Table 1 The expression of pro-inflammatory cytokines in peripheral blood mononuclear cells of HTLV-1 infected individuals

Case	Age	Sex	PVL[a]	%IFN-γ$^+$ in CD4$^+$OX40^{+b}	%IFN-γ$^+$ in CD4$^+$OX40$^-$	%IFN-γ$^+$ in CD4$^+$Tax^{+c}	%IFN-γ$^+$ in CD4$^+$Tax$^-$	% TNF-α$^+$ in CD4$^+$OX40$^+$	% TNF-α$^+$ in CD4$^+$OX40$^-$	% TNF-α$^+$ in CD4$^+$Tax$^+$	% TNF-α$^+$ in CD4$^+$Tax$^-$
HAM/TSP7	68	F	1200	56.3	8.9	74.1	12.4	66.9	26.1	70.8	25.3
HAM/TSP8	68	F	1118	77.2	5.0	91.5	4.7	84.1	10.0	87.6	10.7
HAM/TSP9	71	F	1424	64.8	4.7	80.1	5.3	70.4	18.8	80.5	16.6
mean±SE	69.0±1.0		1247±65	66.1±4.3	6.2±1.0	81.9±3.6	7.5±1.7	73.8±3.7	18.3±3.3	79.6±3.4	17.5±3.0
AC4	74	F	435	61.9	13.8	61.8	13.6	30.8	11.5	25.0	11.1
AC5	76	M	139	55.3	24.9	43.0	27.8	38.3	22.1	47.9	14.3
AC6	71	F	250	47.3	15.0	62.1	34.6	15.8	10.5	34.8	21.9
mean±SE	73.7±1.5		275±61	54.8±3.0	17.9±2.5	55.6±4.5	25.3±4.4	28.3±4.7	14.7±2.6	35.9±4.7	15.8±2.3

HAM/TSP: HTLV-1 associated myelopathy/tropical spastic paraparesis. AC: asymptomatic carrier. PVL: Proviral load.
[a] PVL: HTLV-1 tax copy number per 10^4 peripheral blood mononuclear cells (PBMCs).
[b] "%IFN-γ$^+$ in CD4$^+$OX40^{+} means the frequency of IFN-γ$^+$ cells in the CD4$^+$OX40$^+$ cell gate.
[c] "%IFN-γ$^+$ in CD4$^+$Tax^{+} means the frequency of IFN-γ$^+$ cells in the CD4$^+$Tax$^+$ cell gate.

Table 2 Clinical and laboratory findings of HAM/TSP patients for whom paired CSF and plasma samples were tested for soluble OX40 (sOX40)

Case	Age	Sex	Disease Duration	HTLV-1 proviral load (copies/10^4PBMCs)	HTLV-1 Ab titer (PA)	OMDS*	sOX40 (Plasma)	sOX40 (CSF)
HAM/TSP10	67	F	6 years	698	×4096	7	534.9	52.1
HAM/TSP11	29	F	1 year	1138	×16384	2	394.0	54.1
HAM/TSP12	41	F	5 years	800	×16384	4	1459.0	55.6
HAM/TSP13	62	F	1 month	224	×8192	10	626.6	752.1
HAM/TSP14	75	F	3 months	437	×4096	9	337.6	897.4
HAM/TSP15	66	F	2 months	534	×4096	9	423.5	652.5

*OMDS: Osame Motor Disability Score that graded the motor dysfunction from zero (normal walking and running) to 13 (complete bedridden): 1=normal gait but runs slow; 2=abnormal gait; 3=abnormal gait and unable to run; 4=need support while using stairs; 5=need one hand support in walking; 6=need two hands support in walking; 7=need two hands support in walking but is limited to 10 m; 8=need two hands support in walking but is limited to 5 m; 9=unable to walk but able to crawl on hands and knees; 10=crawls with hands; 11=unable to crawl but can turn sideways in bed; 12=unable to turn sideways but can move the toes.

after culture, suggesting that the anti-OX40 mAb (B-7B5) did not suppress expression of Tax but specifically eliminated OX40-positive HTLV-1 infected cells (Figure 6).

Discussion

Retroviral infection is characterized by chronic immune-system activation and pro-inflammatory cytokine production [40]. HTLV-1 infection is associated with the development of several different inflammatory conditions, including chronic arthritis, pulmonary alveolitis, polymyositis, Sjögren syndrome, and uveitis [41]. The main pathological feature of HAM/TSP is chronic inflammation of the spinal cord, characterized by perivascular cuffing of mononuclear cells accompanied by parenchymal lymphocytic infiltration. Increased spontaneous peripheral blood lymphocyte proliferation with the production of TNF-α

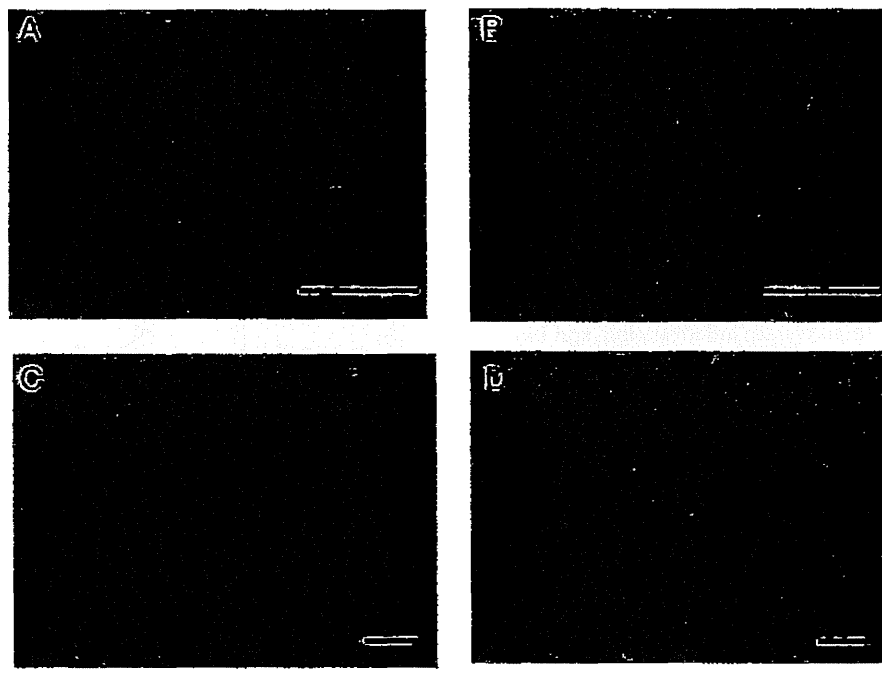

Figure 4 Expression of OX40 in inflammatory mononuclear cells in spinal cord lesions of HAM/TSP patient with short disease duration and progressive symptoms. We studied autopsy specimens from 9 HAM/TSP patients by immunohistochemical staining. **A.** No OX40 positive cells are detected in the spinal cord lesion without active inflammation of a HAM/TSP patient with a long duration of illness. Magnification: ×40. **B.** Many infiltrating mononuclear cells are positively stained by anti-OX40 mAb in the spinal cord lesion with active inflammation of HAM/TSP patient with 2.5 years of illness. Magnification: ×40. **C.** There was reduced or no OX40L protein expression in spinal cord tissues of HAM/TSP patients. OX40L showed only low background staining and there was no OX40L positive staining on inflammatory mononuclear cells in the spinal cord lesions. Magnification: ×20. **D.** Positive control staining for OX40L positive CEM-OX40L cells. Magnification: ×20. Bar: 50 μm.

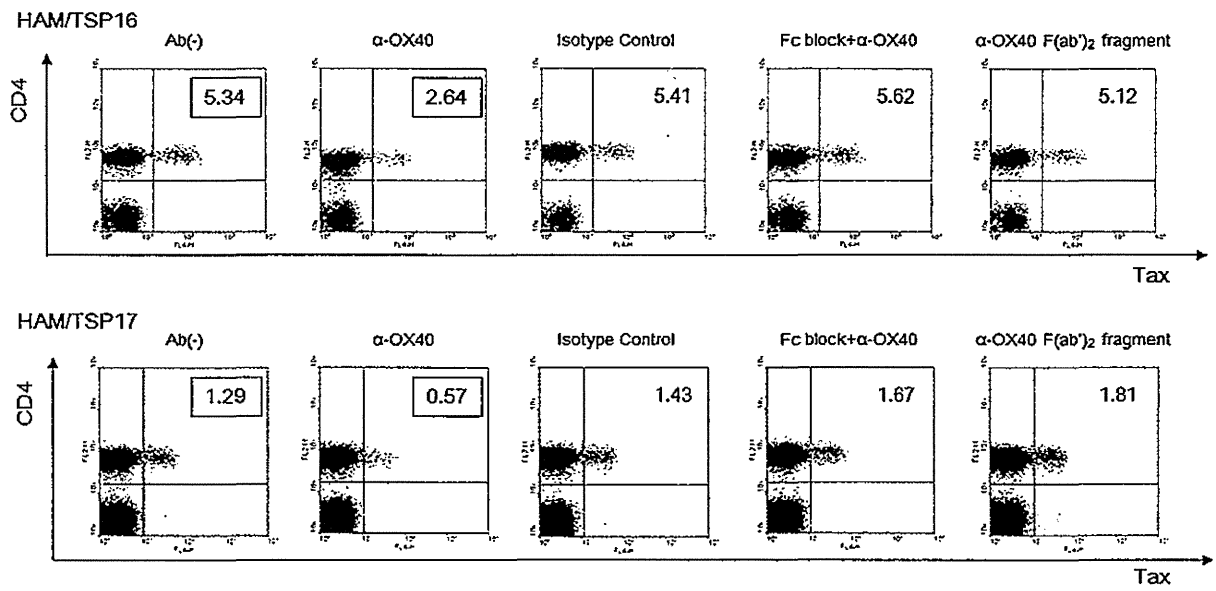

Figure 5 Anti-OX40 monoclonal antibody specifically eliminated naturally infected CD4$^+$ T cells via antibody-dependent cell-mediated cytotoxicity (ADCC) in cultured PBMCs. Anti-OX40 mAb (clone B-7B5) reduces the percentage of Tax-positive cells, whereas the isotype control mAb (clone 2C2: anti-HIV-1 gp21, mouse IgG1) has no effect on Tax expression (1st, 2nd, and 3rd panels from left). Culture of PBMCs with anti-CD16/CD32 (Fc receptor) antibody to block Fc receptors abolishes Tax suppression by anti-OX40 mAb (4th panels from left). F(ab')$_2$ fragment do not suppress Tax exppression (right panels).

and IFN-γ [42,43], high prevalence of autoantibodies, hypergammaglobulinemia, and complement fixing immune complexes have also been reported in HAM/TSP patients [6]. Recent research has shown the importance of OX40-OX40L interactions in the development of immune-mediated diseases. Specifically, a strong reduction in disease severity, or a complete lack of disease, has been reported when OX40 or OX40L is absent or neutralized in animal models. We therefore hypothesized that the OX40-positive subpopulations of chronically activated T cells exist in naturally HTLV-1-infected cells of HAM/TSP patients. These cells may function to accelerate inflammation, and blocking OX40 may have therapeutic potential in the treatment of HAM/TSP.

Previous reports indicated that OX40 is strongly stimulated by the HTLV-1 viral transactivator Tax [15,19,20]. However, these previous findings were obtained by northern blot or western blot analysis using whole cells. Thus, it was not clear if this induction occurs in naturally infected CD4$^+$ T cells of HTLV-1 infected individuals. In the present study, our flow cytometry analysis clearly showed that almost all OX40-positive cells are Tax-positive after short-term culture of naturally HTLV-1-infected cells, suggesting that OX40 is driven exclusively by Tax at the single cell level. In contrast, flow cytometry analysis of JPX-9 cells showed higher percentages of OX40$^+$Tax$^-$ cells, as well as OX40$^+$Tax$^+$ cells, after induction of Tax. Although the reasons for this discrepancy are not clear, it can be caused by differential modulation of surface and intracellular protein expression in JPX-9 cells. Our ELISA analysis indicates the existence of intracellular pools of OX40, suggesting that Tax$^+$OX40$^-$ cells also contain Tax-induced OX40 within JPX-9 cells. While the expression of another co-stimulatory member of the TNFR family, 4-1BB, has also been reported [32], our data indicate that the

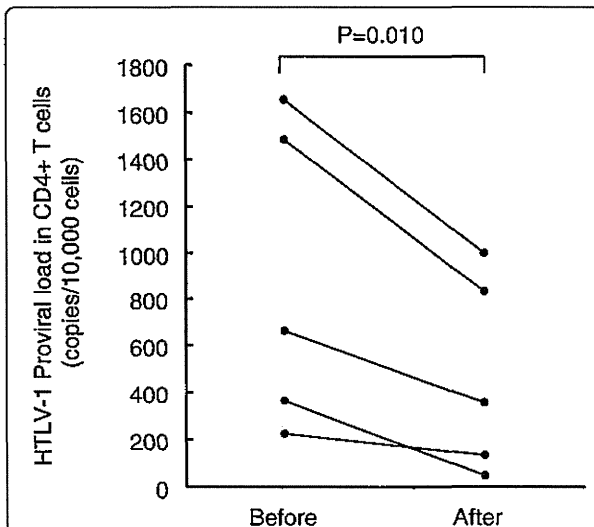

Figure 6 The HTLV-1 proviral load in CD4$^+$ T cells were significantly reduced after cultivation. CD4$^+$ T cells were isolated from PBMCs before and after cultivation, extracted genomic DNA, and measured the HTLV-1 PVL. HTLV-1 PVL in CD4+ T cells was significantly reduced after culture.

expression of OX40 was more specific than the expression of 4-1BB in Tax^+CD4^+ T cells naturally infected with HTLV-1. It has been previously reported that Tax strongly activates the 4-1BB promoter via a single NF-κB site [32] and the OX40 promoter via 2 NF-κB sites [16]; hence, sustained activation of NF-κB leads to increased expression of numerous pro-inflammatory cytokines and growth factors [44] via NF-κB signaling pathways and ultimately leads to chronic inflammation. In support of these observations, our results show that the frequencies of pro-inflammatory cytokine positive cells within the $OX40^+CD4^+$ and Tax^+CD4^+ populations from HAM/TSP patients are significantly higher than $OX40^-CD4^+$ and Tax^-CD4^+ T cells, respectively. These cells may be more likely to cross the blood brain barrier and enter the CNS, attract other cells including pro-inflammatory virus-specific $CD8^+$ cells, and result in bystander damage to the CNS tissue.

The experimental autoimmune encephalomyelitis (EAE) rat model of human MS shows a selective upregulation of the OX40 protein in encephalitogenic myelin basic protein-specific T cells in the spinal cord during onset of the disease [21]. In contrast, T cells isolated from peripheral blood and spleen of the same animal express low levels of OX40 [21]. This is similar to our present finding, where OX40 was markedly expressed in infiltrating mononuclear cells in spinal cord lesions, but not in uncultivated PBMCs from HAM/TSP patients. Because locally produced pro-inflammatory cytokines up-regulate MHC class II molecules on astrocytes and microglia, increase presentation of CNS antigens, and exert a direct cytotoxic effect on oligodendrocytes [45], the observed expression of OX40 in inflammatory mononuclear cells in spinal cord lesions suggest a role for OX40 in inflammation and neuronal damage that occurs in the CNS of HAM/TSP patients. In the rat EAE model, selective depletion of myelin-reactive T cells, by treatment with an anti-OX40 mAb-conjugated immunotoxin, effectively suppressed disease symptoms [21]. The association of clinical progression of HAM/TSP with increased HTLV-1 PVL in individual patients [9] and the strong stimulation of OX40, together with the expression of the viral transactivator Tax in $CD4^+$ T cells, indicates that targeting of OX40 positive T cells by anti-OX40 antibodies may provide a novel therapeutic strategy for the treatment of HAM/TSP.

In the present study, an anti-OX40 monoclonal antibody specifically eliminated naturally infected $CD4^+$ T cells in cultured PBMCs via ADCC. This indicates that effector cells may actively lyse HTLV-1-infected $CD4^+$ T cells that are bound by the anti-OX40 antibody. Indeed, defucosylated humanized anti-CC chemokine receptor 4 (CCR4) mAbs, which exert a strong ADCC effect, were found to be effective and well tolerated as a treatment for patients with relapsed CCR4-positive ATL or peripheral T-cell lymphoma [46]. In the present study, OX40 expression was not observed in T cells of healthy individuals, and its expression was more specific than CCR4 for HTLV-1-infected cells. This finding suggests that specific elimination of HTLV-1-infected T cells by defucosylated humanized anti-OX40 monoclonal antibodies might be a promising future approach for treatment of HAM/TSP.

We also found that plasma sOX40 levels were more elevated in HTLV-1-infected individuals (chronic HAM/TSP patients and ACs) than in NCs. Three rapidly progressive HAM/TSP patients also showed higher levels of sOX40 in the CSF than in the plasma, suggesting the possibility that sOX40 is released at high levels following strong intrathecal immune activation. In contrast, expression of OX40L was absent in HTLV-1-infected lymphocytes even after short term ex vivo cultivation, in active-chronic spinal cord lesions of HAM/TSP patient, and in plasma of HTLV-1 infected individuals. Therefore, OX40 signals might be generated by interactions with OX40L on antigen presenting cells or endothelial cells at specialized sites such as lymphoid organs. In such lesions, similar to other members of the TNF receptor superfamily like 4-1BB, sOX40 may act as an antagonist to membrane-bound receptors and induce signaling in $OX40L^+$ cells to produce cytokines, which in turn drive specific T helper (Th)-cell differentiation and suppress the generation of adaptive Tregs to participate in HAM/TSP pathogenesis.

In conclusion, we demonstrate that OX40 was specifically expressed in $CD4^+$ T cells naturally infected with HTLV-1. These cells have the potential to produce pro-inflammatory cytokines along with the expression of the viral transactivator Tax. Higher levels of sOX40 were found in the CSF than in the plasma of three rapidly progressive HAM/TSP patients, and OX40 was overexpressed in the spinal cord infiltrating mononuclear cells of HAM/TSP patient with active disease. Anti-OX40 mAb was able to specifically eliminate HTLV-1-infected $CD4^+OX40^+Tax^+$ T cells via ADCC. These findings indicate that, in addition to its established role in the regulation of T cell division and survival, OX40 may be a key molecule in the pathogenesis of HAM/TSP, as well as a potential target for immunotherapy.

Methods

Patients

Peripheral blood was studied from 23 patients with a clinical diagnosis of HAM/TSP, 9 ACs and 13 uninfected normal controls (NCs). The diagnosis of HAM/TSP was made according to the World Health Organization diagnostic criteria [47]. In this paper, chronic HAM/TSP means typical cases fulfilling diagnostic criteria and rapidly progressive HAM/TSP is defined by patients' incapacity to walk unaided within three months after

symptoms' onset. This study was approved by the Institutional Review Board of the University of the Ryukyus with license number H21-1-9. All patients provided written informed consent for the collection of samples and subsequent analysis. The CSF and plasma samples were collected before starting therapy. Control subjects of other neurological diseases were MS (n=12), aseptic meningitis (n=8), systemic lupus erythematosus (SLE) with neurological manifestations (n=5), chronic inflammatory demyelinating polyneuropathy (CIDP) (n=9), Guillain-Barré syndrome (GBS) (n=6), and amyotrophic lateral sclerosis (ALS) (n=9). The specimens were stored at −80°C until use.

Cell culture

Six HTLV-1 infected T-cell lines (HUT-102, MT-1, MT-2, MT-4, SLB-1, C5/MJ) and two HTLV-1-uninfected T-cell lines (CEM-OX40L, CEM-OX40) were used in this study. CEM-OX40 and CEM-OX40L cell lines are stable CEM-derived cell lines expressing the human OX40 or OX40L, respectively. The Tax-inducible JPX-9 cell line is a derivative of the Jurkat HTLV-1 negative human T cell leukemia cell line, which expresses biologically active Tax protein under the control of the metallothionein promoter [29]. These cells were cultured in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum (FCS), 50 U/ml penicillin, and 50 μg/ml streptomycin (Wako) at 37°C in 5% CO_2.

Preparation of PBMC samples

Fresh peripheral blood mononuclear cells (PBMCs) were isolated on a Histopaque-1077 (Sigma) density gradient centrifugation, washed twice in RPMI 1640 with 10% heat inactivated FCS, and stored in liquid nitrogen as stocked lymphocytes until use. $CD4^+$ T cells were isolated from PBMCs by positive immunoselection with the Dynal® CD4-positive isolation kit (Invitrogen), according to the manufacturer's protocol. In brief, PBMCs were incubated with anti-CD4-coated beads for 30 min at 4°C under gentle tilt rotation. Captured $CD4^+$ cells were collected with a magnet (Dynal MPC-S) and detached from beads with DETACHaBEAD CD4/CD8® (Invitrogen). Purity was >99% $CD4^+$ T cells, as determined by flow cytometry (data not shown). To induce cytokine production by $OX40^+CD4^+$ T cells, PBMCs were cultivated for 12 hours, then 0.1 ng/ml phorbol myristate acetate (PMA) (Sigma) and 0.5 μg/ml A23187 (Sigma) and 2 mM monensin (Sigma) were added to the culture medium and further cultivated for 5 hours.

Monoclonal antibodies and reagents

We produced the following monoclonal antibodies (mAbs) in our laboratory: mouse IgG1 mAbs anti-human OX40L (clones 5A8, 8F4), anti-human OX40 (clones B-7B5 and 17D8), anti-HIV-1 p24 (clone 2C2 and NP24), and mouse IgG3 mAb anti-HTLV-1 Tax (clone Lt-4) [48] as well as rat IgG2b mAbs anti-human OX40 (clone W4-54), anti-human OX40L (clone W18) and isotype control anti-HCV (clone MO-8). Some of these mAbs were labeled using FITC, Cy5, or HRP using commercial labeling kits (Dojin or Amersham, Japan) according to the manufacturers' instructions. Biotinylated recombinant soluble human OX40L (sOX40L in a form of murine CD8-fusion protein) was purchased from Ancell (Bayport, MN) and used with PE-streptavidin (Biolegend) for staining. Recombinant human OX40 ligand/TNFSF4 and recombinant human OX40/TNFRSF4/Fc Chimera were purchased from R&D Systems (Minneapolis, MN) and used for the standard curve in sOX40L and sOX40 ELISA, respectively.

Immunohistochemistry

Immunohistochemical staining of the spinal cord specimens from HAM/TSP patients was performed on buffered formalin-fixed paraffin-embedded sections using EnVision (DAKO) method for signal detection as described previously [36]. The clinical and pathological characteristics of the patients are described elsewhere [36-39]. The monoclonal antibodies to OX40 (clone B-7B5) and OX40L (clone 8F4) were used at a final concentration of 1 μg/ml.

Flow cytometry

Cell surface staining

After thawing, cells were washed three times with phosphate-buffered saline (PBS) and fixed in PBS containing 2% paraformaldehyde (Sigma) for 20 minutes at 4°C. Fixed cells were washed with PBS containing 7% of normal goat serum (Sigma) and then incubated for 15 minutes at room temperature with various combinations of fluorescence-conjugated mAbs as follows: phycoerythrin-cyanin 5.1 (PC5)-labeled anti-CD4 (13B8.2), PC5-labeled anti-CD8 (B9.11), phycoerythrin (PE)-labeled anti-CD4 (13B8.2) (Beckman Coulter), PE-labeled anti-4-1BB (4B4) (eBioscience), fluorescein isothiocyanate (FITC)-labeled anti-OX40 (B-7B5) and OX40L (5A8). Isotype matched mouse immunoglobulins were used as a control. After the staining procedure, the cells were washed twice and analyzed by standard flow cytometry using a FACS Calibur and Cell Quest software (BD).

Concomitant detection of intracellular and cell surface molecules

For intracellular staining of Tax and/or cytokines, surface stained cells were washed and permeabilized with PBS/7% normal goat serum containing 0.2% saponin (Sigma) (PBS-SAPO) for 10 minutes at room temperature. Permeabilized cells were then washed twice and resuspended in PBS-SAPO containing FITC or cyanin 5 (Cy5)-labeled anti-Tax

mAb (Lt-4), PE-labeled anti TNF-α (BD Pharmingen) or PE-labeled IFN-γ (BD Pharmingen) mAb for 20 minutes at room temperature. Finally, the cells were washed twice and analyzed by flow cytometry.

Flow cytometry based binding assay

To determine whether cell surface OX40 is functional, aliquots of Fc-blocked cells were incubated with biotinylated recombinant soluble OX40L at a concentration of 2.5 mg/ml for 30 min on ice, followed by staining with PE-streptavidin (Biolegend) for 30 min on ice. After the staining procedure, the cells were washed twice and analyzed by flow cytometry.

ELISA

Cell lysates were prepared by lysis of 2×10^7 cells in 1ml of a lysis buffer (10 mM Tris–HCl, pH8.0, 140 mM NaCl, 3 mM MgCls, 2 mM phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40) on ice for 20 min, followed by centrifugation at 13,000 × g for 10 min at 4°C. Both OX40 and OX40L levels in cell lysates, culture supernatants, plasma and CSF were assayed by in house made sandwich ELISA using monoclonal antibodies against OX40 (clone 17D8 for capture and W4-54 for detection) and OX40L (clone 8F4 for capture and W18 for detection). Briefly, 96-well Immuno Module/Strip Plates (Nunc) was coated with either anti-OX40 monoclobal antibody (clone 17D8) or anti-OX40L monoclobal antibody (clone 8F4) at 4°C overnight, then blocked with 1% casein in 0.02% thimerosal-PBS at room temperature for 30 min. After washing plates three times with wash buffer (PBS with 0.05% Tween 20, pH 7.5), 50 μl of irrelevant mouse IgG1 (anti-HIV1 p24 mAb NP24) was added into each well as a blocking antibody. OX40 or OX40L standard was diluted to 4,000 pg/ml in dilution buffer (PBS with 0.1% BSA, 0.5% Triton X100, 0.05% Tween20), and two-fold serial dilutions were performed ranged from 4,000 to 16 pg/ml. Then 50 μl of the diluted standard or samples (cell lysates, culture supernatants, plasma and CSF) were added into 96-well plates and incubated one hour at room temparature. After washing plates three times, 50 μl each of diluted (0.2 μg/μl) anti-OX40 monoclobal antibody (clone W4-54) or anti-OX40L monoclobal antibody (clone W18) conjugated to HRP was added as detection antibody and incubated for one hour at room temperature. Color reactions using alkaline-phosphatase substrate (Sigma-Aldrich) were then evaluated by Model 680 Microplate Reader (Bio-Rad) reading at 450 nm with reference at 630 nm, and the data was analyzed using the Microplate manager III software (Bio-Rad). Results are shown as mean ± SE for duplicate wells. Human interleukin-2 soluble receptor alpha (IL-2sRα) was measured by ELISA according to the manufacturer's instruction (Quantikine Human IL-2sRα Immunoassay, R&D Systems, Inc. MN).

Genomic DNA, RNA extraction and cDNA synthesis

Genomic DNA was extracted from the frozen PBMCs by QIAamp blood kit (QIAGEN, Tokyo, Japan). RNA from 1×10^5 enriched $CD4^+$ T cells was extracted using RNeasy Mini Kit with on-column DNase digestion (QIAGEN, Tokyo, Japan) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using PrimeScript® RT reagent Kit (Takara, Kyoto, Japan). All reaction procedures were performed as suggested by the manufacturer.

Quantification of HTLV-1 proviral load and anti-HTLV-1 antibody titers

To examine the HTLV-1 PVL, we carried out a quantitative PCR method using Thermal Cycler Dice® Real Time System (Takara, Japan) with 100 ng of genomic DNA (roughly equivalent to 10^4 cells) from PBMCs samples as reported previously [8]. Based on the standard curve created by four known concentrations of template, the concentration of unknown samples were determined. Using β-actin as an internal control, the amount of HTLV-1 proviral DNA was calculated by the following formula: copy number of HTLV-1 tax per 1×10^4PBMCs = $[(copy\ number\ of\ tax)/(copy\ number\ of\ \beta-actin/2)] \times 10^4$. All samples were performed in triplicate. Serum HTLV-1 antibody titers were determined by a particle agglutination method (Serodia-HTLV-1®, Fujirebio, Japan).

Real-Time RT-PCR analysis

We used the real-time RT-PCR method to carry out a quantitative analysis of the expression of the tax and OX40 mRNA by using Thermal Cycler Dice® Real Time System (Takara, Japan) as reported previously [49]. HTLV-1 tax or OX40 mRNA load was calculated by the following formula: HTLV - 1 tax mRNA load = value of tax/value of HPRT (Hypoxanthine Phosphorisbosytransferase). OX40 mRNA load = value of OX40/value of HPRT. We used aliquots of the same standard MT-2 cDNA preparation for all assays and the correlation values of standard curves were always more than 99%. The sequences of primers for tax mRNA detection were as follows: 5'- ATC CCG TGG AGA CTC CTC AA-3' and 5'- ATC CCG TGG AGA CTC CTC AA-3', and the probe that surrounded the splice junction site of tax mRNA was 5'- TCC AAC ACC ATG GCC CAC TTC CC-3'. The sequences of primers for OX40 mRNA detection were as follows: 5'-AAC CAG GCC TGC AAG CCC T-3' and 5'-GTC CCT GTC CTC ACA GAT T-3', and the probe that span the junction between exon 4 and 5 was 5'- ACC AAC TGC ACC TTG GCT GGG AAG CA-3'. We used the HPRT primers and probe set (Applied Biosystems) for internal calibration. All assays were performed in triplicate.

Statistical analysis

To test for significant differences among the cell populations between three different groups of subjects (HAM/TSP, ACs and NCs), the Kruskal-Wallis test was employed. For multiple comparisons, we used Sheffe's F to analyze statistical difference. Correlations between variables were examined by Spearman rank correlation analysis. We made paired comparison of changes in HTLV-1 PVL in $CD4^+$ T cells before and after PBMCs cultivation by using a paired t-test. The results represent the mean ± SE where applicable. Values of $p<0.05$ were considered statistically significant.

Additional files

Additional file 1: Figure S1. OX40 was expressed on the surface of Tax^1 $CD4^1$ T cells from HTLV-1 infected individuals. OX40 was detected on $CD4^1$ T cells of HAM/TSP patients (HAM/TSP3, 4) and AC (AC1) with anti-OX40 mAb (clones B-7B5) after 16 hours in vitro cultivation in the absence of any growth factors or mitogen (center panels). OX40 was expressed almost exclusively in naturally infected $CD4^1$ T cells that also expressed Tax (right panels). **FigureS2.** The expression of 4-1BB on $CD4^1$ T cells from HAM/TSP patients. **A.** 4-1BB was detected on both $CD4^1$ and CD4- T cells of HAM/TSP patients with anti-4-1BB mAb (clone 4B4, eBioscience) after 16 hours in vitro cultivation in the absence of any growth factors or mitogen. **B.** Tax protein was detected in $CD4^1$ T cells after 16 hours in vitro cultivation. **C.** The expression of 4-1BB was associated with the expression of Tax. **Figure S3.** Functional OX40 is specifically expressed on the surface of T cells naturally infected with HTLV-1. To determine if cell surface OX40 is functional, flow cytometry based binding assays have been carried out. Aliquots of Fc-blocked cells were incubated with biotinylated recombinant soluble OX40L at a concentration of 2.5 mg/ml for 30 min on ice. Then cells were washed and stained with PE-streptavidin (Biolegend) and PC5-labeled anti-CD4 for 30 min on ice. After washing, the cells were fixed and processed to detect concomitantly Tax (see Methods). The frequency of $CD4^1$ T cells that were positively stained with biotinylated recombinant soluble OX40L and PE-streptavidin was similar to the percentage of $CD4^1$ T cells stained by anti-OX40 mAb, indicating that these cells expressed functional OX40.

Additional file 2: Table S1. Ex vivo frequency of OX40 and Tax positive T cells in peripheral blood mononuclear cells from HTLV-1 infected individuals.

Abbreviations

HTLV-1: Human T-cell leukemia virus type-1; ATL: Adult T-cell leukemia; HAM/TSP: HTLV-1-associated myelopathy/tropical spastic paraparesis; ACs: Asymptomatic carriers; NCs: Normal uninfected healthy controls; MS: Multiple sclerosis; CSF: Cerebrospinal fluid; OINDs: Other inflammatory neurological diseases; ADCC: Antibody-dependent cellular cytotoxicity; PVL: Proviral load; CNS: Central nervous system; SLE: Systemic lupus erythematosus; CIDP: Chronic inflammatory demyelinating polyneuropathy; GBS: Guillain-Barré syndrome; ALS: Amyotrophic lateral sclerosis; HPRT: Hypoxanthine phosphoribosyltransferase.

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; TM, SI, TT, YO, and HT provided clinical samples and assembled clinical database; RT, SA, FU, and SI performed experiments, analyzed and interpreted data; YT made contribution to the conception and design of the study. All authors read and approved the final manuscript.

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A novel and simple method for generation of human dendritic cells from unfractionated peripheral blood mononuclear cells within 2 days: its application for induction of HIV-1-reactive CD4+ T cells in the hu-PBL SCID mice

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Because dendritic cells (DCs) play a critical role in the regulation of adaptive immune responses, they have been ideal candidates for cell-based immunotherapy of cancers and infections in humans. Generally, monocyte-derived DCs (MDDCs) were generated from purified monocytes by multiple steps of time-consuming physical manipulations for an extended period cultivation. In this study, we developed a novel, simple and rapid method for the generation of type-1 helper T cell (Th1)-stimulating human DCs directly from bulk peripheral blood mononuclear cells (PBMCs). PBMCs were cultivated in the presence of 20 ng/ml of granulocyte-macrophage colony-stimulating factor, 20 ng/ml of interleukin-4 (IL-4) and 1,000 U/ml of interferon-β for 24 h followed by 24 h maturation with a cytokine cocktail containing 10 ng/ml of tumor necrosis factor-α (TNF-α), 10 ng/ml of IL-1β and 1 μg/ml of prostaglandin E2. The phenotype and biological activity of these new DCs for induction of allogeneic T cell proliferation and cytokine production were comparable to those of the MDDCs. Importantly, these new DCs pulsed with inactivated HIV-1 could generated HIV-1-reactive CD4+ T cell responses in humanized mice reconstituted with autologous PBMCs from HIV-1-negative donors. This simple and quick method for generation of functional DCs will be useful for future studies on DC-mediated immunotherapies.

Keywords: dendritic cell, short-term culture, Th1-inducing DCs, anti-HIV-1 T cell response, hu-PBL-SCID

INTRODUCTION

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) which play a critical role in the regulation of the adaptive immune response through activation and polarization of naive T cells (Banchereau et al., 2000). Since small numbers of activated DCs are highly efficient in generating immune responses against infections and cancers (Moll and Berberich, 2001; Steinman and Banchereau, 2007), the DC therapy represents a new and promising immunotherapeutic approach for treatment of advanced cancers as well as for prevention of infectious diseases. Indeed, the current clinical trials with *ex vivo*-generated DCs (so-called DC vaccine) will yield precious information regarding their potentials as vectors for immunotherapy (Gilboa, 2007; Connolly et al., 2008; Ezzelarab and Thomson, 2011). However, the general protocols to generate DCs are complicated and time consuming. Moreover, since different *ex vivo* DC generation methods affect the DC phenotype and function (Kalantari et al., 2011), it is critical to choose appropriate method for generating functional DCs. In general, the DC precursor monocytes are purified from PBMCs by adherence (Jonuleit et al., 2001), elutriation (Berger et al., 2005) or positive or negative selection using immunomagnetic beads (Babatz et al., 2003). These enriched monocytes are then induced to differentiate into DCs by 5 days-*in vitro* cultivation in medium supplemented with granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 followed by a 2-days-maturation procedure (Sallusto and Lanzavecchia, 1994; Gilboa, 2007; Dauer et al., 2008). However, a lines of evidence are increasing that mature monocyte-derived DCs can be generated even after short-term cell culture for 2–3 days (Dauer et al., 2003a,b; Jarnjak-Jankovic et al., 2007; Zhang et al., 2008; Tawab et al., 2009).

In this study, in an attempt to simplify the methods currently being used for optimal DC generation and to develop a standardized method of preparing effective myeloid DC vaccine for immunotherapies, we explored the efficacy of using unfractionated PBMCs as a source of DC precursors and short-term *in vitro* cell culture just for 2 days.

MATERIALS AND METHODS

REAGENTS

The media used were RPMI 1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal calf serum (FCS; Sigma, St. Louis, MO, USA), 100 U of penicillin per ml, and 100 μg of streptomycin per ml (hereafter called RPMI medium) and Iscove's modified Dulbecco's medium (Lifetechnologies, Grand Island,

NY, USA) supplemented with 10% FCS with the same antibiotics (hereafter called Iscove's medium). Aldrithiol-2 (AT-2) and low-endotoxin bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO, USA). The recombinant human cytokines used included IL-4, GM-CSF, TNF-α and IL-1β (PeproTech, London, UK). Enzyme-linked immunosorbent assay (ELISA) kits for the quantitation of human IFN-γ, human IL-4, human IL-10 and human IL-12 (detecting IL-12 p75 heterodimer) were purchased from Biolegend. The human monocyte negative isolation kits and the human T cell isolation kits were purchased from Invitrogen (Carlsbad, CA, USA). The human naive $CD4^+$ T cell isolation kit was purchased from Miltenyi Biotec (Gladbach, Germany). The Vybrant CFDA SE Cell Tracer Kit was purchased from Invitrogen.

GENERATION OF DCs

Human PBMCs were isolated from heparinized peripheral blood obtained from normal healthy adult volunteer donors by standard density gradient centrifugation. Cells at the interface were collected and washed three times in cold phosphate-buffered saline (PBS) containing 0.1% low-endotoxin BSA and 2 mM Na_2EDTA. For select experiments, monocytes were purified from PBMCs using the $CD14^+$ monocyte negative isolation kit (Invitrogen, Carlsbad, CA, USA). An aliquot of cells from each monocyte preparation was examined by flow cytometry and found to contain >90% $CD14^+$ cells. To obtain immature MDDCs (iMDDCs), PBMCs (2.5×10^6 cells/ml) or the purified monocytes (5×10^5 cells/ml) were cultured in RPMI medium containing 20 ng/ml of human GM-CSF and 20 ng/ml of human IL-4 at 37°C in 24-well plates in a 5% CO_2 humidified incubator for 5 days. In other experiments, iDCs were generated from either purified monocytes or whole PBMCs by cultivation in RPMI medium containing GM-CSF (20 ng/ml), IL-4 (20 ng/ml) and IFN-β (1,000 U/ml) for 1 day. These iDCs were matured by incubation in the presence of either 10 ng/ml of LPS (Sigma) or a cocktail containing 10 ng/ml of TNF-α, 10 ng/ml of IL-1β and 1 μg/ml of prostaglandin E2 (PGE2; TIP cocktail) for 1–2 days.

FLOW CYTOMETRY

Aliquots of the cells to be analyzed were incubated in PBS containing 0.1% BSA and 0.1% sodium azide (FACS buffer) supplemented with 2 mg/ml normal human IgG on ice for 15 min to block Fc receptors. The cell suspension was then incubated with a predetermined optimal concentration of the appropriate fluorescent dye-labeled mAbs against human cell surface markers on ice for 30 min. The fluorescent dye-labeled monoclonal antibodies (mAbs) against human cell surface molecules used included anti-CD3, anti-CD4, anti-CD8, anti-CD14, CD20, anti-CD80, anti-HLA-DR, and isotype-matched control mAbs (Beckman Coulter, Fullerton, CA, USA), and anti-CD11c, anti-CD86, and anti-CD83 (BioLegend, San Diego, CA, USA). After washing with FACS buffer, cells were fixed in 1% paraformaldehyde (PFA) containing FACS buffer. The cells were then analyzed on FACS-Calibur flow cytometer with CellQuest software (BD Pharmingen, San Diego, CA, USA). Isotype-matched mAbs were utilized as controls to stain an aliquot of the cells to be analyzed for purposes of establishing gates and for determination of the frequency of positively stained cells.

HIV-1 PREPARATION AND INACTIVATION

$HIV\text{-}1_{IIIB}$ (virus that only use CXCR4 as chemokine co-receptor, termed X4) was harvested from Molt-4/IIIB cell cultures. Batches of each HIV-1 preparation were inactivated with Aldrithiol-2 (AT-2; Sigma) as described previously (Yoshida et al., 2003). AT-2 was removed by three successive ultrafiltration in PBS using 100-kDa-cut-off centrifugal filtration devices (Centriprep 100; Amicon, Beverly, MA, USA). Then AT-2–inactivated HIV-1 (iHIV) was purified by pelleting down the virus at 20,000 × *g* for 2 h three times in 0.1% BSA-PBS. The virus pellet was resuspended in 0.1% BSA-PBS, aliquoted, and stored at −80°C until use. The concentration of HIV-1 was estimated by measuring levels of HIV-1 p24 antigen with our in-house p24 ELISA kit (Tanaka et al., 2010). As previously described (Yoshida et al., 2003), activated human PBMCs incubated with an aliquot of 1 μg/ml of the AT-2-treated HIV-1 preparation failed to demonstrate the presence of any detectable infectious virions (data not shown).

STIMULATION OF T CELLS

Enriched populations of naive $CD4^+$ T cells and bulk T cells with >90% purity were isolated from normal human PBMCs by using appropriate negative cell isolation kits. These T cells (4×10^4 cells/well) were first labeled with carboxy-fluorescein diacetate succinimidyl ester (CFSE) according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA), then co-cultured with allogeneic DCs at a T cells: DCs ratio of 50:1 in 100 μl of RPMI medium supplemented with 20 U/ml human IL-2 in 96-well, U-bottomed plates. Cell proliferation and cytokine production were determined on day 4.

hu-PBL-SCID MICE

The BALB/c-$rag2^{-/-}\gamma c^{-/-}$ mice lacking T cells, B cells and natural killer (NK) cells ($Rag2^{-/-}$ mice; Traggiai et al., 2004) were used in this study. The mice were kept in the specific-pathogen-free and P3 animal facilities of the Laboratory Animal Center, University of the Ryukyus. The protocols for the care and use of mice engrafted with human PBMCs and autologous DCs sensitized with inactivated HIV-1 or ovalbumin (OVA) were approved by the committee on animal research of the University of the Ryukyus prior to initiation of the study. Matured DCs (5×10^5 cells) pulsed with either AT-2-inactivated HIV-1 (40 ng of p24) or 100 μg of OVA in 100 μl of RPMI medium for 2 h at 37°C were mixed with autologous fresh PBMCs (3×10^6 cells) in a final volume of 100 μl in serum-free RPMI medium, and the were directly injected into the spleen of Rag2 mice as previously described (Yoshida et al., 2003). One week later, the same number of DCs pulsed with the same antigens were inoculated again into the spleen. One week later, mice were sacrificed, blood was collected by cardiocentesis, and human $CD4^+$ T cells were enriched from splenocytes using a human $CD4^+$ T cell isolation kit according to the manufacturer's instructions. For the measurement of antigen-specific human cellular immune responses, human $CD4^+$ T cell (2×10^5 cells) collected from the spleens of immunized $Rag2^{-/-}$ mice were cultured for 2 days with autologous monocytes (2×10^5 cells) in the presence or absence of inactivated HIV containing 40 ng/ml of p24 in 500 μl of RPMI medium supplemented with 20 U/ml of IL-2 in individual wells of a 48-well plate at 37°C. The concentration

of human IFN-γ or IL-4 produced in the culture supernatants was determined with ELISA kits.

STATISTICAL ANALYSIS

Data were analyzed by Student's *t* test with the with Prism software (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

GENERATION OF MYELOID MATURE DCs DIRECTLY FROM PBMCs WITHIN 2 DAYS

In order to reduce the cost, labor and any loss of potential precursors from PBMCs, we have previously established a novel culture method for generating functional human DCs from unfractionated PBMC in which whole PBMCs were cultured in the presence of IL-4 and GM-CSF for 5 days followed by a 2-day maturation in media containing poly I:C and IL-1β (Kodama et al., 2010). However, there were considerable lot variations in commercial poly:IC in the DC-maturation activity (data not shown). Therefore, we tested a previously reported maturation cytokine cocktail containing TNF-α, IL-1β, IL-6 and PGE2 (Jonuleit et al., 1997). In a preliminary study, we found that IL-6 was not necessary to mature DCs from purified monocytes in the present cell culture conditions, probably due to the use of serum-containing media. Thus, we used a cytokine cocktail

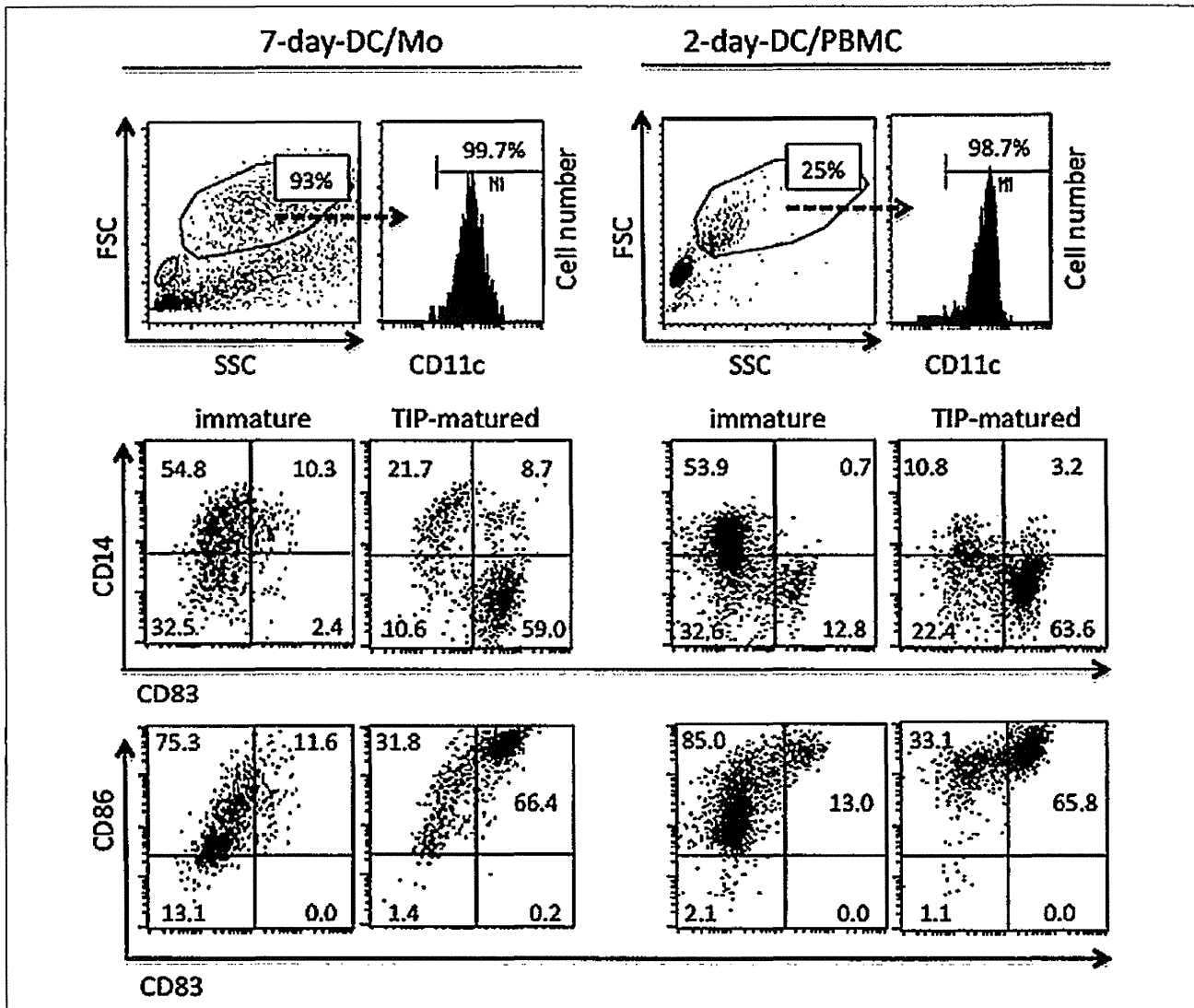

FIGURE 1 | Generation of functional human myeloid DCs directly from PBMCs *in vitro* within 2 days. MDDCs generated purified monocytes for 7 days (7-day-DC/Mo) or whole PBMCs cultured in medium containing GM-CSF, IL-4 and IFN-β (2-day-DC/PBMC), either non-treated or treated with the maturation cocktail containing TNF-α, IL-1β and PGE2 (TIP) were examined for the expression of mature DC markers by a flow cytometry. Cells were stained with antibodies to CD11c, CD14, CD83 and CD86, and analyzed on a subpopulation gated for FSC^{high}, SSC^{high} and $CD11c^{+}$. The percentages of the positive cells in the gated population were shown in the dot plots. Data shown are representative of three independent experiments using blood from three different donors.

containing 10 ng/ml of TNF-α, 10 ng/ml of IL-1β and 1 μg/ml of PGE2 (hereafter called TIP cocktail) throughout the present study.

Based on our previous report that monocytes can be differentiated into mature DCs within 2 days (Zhang et al., 2008), we tested whether Th1-inducing DCs could be generated from unfractionated PBMCs. PBMCs (2.5×10^6 cells/ml) were cultured in RPMI medium containing GM-CSF (20 ng/ml), IL-4 (20 ng/ml) and IFN-β (1,000 U/ml) for 1 day followed by additional 1 day cultivation in the presence or absence of the TIP cocktail. The phenotypes of $CD11c^+$ large cells in these 2-day PBMC cultures were compared with those of MDDCs derived from purified monocyte for 7 days (7-day-DC/Mo; **Figure 1**). The proportion of FSC^{high} and SSC^{high} cells in the 2-day-DC/PBMC culture was 20~25% of total viable cells depending on donors and these cells expressed CD11c (data not shown). After maturation with the TIP cocktail, similar to the 7-day-DC/Mo, the large $CD11c^+$ cells in the 2-day PBMC cultures became $CD14^{low}$, $CD86^{high}$ and $CD83^{high}$, a typical marker of matured myeloid DCs (Ohshima et al., 1997). The other viable cell populations in the 2-day PBMC cultures were $CD3^+$ T cells (54.0~59.2%), $CD56^+$ NK cells (8.4~9.3%) and $CD19^+$ B cells (6.5~8.6%; $n = 3$). These data showed that the present culture method was applicable to generate myeloid mature DCs from bulk PBMCs within 2 days (2-day-DC/PBMC).

Then we tested cytokine production by these 2-day-DC/PBMC. Interestingly, in contrast to the DCs matured in the presence of LPS, the production of IL-12 and IL-10 by the TIP matured 2-day-DC/P was minimum (**Figure 2**). To investigate whether the 2-day-DC/PBMC were immunologically functional, we examined their ability to stimulate allogeneic T cell proliferation. Like the MDDCs (7-day-DC/Mo), the 2-day-DC/PBMC could stimulate allogeneic T cell proliferation (**Figure 3A**). Then we quantitated the levels of IFN-γ and IL-4 in the culture supernatants from allogeneic $CD4^+$ T cells co-cultured with various DCs. As shown in **Figure 3B**, among the four DC preparations including the 7-day-DC/Mo, 7-day-DCs from PBMCs (7-day-DC/PBMC), 2-day-DCs from monocytes (2-day-DC/Mo) and 2-day-DC/PBMC, the 2-day-DC/PBMC were most potent in induction of IFN-γ production. The bulk 2-day-DC/PBMC alone did not produce detectable IFN-γ (<20 pg/ml) in the present culture conditions (data not shown). The levels of IL-4 and IL-10 were below detection (<5 pg/ml) in all the samples tested (data not shown). These results indicated that the 2-day-DC/PBMC had a potential to induce Th1 response.

INDUCTION OF HIV-1-REACTIVE HUMAN $CD4^+$ T CELL RESPONSES IN hu-PBL-SCID MICE

Finally, we examined whether the short-term generated 2-day-DC/PBMC could induce HIV-1-reactive immune responses *in vivo* in comparison to MDDCs (7-day-DC/Mo) using our hu-PBL-SCID mice model (Yoshida et al., 2003). SCID mice were *intra-splenically* transplanted with DCs loaded with AT-2-inactivated HIV-1 together with autologous fresh PBMCs. On day 7, these mice were received an *intra-splenic* booster injection with similarly prepared antigen-pulsed DCs. Seven days after the booster injection, mice were sacrificed and examined for antigen-specific human immune responses. **Figure 4** showed that after *in vitro* re-stimulation with autologous APCs pulsed with inactivated HIV-1, enriched human $CD4^+$ T cells from two out of three mice immunized with MDDCs (7-day-DC/Mo) pulsed with HIV-1 and those from three out of four mice immunized with 2-day-DC/PBMC pulsed with HIV-1 produced IFN-γ in antigen-dependent way, indicating that the 2-day-DC/PBMC could induce HIV-1 antigen-reactive human T responses *in vivo* as potent as MDDCs. In the re-stimulated culture supernatants, no IL-4 or IL-10 was detected (<5 pg/ml) using ELISA (data not shown). In addition, no detectable antibodies against HIV-1 were detected

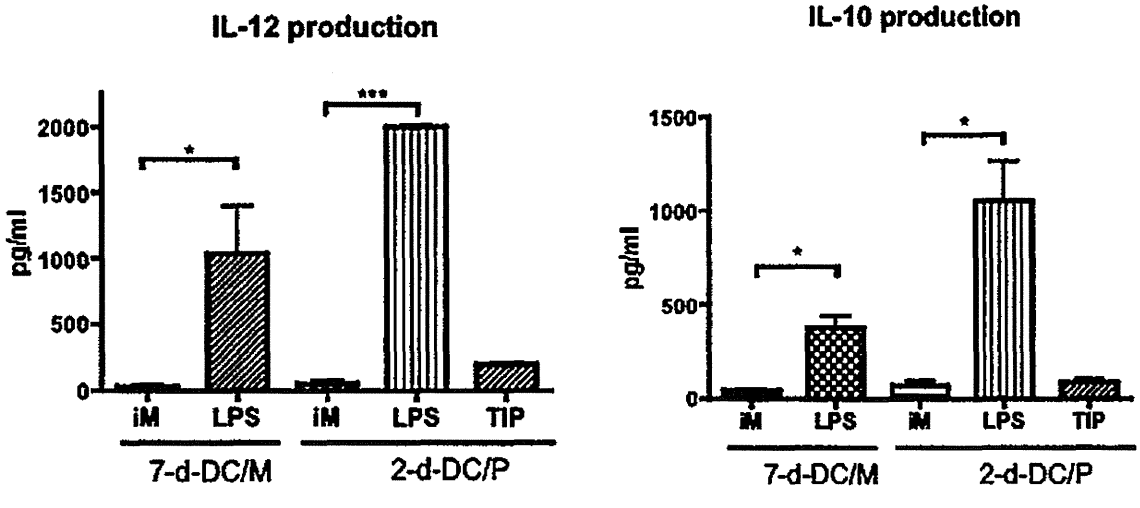

FIGURE 2 | Production of cytokines by the 2-day-DC/PBMC. The levels of IL-12 (bioactive p70 heterodimer) and IL-10 in the supernatants from MDDCs (7-d-DC/M) or 2-day-DC/PBMC (2-d-DC/P) either non-matured (iM) or matured with LPS or the TIP cocktail were determined by ELISA. Data are mean ± SD of triplicate cultures. $*P < 0.05$, $***P < 0.001$. Data shown are representative of three independent experiments using blood from two different donors.

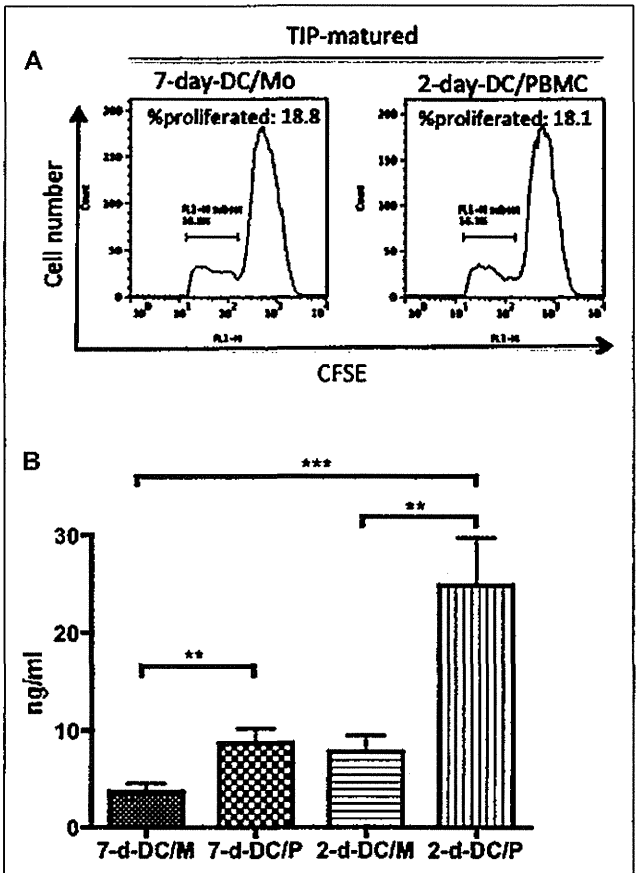

FIGURE 3 | High levels of production of IFN-γ by allogeneic T cells stimulated with 2-day-DCs generated from PBMCs. Matured MDDCs (7-day-DC/Mo) and 2-day-DCs generated from PBMCs (2-day-DC/PBMC) were co-cultured with CFSE-labeled allogeneic $CD4^+$ T cells at DC to T cell ratio of 1:50 for 4 days. The cell number of DCs in 2-day-DC/PBMC was manually counted using a Burker-Turk hemo-cytometer in which only large cells were counted. **(A)** Percentages of proliferated cells were examined by flow cytometry. **(B)** The levels of IFN-γ produced in the supernatants were quantitated by ELISA. In addition to the DCs shown in **(A)**, MDDCs generated from PBMCs for 7 days (7-d-DC/P) and 2-day-DCs generated from purified monocytes (2-d-DC/M) were also tested. Data shown are representative of three independent experiments using blood from two different donors.

as determined by using a commercial Western blot assay kit in plasma samples from all the DCs-HIV-1-immunized mice (data not shown).

Altogether, these results demonstrated that human myeloid DCs directly generated from PBMCs by the present short-term cultivation method were potent in induction of functional Th1 responses both *in vitro* and *in vivo*.

DISCUSSION

In the present study, we have developed a novel, simple and rapid protocol for generating Th1-stimulating human myeloid DCs directly from unfractionated PBMCs. These 2-day-DC/PBMC were potent in both stimulating allogeneic T cells *in vitro* and inducing HIV-1-reactive Th1 responses in hu-PBL-SCID mice. The use of whole PBMCs as DC precursors might reduce any loss of monocytes in the step of purification by adherence (Jonuleit et al., 2001), elutriation (Berger et al., 2005) or positive or negative selection using immunomagnetic beads (Babatz et al., 2003). One possible concern on using whole PBMCs was that the non-monocyte cells, such as T, B or NK cells, in the PBMCs might interfere with differentiation and function of DCs. However, in the present study there was no obvious difference in DC maturation and function between in PBMC and purified monocyte cultures.

For the final maturation, we used a cytokine cocktail containing TNF-α and IL-1β and PGE2 (TIP coctail). Simultaneous use of these three reagents in TIP was essential for maturation of DCs since use of the reagents either in single or in two combinations failed to mature DCs (data not shown). In general, IL-6 that is included in the maturation cytokine cocktail TNF-α and IL-1β and PGE2 to mature DCs was not necessary in the present culture conditions. The reason remains to be studied, but it is possible that IL-6 is required in serum-free culture conditions. The present 2-day-DC/PBMC matured by TIP produced lower IL-12 than those matured by LPS. Low levels production of IL-12 might be ascribed to the use of PGE2 that inhibits bioactive IL-12 heterodimer production (Kalinski et al., 2001; Kalim and Groettrup, 2013). Despite of the low level production of IL-12, the TIP-matured 2-day-DC/PBMC were potent in stimulating IFN-γ, but not IL-4 or IL-10, production by allogeneic T cells. The reason for higher potentials of 2-day-DC/PBMC to induce Th1 cells than MDDCs remains to be clarified. It is speculated that natural DCs contained in the 2-day-PBMC-derived DCs might enhance the activation. Indeed, 2-day-DC/PBMC culture generated from $CD14^+$ cell-depleted PBMCs were able to stimulate allogeneic $CD4^+$ T cells to a lesser extent (data not shown). However, we cannot clearly determine if the stimulation was mediated by remaining monocytes. Further study is required to solve this issue. Importantly, as the previous study (Yoshida et al., 2003), the present study showed the induction of primary HIV-1-specific human $CD4^+$ T cell immune responses in hu-PBL-SCID mice by DC-based immunization, demonstrating that the present 2-day-PBMC-derived DCs might have a potential for clinical use in DC-based immunization in humans against HIV-1. It was of interest that the levels of IFN-γ production were higher in $CD4^+$ T cells immunized with 2-day-DC/PBMC than those immunized with 7-day-DC/Mo. It is possible that 2-day-DC/PBMC could live longer than 7-day-DC/Mo *in vivo* to stimulate antigen-specific $CD4^+$ T cells. In addition, because myeloid DCs are susceptible to HIV-1 infection (Knight et al., 1990), the use of these IFN-β-treated DCs will be beneficial for HIV-1-infected individuals.

In conclusion, the present study provided a new method to generate functional human myeloid DCs directly from PBMCs in a short-term culture period. These DCs will be useful for studies exploring potentials of DC-based immunization for not only infectious diseases but also cancers *in vitro* and *in vivo*.

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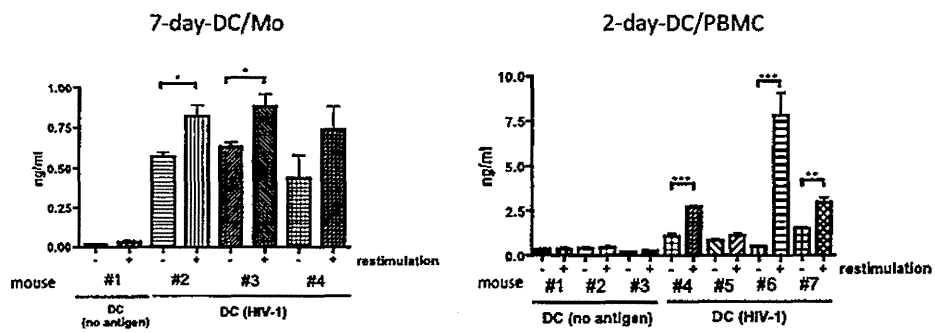

FIGURE 4 | Functional activity of the 2-days-DCs in hu-PBL-SCID mice. Fresh autologous PBMCs from normal human donors were transferred into the Rag2$^{-/-}$ mouse spleen together with autologous mature MDDCs (7-day-DC/Mo) or 2-days-PBMC-derived DCs (2-day-D/PBMC) pulsed with no antigen (no antigen) or AT-2-inactivated HIV-1 (40 ng of p24). On day 7 after the first transplantation, these mice were received an *intra splenic* booster injection with similarly prepared DCs. Seven days after the booster injection, mice were sacrificed and human CD4$^+$ T cells were purified from splenocytes. These CD4$^+$ T cells were co-cultured with autologous APCs (adherent PBMCs) in the presence or absence of antigens (restimulation) for 2 days at 37°C. IFN-γ levels produced in the culture supernatants were measured by ELISA. Data show mean ± SD of triplicate cultures. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Data shown are representative of three independent experiments using blood from two different donors.

AUTHOR CONTRIBUTIONS

Akira Kodama designed and performed the experiments, analyzed the data and wrote the paper. Reiko Tanaka and Mineki Saito performed the experiments, analyzed the data and wrote the paper. Aftab A. Ansari participated in the design of the study and helped to draft the manuscript. Yuetsu Tanaka designed and supervised the research, performed experiments and wrote the paper. All authors checked the final version of this manuscript.

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Elimination of Human T Cell Leukemia Virus Type-1-Infected Cells by Neutralizing and Antibody-Dependent Cellular Cytotoxicity-Inducing Antibodies Against Human T Cell Leukemia Virus Type-1 Envelope gp46

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Abstract

Human T cell leukemia virus type-1 (HTLV-1) is prevalent worldwide with foci of high prevalence. However, to date no effective vaccine or drug against HTLV-1 infection has been developed. In efforts to define the role of antibodies in the control of HTLV-1 infection, we capitalized on the use of our previously defined anti-gp46 neutralizing monoclonal antibody (mAb) (clone LAT-27) and high titers of human anti-HTLV-1 IgG purified from HAM/TSP patients (HAM-IgG). LAT-27 and HAM-IgG completely blocked syncytium formation and T cell immortalization mediated by HTLV-1 *in vitro*. The addition of these antibodies to cultures of $CD8^+$ T cell-depleted peripheral blood mononuclear cells (PBMCs) from HAM/TSP patients at the initiation of culture not only decreased the numbers of Tax-expressing cells and the production of HTLV-1 p24 but also inhibited the spontaneous immortalization of T cells. Coculture of *in vitro*-HTLV-1-immortalized T cell lines with autologous PBMCs in the presence of LAT-27 or HAM-IgG, but not an $F(ab')_2$ fragment of LAT-27 or non-neutralizing anti-gp46 mAbs, resulted in depletion of HTLV-1-infected cells. A 24-h ^{51}Cr release assay showed the presence of significant antibody-dependent cellular cytotoxicity (ADCC) activity in LAT-27 and HAM-IgG, but not $F(ab')_2$ of LAT-27, resulting in the depletion of HTLV-1-infected T cells by autologous PBMCs. The depletion of natural killer (NK) cells from the effector PBMCs reduced this ADCC activity. Altogether, the present data demonstrate that the neutralizing and ADCC-inducing activities of anti-HTLV-1 antibodies are capable of reducing infection and eliminating HTLV-1-infected cells in the presence of autologous PBMCs.

Introduction

HUMAN T CELL LEUKEMIA VIRUS type-1 (HTLV-1) is the first human retrovirus that was etiologically associated with adult T cell leukemia (ATL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP).[1–4] HTLV-1 is prevalent worldwide with foci of high prevalence in southwest Japan, the Caribbean islands, South America, and a part of Central Africa. The total number of HTLV-1 carriers is currently estimated to be 10–20 million.[5] The majority of HTLV-1 carriers remain asymptomatic throughout their lives, and approximately 5% of HTLV-1-infected individuals will develop either ATL or HAM/TSP after prolonged latency periods.

HTLV-1 is transmitted through contact with bodily fluids containing infected cells most often from mother to child through breast milk or via blood transfusion. It has been previously established that HTLV-1 efficiently spreads from cell to cell via the formation of virological synapses.[6] More recently, however, the formation of extracellular HTLV-1 viral particles similar to the formation of bacterial films has also been shown to be effective in viral transmission.[7] HTLV-1-antigen-expressing cells are difficult to detect at least in fresh peripheral blood mononuclear cells (PBMCs) from HTLV-1-infected individuals.[8] However, when these PBMCs are isolated from the blood and cultured *in vitro*, some T cells begin to produce HTLV-1 antigen[9,10] followed by spontaneous immortalization of the cells in media containing interleukin-2 (IL-2).[11]

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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

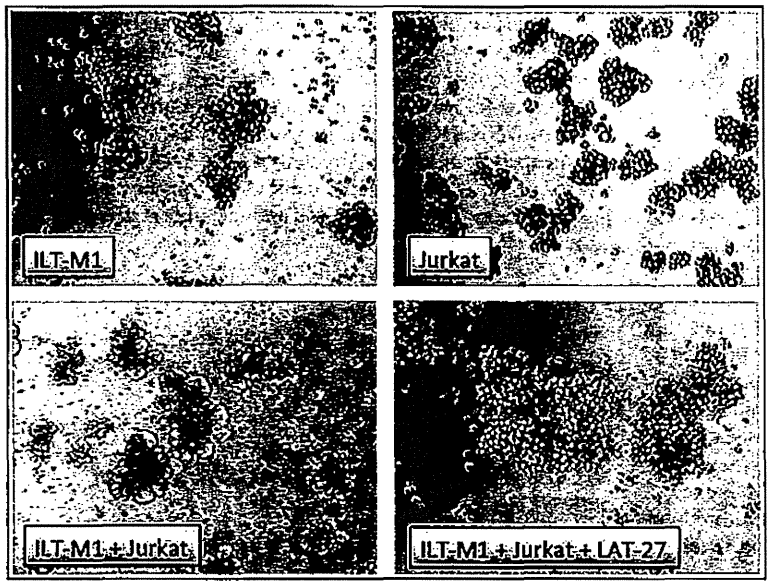

FIG. 1. Human T cell leukemia virus type-1 (HTLV-1)-mediated syncytia formation. $HTLV\text{-}1^+$ ILT-M1 and $HTLV\text{-}1^-$ Jurkat cells were either cultured alone or cocultured at a cell-to-cell ratio of 1:1 in the presence or absence of 10 μg/ml LAT-27 for 18 h. Syncytia were microscopically observed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

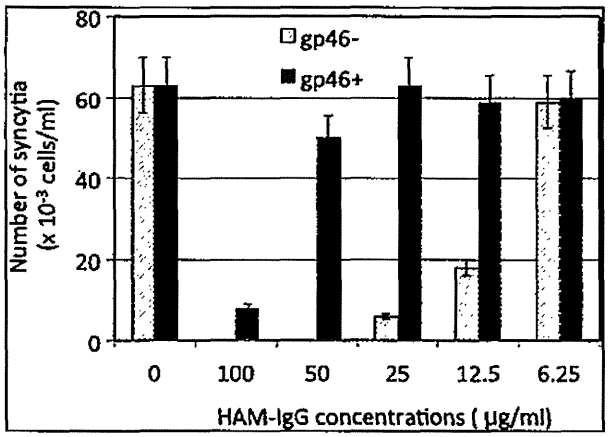

FIG. 2. Anti-gp46 antibodies are major HTLV-1 neutralizing antibodies in HAM-IgG. HAM-IgG at graded concentrations (0~100 μg/ml) was preincubated with either affinity-purified gp46 antigen (black bars) at 10 μg/ml for 10 min or incubated with medium alone (gray shaded bars, labeled as "gp46-") and tested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

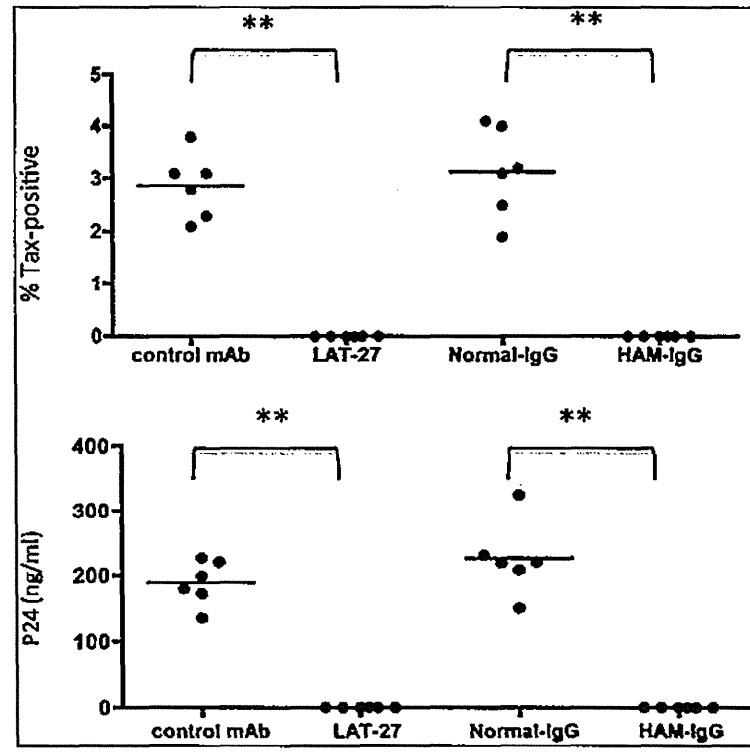

FIG. 3. LAT-27 and HAM-IgG completely block HTLV-1-mediated T cell immortalization *in vitro*. Activated peripheral blood mononuclear cells (PBMCs) from normal donors were seeded into six wells of 96-well U-bottom plates (1×10^5 cells/0.1 ml/well) and cocultured with an equal number of mitomycin C-treated ILT-M1 cells in the presence or absence of 10 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).