cells. New research published in *Nature Medicine* has shown that $CD25^{low}FoxP3^{+}CD4^{+}$ T cells can differentiate into Th17 cells *in vivo*, and that these cells play an important role in the pathogenesis of autoimmune arthritis. Considering the role of autoreactive T cells particularly Th17 cells in MS, such exFoxP3 Th17 cells derived from $FoxP3^{+}$ T cells might also be able to control the initiation and progression of MS. (Clin. Exp. Neuroimmunol doi: 10.1111/cen3.12114, April 2014)

Multiple sclerosis (MS) is an autoimmune inflammatory disorder of the central nervous system (CNS), and is considered to be mainly T cell-mediated.[1] Accumulating evidence suggests that regulatory T (Treg) cells and interleukin (IL)-17-producing helper T (Th17) cells play a key role in the development and progression of MS.[2] Because the pathogenesis of MS shows that maintenance of immunological self-tolerance and its alteration can cause CNS autoimmunity, the balance between self-reactive T cells and cells that can suppress self-reactive T cells is a key to control the onset and progression of MS. Treg cells are essential for the maintenance of immune tolerance through control of the number and function of self-reactive T cells in the periphery.[3] Treg cells can be classified as two main subsets; that is, natural Treg (nTreg) cells and inducible Treg (iTreg) cells, according to their surface phenotype or their cytokine secretion profile. nTreg cells develop in the thymus and are detected in the periphery; they are characterized by their stable expression of the forkhead box protein 3 (FoxP3) transcriptional regulator and high surface expression of the IL-2 receptor α chain (CD25). iTreg cells are induced in the periphery from non-regulatory T cells and may or may not express FoxP3. Dysfunction of Treg cells is associated with the development of various organ-specific autoimmune diseases, including MS.

The study by Komatsu et al.[4] recently published in *Nature Medicine* has shown that $CD25^{low}FoxP3^{+}CD4^{+}$ T cells can differentiate into Th17 cells *in vivo*, and that these cells play an important role in the pathogenesis of autoimmune arthritis. By using genetically engineered mouse models, the authors elegantly showed that $CD25^{low}FoxP3^{+}$ T cells, but not $CD25^{high}FoxP3^{+}$ T cells, can convert into pathogenic Th17 cells (exFoxP3 Th17 cells), which have lost the ability to express FoxP3 under local inflammatory conditions as a result of IL-6 secreted from arthritic synovial fibroblasts at the affected joints. Furthermore, the authors also showed that there is a positive feedback loop at the arthritic joints, which is regulated by pathogenic, self-reactive exFoxP3 Th17 cells generated from $CD25^{low}FoxP3^{+}CD4^{+}$ T cells. To be precise, the production of IL-6 from arthritic synovial fibroblasts induces not only the secretion of more IL-6 from these fibroblasts, but also the production of IL-17 by exFoxP3 Th17 cells, which in turn induces the secretion of more IL-6 from synovial fibroblasts. This positive feedback loop only occurred in collagen-immunized mice that harbor collagen-specific T cells, but not in ovalbumin-immunized mice, indicating that this phenomenon is self-antigen driven. Finally, the authors observed $IL\text{-}17^{+}FoxP3^{+}$ T cells, which might appear to be at the transition stage during the conversion of $Foxp3^{+}$

T cells into exFoxP3 Th17 cells in the synovium of patients with rheumatoid arthritis.

In human MS, although the number of $CD25^{high}FoxP3^{+}CD4^{+}$ Treg cells does not differ between patients with MS and healthy controls, these cells are functionally impaired in terms of their ability to suppress self-reactive T cells.[5] Meanwhile, a recent report by Bailey-Bucktrout et al.[6] clearly showed that FoxP3 expression is lost in self-reactive Treg cells during the course of inflammation in experimental autoimmune encephalomyelitis. Furthermore, it has been reported that available immune-modulatory drugs for MS, such as interferon-β1a (IFN-β1a)[7] and glatiramer acetate,[8] exert their effects in part through cells with a regulatory phenotype. Because the report by Komatsu et al. clearly showed the importance of such FoxP3 instability in the generation of pathogenic self-reactive exFoxP3 Th17 cells, it would be interesting to clarify whether exFoxP3 Th17 cells have also a causal role in MS. Furthermore, Komatsu et al. showed the increased expression of CC chemokine receptor 6 (CCR6) on exFoxP3 Th17 cells that originated from a subpopulation of peripherally-derived iTreg cells, not from thymus-derived nTreg cells nor activated conventional T cells. It has been reported that the expression of retinoid acid-related orphan receptor γt (RORγt), CCR6 and IL-17 in Treg cells was observed in the context of Th17 cell responses, whereas the expression of T-bet, CXC chemokine receptor 3 (CXCR3) and IFN-γ in Treg cells was detected at sites of Th1 responses. Interestingly, a recent report showed that there was an increase in iTreg cells that lost their ability to express FoxP3 in both human T-cell leukemia virus type-1 bZIP factor (HTLV-1 HBZ) transgenic mice and patients with HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP); furthermore, these cells became IFN-γ-producing cells as a source of pro-inflammatory $CD4^{+}$ T cells.[9] These results suggest that the generation of exFoxP3 cells from iTreg cells is a common mechanism of autoimmune and virus-induced chronic inflammations.

On the basis of these findings, the next generation of MS therapies should be targeted to promote the stability and function of Treg cells as well as to reduce inflammation by suppressing pro-inflammatory cytokines. Although further studies are required to address several issues; for example, translating rodent data to human therapy and potential adverse effects of non-specific immunological manipulation, the report by Komatsu et al. provides a rational basis for the functional reconstitution or augmentation of Treg cells as a component of an integrated therapy for autoimmune diseases, such as MS.

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RETROVIROLOGY

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

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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

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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/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.

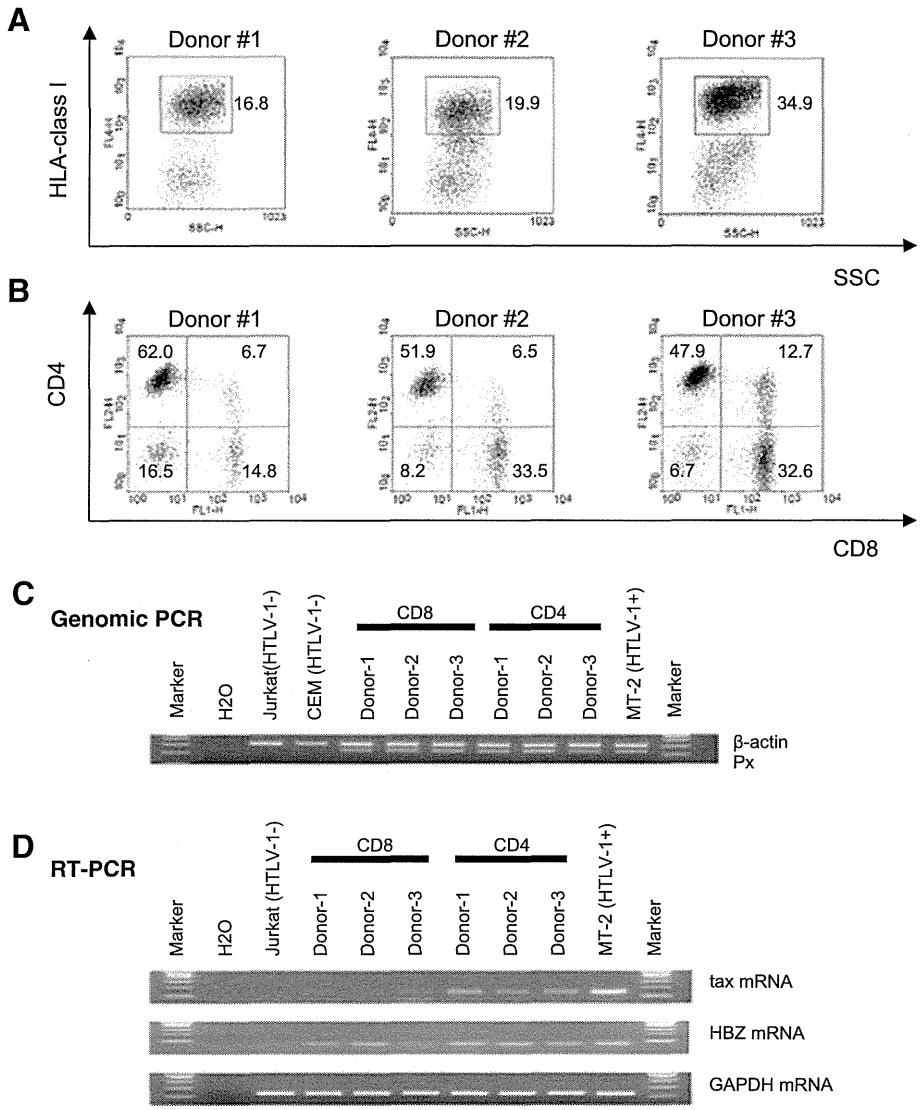

Figure 1 In vivo infection of HTLV-1 in engrafted human PBMCs in hu-PBMC-NOG-spl mice. A. Live cells were gated on their forward and side light scatter characteristics, and then cell surface markers within the HLA-class I-positive population were analyzed. **B**. There tended to be higher frequencies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

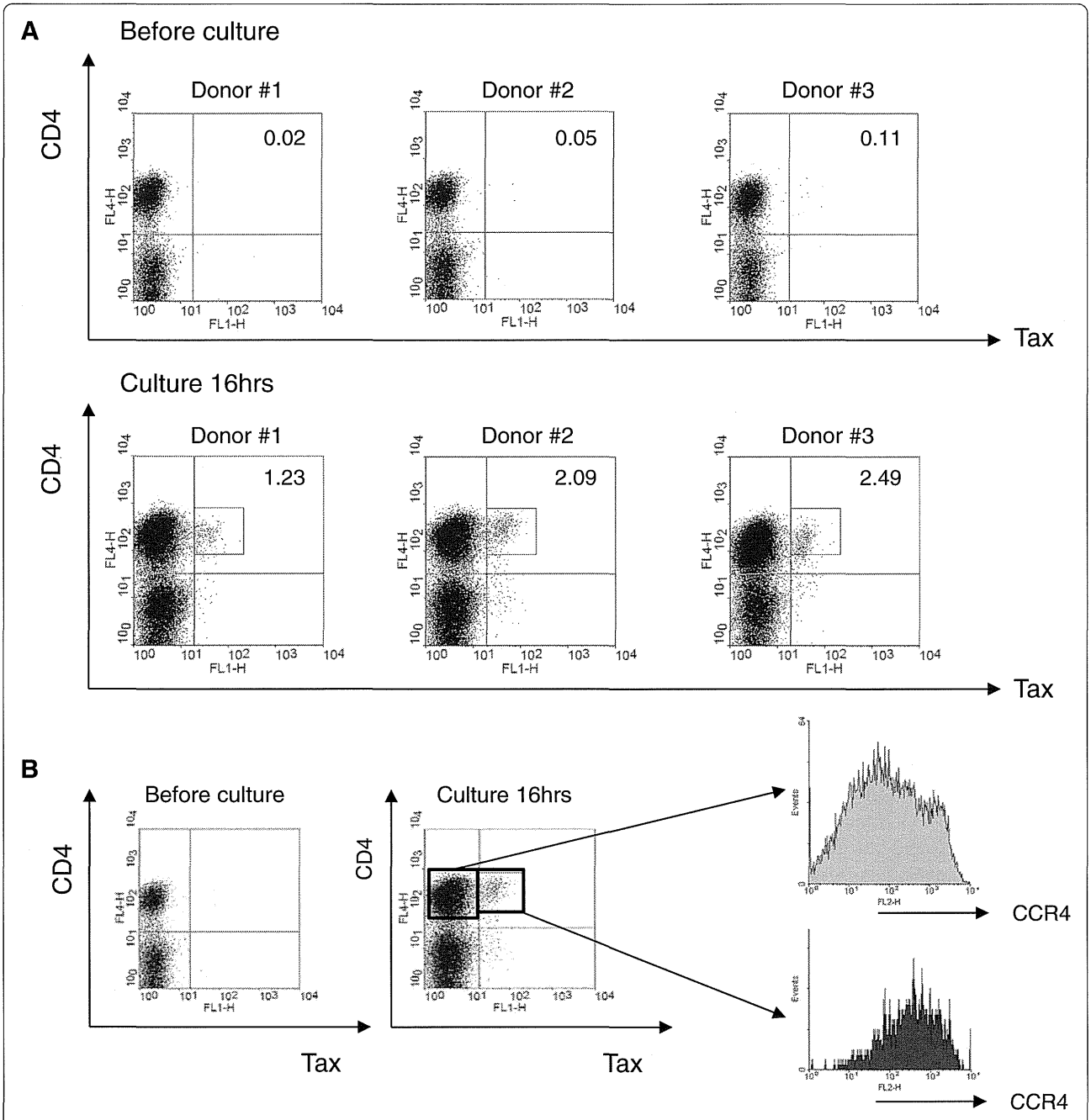

Figure 2 Characteristics of HTLV-1-infected human T cells recovered from hu-PBMC-NOG-spl mice. Tax protein expression in human lymphocytes recovered from the spleens of hu-PBMC-NOG-spl mice by flow cytometry. **A**. Human lymphocytes recovered from mouse spleens express very low levels of Tax protein (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