HAM/TSP patients are significantly higher than OX40$^-$ CD4$^+$ and Tax$^-$CD4$^+$ T cells, respectively ($p < 0.001$, Student's *t*- test) (Figure 2F and Table 1).

Increased expression of OX40 in vivo in rapidly progressive HAM/TSP patients

To investigate if OX40 expression is associated with in vivo pathogenesis of HAM/TSP, we first measured the plasma concentration of sOX40 and sOX40L in 20 chronic HAM/TSP patients, 9 ACs, and 13 NCs by ELISA by using monoclonal antibodies generated in our laboratory (Figure 3A). None of the samples had detectable levels of sOX40L (data not shown), but we could readily detect sOX40. The median level of sOX40 in NCs was 149.5 pg/ml (range 13–328 pg/ml). Significantly higher sOX40 levels were found in chronic HAM/TSP patients (median 395.2 pg/ml, range 113–1295 pg/ml) and ACs (median 423.8 pg/ml, range 201–881 pg/ml) than in NCs (p=0.0043 for differences between HAM/TSP and NCs, p=0.0020 for differences between ACs and NCs). The difference between chronic HAM/TSP patients and ACs was not statistically significant. No positive correlation was found between sOX40 in the plasma and HTLV-1 PVL in infected individuals (i.e., chronic HAM/TSP patients and ACs) (Spearman's rank correlation coefficient n=29, r=0.031, P=0.873; Figure 3B). We then tested disease specificity by measuring the levels of sOX40 in the CSF from both rapidly progressive and chronic HAM/TSP patients, and in patients with other neurological disorders, with and without inflammation (e.g., 12 MS, 8 aseptic meningitis, 5 systemic lupus erythematosus with neurological manifestations, 9 chronic inflammatory demyelinating polyneuropathy, 6 Guillain-Barré syndrome, and 9 amyotrophic lateral sclerosis patients). As shown in Figure 3C, CSF sOX40 levels were markedly increased in patients with rapidly progressive HAM/TSP (n=3) and aseptic meningitis (n=8). The CSF sOX40 levels in other HTLV-1-infected inflammatory neurological diseases, i.e. any inflammatory neurological disorders except for HAM/TSP that occurred in HTLV-1 infected individuals, (HTLV-1+ OINDs, n=6) was not significantly different from chronic HAM/TSP (n=20), whereas the sOX40 level of HTLV-1+ OINDs was significantly increased compared to non-infected OINDs (HTLV-1- OINDs, n=26; Figure 3D).

Of the HAM/TSP patients studied, paired CSF and plasma samples, i.e., blood and CSF were collected on the same day, were available for six patients. HAM/TSP patients No.10-12 had a lower concentration of sOX40 in the CSF than in the plasma (Table 2), and the patients showed a typical clinical course of HAM/TSP (i.e. slowly progressive symmetrical myelopathy) and had no history of rapid exacerbation. In contrast, HAM/TSP patients No.13-15, who had higher concentrations of sOX40 in the CSF than in the plasma, showed a rapidly progressive clinical course (i.e. patients became unable to walk within three months after onset of initial symptoms).

Expression of OX40 in inflammatory mononuclear cells in spinal cord lesions of HAM/TSP patient with short disease duration and progressive symptoms

We also examined autopsy specimens from HAM/TSP patients by immunohistochemical staining. Although there was reduced or no OX40 protein expression in HAM/TSP patients who had a long duration of illness and who no longer had active inflammation (a representative example is shown in Figure 4A), we observed marked OX40 expression in inflammatory round-shaped mononuclear cells around the blood vessels in spinal cord lesions from one HAM/TSP patient (Figure 4B). This patient (patient 1 in refs [35-38], who had a shorter disease duration of up to 2.5 years after the onset of neurological symptoms) showed predominant infiltration of CD4$^+$ T cells [36] that also expressed tax mRNA [38], pro-inflammatory cytokines [37], and matrix metalloproteinases [39]. In contrast, we observed only low background staining for OX40L in spinal cord tissues of all the HAM/TSP patients examined (a representative example is shown in Figure 4C) compared to positive control (Figure 4D).

Anti-OX40 monoclonal antibody specifically eliminated naturally infected CD4$^+$ T cells via antibody-dependent cell-mediated cytotoxicity (ADCC) in cultured PBMCs

We investigated the role of OX40 in HTLV-1 naturally infected CD4$^+$ T cells, by testing the effects of an anti-human OX40 mAb on Tax expression. As shown in Figure 5, anti-OX40 mAb (clone B-7B5) reduced the percentage of Tax-positive cells, whereas the isotype control mAb (clone 2C2: anti-HIV-1 gp21, mouse IgG1) had no effect on Tax expression (Figure 5, 1st, 2nd, and 3rd panels from left). Culture of PBMCs with anti-CD16/CD32 (Fc receptor) antibody to block Fc receptors abolished Tax suppression by anti-OX40 mAb (Figure 5, 4th panels from left), suggesting that the effect of the anti-OX40 mAb (B-7B5) is mainly mediated by ADCC. We further tested the effects of the F(ab')$_2$ fragment of anti-OX40 mAb (B-7B5) and found that the F(ab')$_2$ fragment did not suppress Tax expression; this finding supports an ADCC mechanism of action of the anti-OX40 mAb (Figure 5, right panels).

Anti-OX40 monoclonal antibody specifically eliminated OX40-positive HTLV-1 infected cells in cultured PBMCs

We examined whether suppression of OX40 expression either reduced the frequency of Tax-positive cells or selectively eliminated HTLV-1-infected cells by isolating CD4$^+$ T cells from PBMCs before and after culture, extracting genomic DNA, and measuring HTLV-1 PVL. HTLV-1 PVL in CD4$^+$ T cells was significantly reduced

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

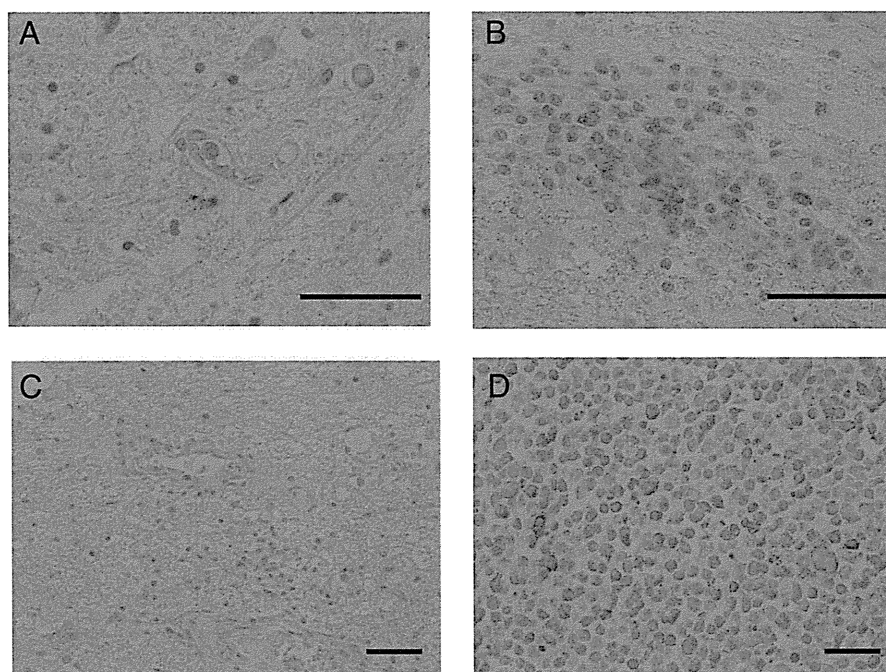

Figure 4 Expression of OX40 in inflammatory mononuclear cells in spinal cord lesions of HAM/TSP patient with short disease duration and progressive symptoms. We studied autopsy specimens from 9 HAM/TSP patients by immunohistochemical staining. **A.** No OX40 positive cells are detected in the spinal 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.

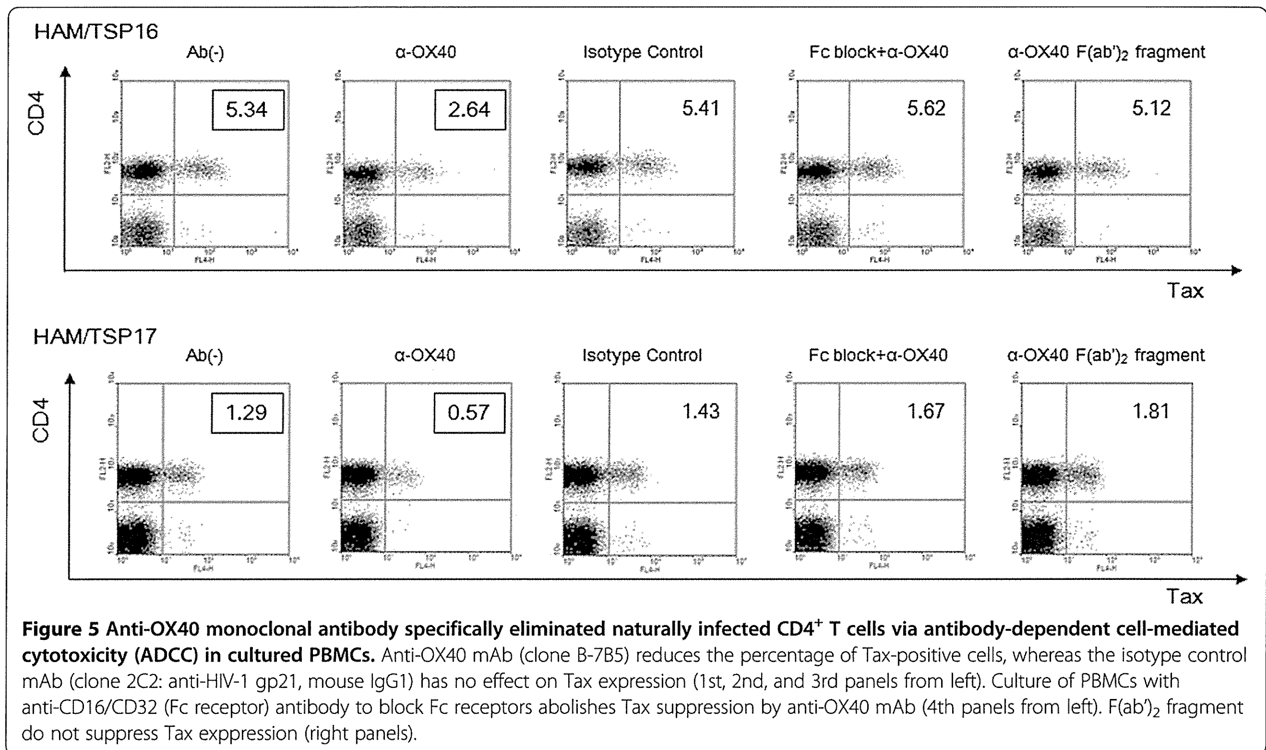

Figure 5 Anti-OX40 monoclonal antibody specifically eliminated naturally infected CD4$^+$ T cells via antibody-dependent cell-mediated cytotoxicity (ADCC) in cultured PBMCs. Anti-OX40 mAb (clone B-7B5) reduces the percentage of Tax-positive cells, whereas the isotype control mAb (clone 2C2: anti-HIV-1 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

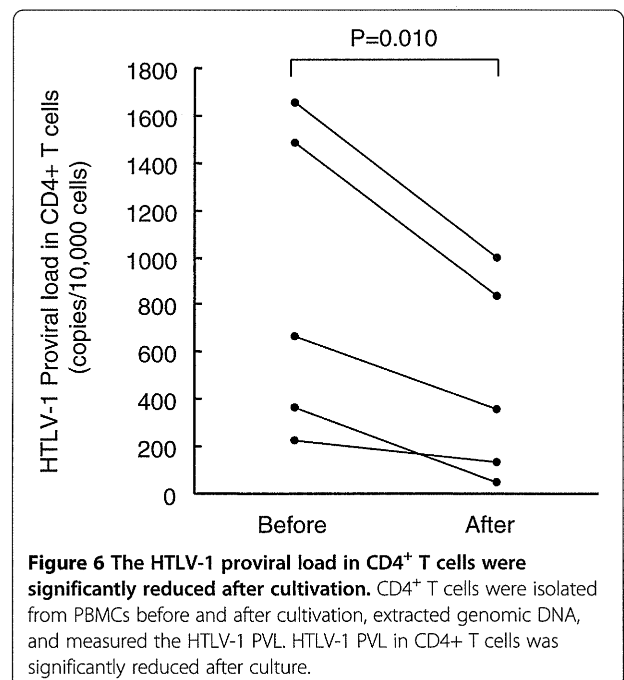

Figure 6 The HTLV-1 proviral load in CD4$^+$ T cells were significantly reduced after cultivation. CD4$^+$ T cells were isolated from PBMCs before and after cultivation, extracted genomic DNA, and measured the HTLV-1 PVL. HTLV-1 PVL in CD4+ T cells was significantly reduced after culture.

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 $1{\times}10^5$ 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^+ $CD4^+$ T cells from HTLV-1 infected individuals. OX40 was detected on $CD4^+$ 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 $CD4^+$ T cells that also expressed Tax (right panels). **FigureS2.** The expression of 4-1BB on $CD4^+$ T cells from HAM/TSP patients. **A**. 4-1BB was detected on both $CD4^+$ 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 $CD4^+$ 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 $CD4^+$ T cells that were positively stained with biotinylated recombinant soluble OX40L and PE-streptavidin was similar to the percentage of $CD4^+$ 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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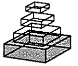

A novel and simple method for generation of human dendritic cells from unfractionated peripheral blood mononuclear cells within 2 days: its application for induction of HIV-1-reactive CD4$^+$ T cells in the hu-PBL SCID mice

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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-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-rag$2^{-/-}$γc$^{-/-}$ mice lacking T cells, B cells and natural killer (NK) cells (Rag$2^{-/-}$ 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 Rag$2^{-/-}$ 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

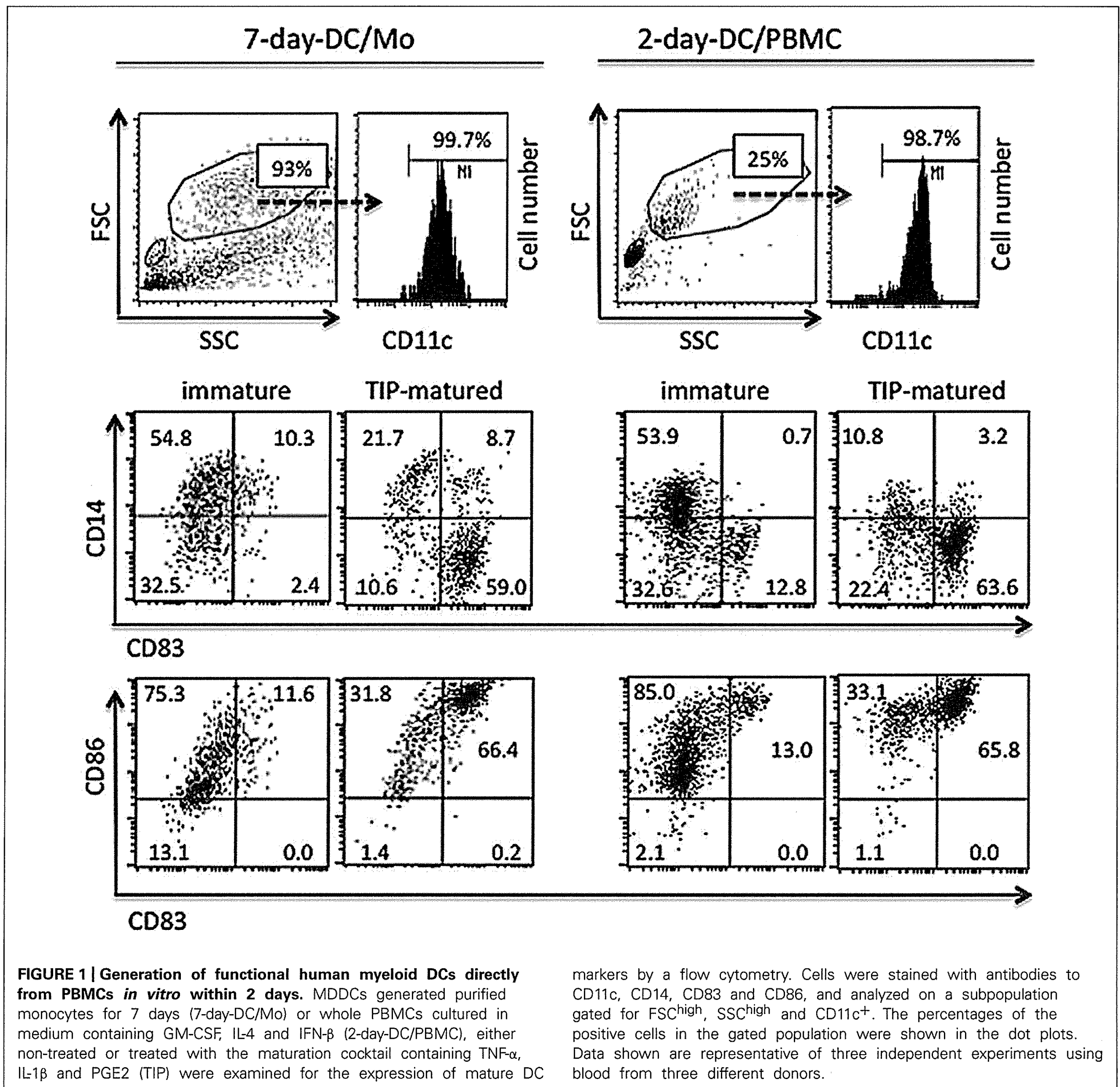

FIGURE 1 | Generation of functional human myeloid DCs directly from PBMCs *in vitro* within 2 days. MDDCs generated purified monocytes for 7 days (7-day-DC/Mo) or whole PBMCs cultured in medium containing GM-CSF, IL-4 and IFN-β (2-day-DC/PBMC), either non-treated or treated with the maturation cocktail 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

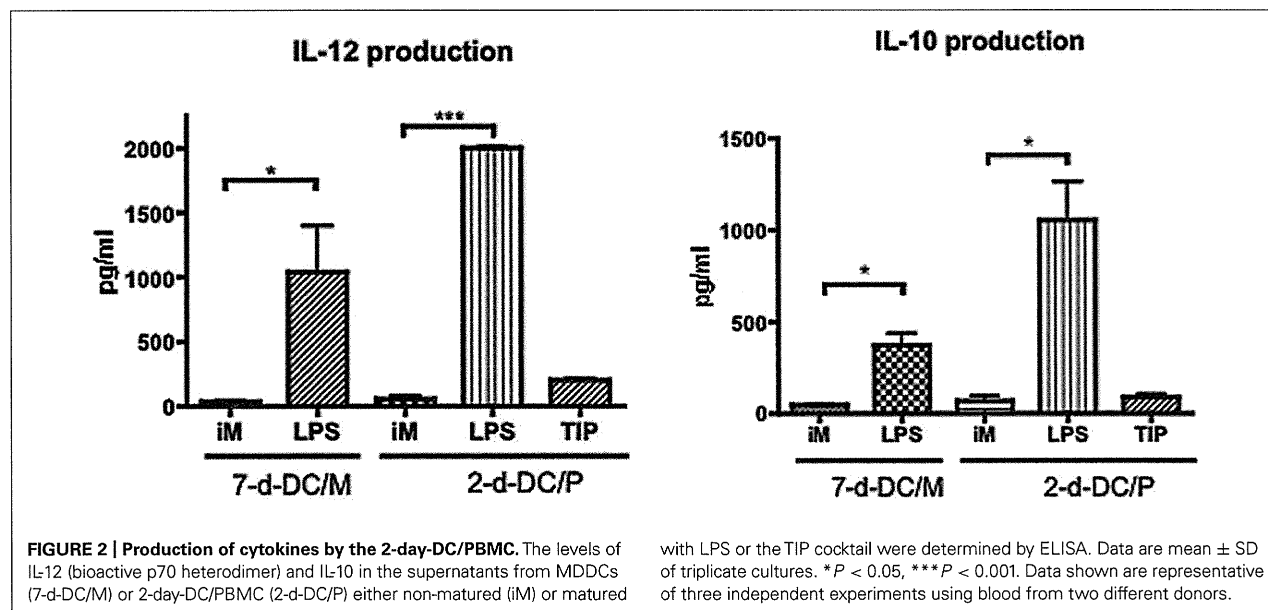

FIGURE 2 | Production of cytokines by the 2-day-DC/PBMC. The levels of IL-12 (bioactive p70 heterodimer) and IL-10 in the supernatants from MDDCs (7-d-DC/M) or 2-day-DC/PBMC (2-d-DC/P) either non-matured (iM) or matured with LPS or the TIP cocktail were determined by ELISA. Data are mean ± SD of triplicate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

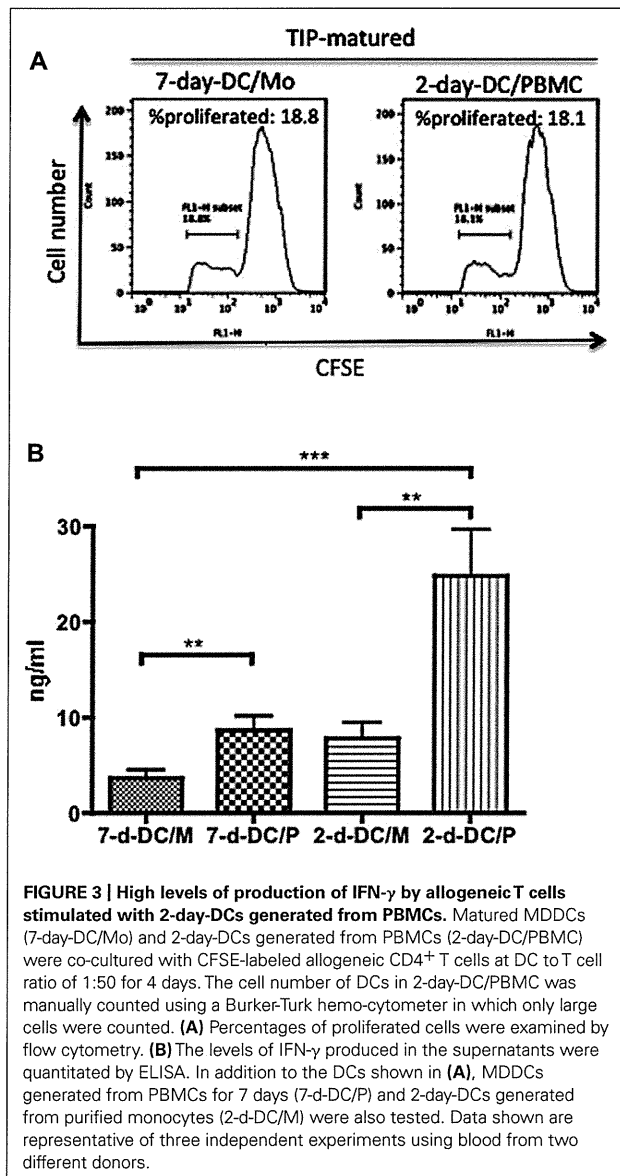

FIGURE 3 | High levels of production of IFN-γ by allogeneic T cells stimulated with 2-day-DCs generated from PBMCs. Matured MDDCs (7-day-DC/Mo) and 2-day-DCs generated from PBMCs (2-day-DC/PBMC) were co-cultured with CFSE-labeled allogeneic CD4$^+$ T cells at DC to T cell ratio of 1:50 for 4 days. The 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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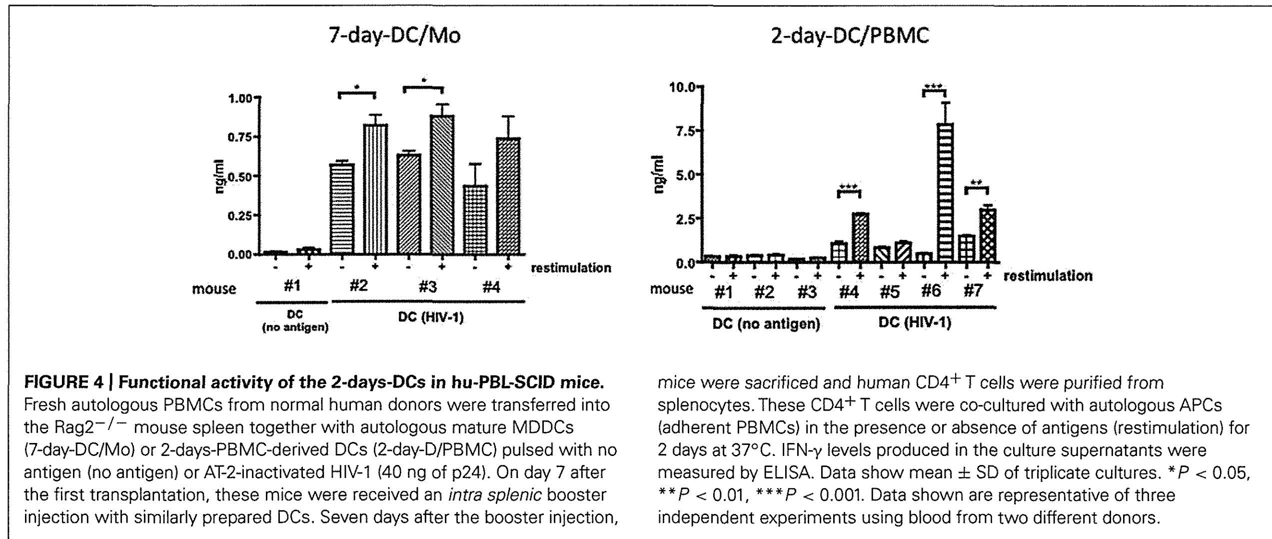

FIGURE 4 | Functional activity of the 2-days-DCs in hu-PBL-SCID mice. Fresh autologous PBMCs from normal human donors were transferred into the Rag2$^{-/-}$ mouse spleen together with autologous mature MDDCs (7-day-DC/Mo) or 2-days-PBMC-derived DCs (2-day-D/PBMC) pulsed with no antigen (no antigen) or 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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REVIEW

Neuroimmunological aspects of human T cell leukemia virus type 1-associated myelopathy/tropical spastic paraparesis

Mineki Saito

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Abstract Human T cell leukemia virus type 1 (HTLV-1) is a human retrovirus etiologically associated with adult T cell leukemia/lymphoma and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). Only approximately 0.25–4 % of infected individuals develop HAM/TSP; the majority of infected individuals remain lifelong asymptomatic carriers. Recent data suggest that immunological aspects of host–virus interactions might play an important role in the development and pathogenesis of HAM/TSP. This review outlines and discusses the current understanding, ongoing developments, and future perspectives of HAM/TSP research.

Keywords HTLV-1 · HAM/TSP · Host immune response

Introduction

Human T cell leukemia virus type 1 (HTLV-1) is a replication-competent human retrovirus associated with two distinct types of disease: a malignancy of mature $CD4^+$ T cells called adult T cell leukemia/lymphoma (ATL) (Hinuma et al. 1981; Poiesz et al. 1980; Yoshida et al. 1984) and a chronic inflammatory central nervous system disease HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Gessain et al. 1985; Osame et al. 1986). Like human immunodeficiency virus (HIV), HTLV-1 is never eliminated from the host despite vigorous cellular and humoral immune responses. However, in contrast to HIV infection, few with HTLV-1 develop disease; only approximately 2–3 % of infected persons develop ATL (Tajima 1990), another 0.25–4 % develop HAM/TSP (Hisada et al. 2004; Kramer et al. 1995; Nakagawa et al. 1995; Osame et al. 1990), and the majority of infected individuals remain lifelong asymptomatic carriers (ACs). Therefore, evaluation of the individual risk of developing disease in ACs would certainly be of considerable importance, especially in HTLV-1 endemic areas.

The viral, host, and environmental risk factors as well as the host immune response against HTLV-1 infection appear to regulate the development of HTLV-1-associated diseases (Bangham and Osame 2005). In particular, a strong immune response, especially the cytotoxic T lymphocyte (CTL) response, to HTLV-1 is seen in patients with HAM/TSP and suggested to be strongly associated with the pathogenesis of HTLV-1-associated diseases (Matsuura et al. 2010; Saito et al. 2012). For more than two decades, the investigation of HTLV-1-mediated immunopathogenesis has focused on Tax, an HTLV-1-encoded viral oncoprotein, because Tax activates many cellular genes by binding to groups of transcription factors and co-activators and is necessary and sufficient for cellular transformation. However, recent reports have identified that another regulatory protein, HTLV-1 basic leucine zipper factor (HBZ), also has a critical role in the development of ATL and HAM/TSP (Matsuoka and Jeang 2011). This review summarizes past and recent studies of HAM/TSP, attempting to answer the following fundamental questions: Why do some HTLV-1-infected people develop disease whereas the vast majority remain healthy? How does HTLV-1 persist in the individual host despite a strong host immune response? How is the inflammatory lesion in HAM/TSP initiated and maintained?

History and epidemiology of HTLV-1

HTLV-1 belongs to the *Deltaretrovirus* genus of the *Orthoretrovirinae* subfamily and infects 10–20 million people worldwide (de The and Bomford 1993; Proietti et al. 2005; Uchiyama 1997). HTLV-1 can be transmitted through sexual contact (Roucoux et al. 2005), intravenous drug use (Proietti et al. 2005), and breastfeeding from mother to child (Hino

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et al. 1985; Kinoshita et al. 1987). At present, the infection is endemic in southwest Japan, the Caribbean, Sub-Saharan Africa, and South America, with smaller foci in Southeast Asia, South Africa, and northeast Iran (Verdonck et al. 2007). HTLV-1 was initially isolated in 1980 from two T cell lymphoblastoid cell lines and the blood of a patient originally thought to have a cutaneous T cell lymphoma (Poiesz et al. 1980). It was the first retrovirus ever associated with cancer in a human. Three years before the isolation of HTLV-1, Takatsuki et al. reported ATL, a rare form of leukemia endemic to southwest Japan, as a distinct clinical entity (Uchiyama et al. 1977). In 1981, Hinuma et al. clearly demonstrated that ATL was caused by a new human retrovirus, originally termed ATLV (Hinuma et al. 1981; Miyoshi et al. 1981). Since then, ATLV and HTLV have been shown to be identical, and a single name, HTLV-1, has been adopted. In the mid-1980s, epidemiological data linked HTLV-1 infection to a chronic progressive neurological disease, which was termed tropical spastic paraparesis in the Caribbean (Gessain et al. 1985) and HTLV-1-associated myelopathy in Japan (Osame et al. 1986). HTLV-1-positive TSP and HAM were subsequently found to be clinically and pathologically identical, and the disease was given a single designation as HAM/TSP (Hollsberg and Hafler 1993). To date, more than 3,000 cases of HAM/TSP have been reported in HTLV-1 endemic areas. Sporadic cases have also been described in non-endemic areas such as the USA and Europe, mainly in immigrants from an HTLV-1 endemic area (Araujo and Silva 2006). HTLV-1 can cause other chronic inflammatory diseases such as uveitis (Mochizuki et al. 1992), arthropathy (Nishioka et al. 1989), pulmonary lymphocytic alveolitis (Maruyama et al. 1988; Sugimoto et al. 1989; Sugimoto et al. 1987), polymyositis (Higuchi et al. 1992; Morgan et al. 1989), Sjögren syndrome (Terada et al. 1994), and infective dermatitis (LaGrenade et al. 1990), although there is no clear evidence for an etiological role of HTLV-1 in these diseases.

Clinical and pathological features of HAM/TSP

HAM/TSP is a chronic progressive myelopathy characterized by spastic paraparesis, sphincter dysfunction, and mild sensory disturbance in the lower extremities (Nakagawa et al. 1996). The period from initial HTLV-1 infection to the onset of HAM/TSP is assumed to range from months to decades, a shorter time than that for the onset of ATL (Nakagawa et al. 1995; Olindo et al. 2006). HAM/TSP occurs both in vertically infected individuals and in those who become infected later in life, that is, through sexual contact almost exclusively from male to female, intravenous drug use, contaminated blood transfusions, etc. The mean age at onset is 43.8 years, and like autoimmune diseases, the frequency of cases of HAM/TSP is greater in women than in men (the male-to-female ratio of occurrence is 1:2.3) (Nakagawa et al. 1995). In addition to HTLV-1 antibody (Ab) positivity both in serum and cerebrospinal fluid (CSF), the presence of atypical lymphocytes (the so-called "flower cells") in peripheral blood and CSF, a moderate pleocytosis, and raised protein content in CSF is observed in patients with HAM/TSP (Araujo and Silva 2006). Oligoclonal immunoglobulin bands in the CSF; raised concentrations of inflammatory markers such as neopterin, tumor necrosis factor (TNF)-α, interleukin (IL)-6, and interferon (IFN)-γ; and increased intrathecal Ab synthesis specific for HTLV-1 antigens have also been described (Jacobson 2002). Clinical progression of HAM/TSP is associated with an increase in the proviral load (PVL) in individual patients, and a high ratio of PVL in CSF cells/peripheral blood mononuclear cells (PBMCs) is also significantly associated with clinically progressive disease (Takenouchi et al. 2003). Thus, a pro-inflammatory environment associated with increased numbers of HTLV-1-infected cells is a characteristic immunological profile of HAM/TSP.

Pathological analysis of HAM/TSP autopsy materials showed the loss of myelin and axons in the lateral, anterior, and posterior columns of the spinal cord. These lesions are associated with perivascular and parenchymal lymphocytic infiltration with the presence of foamy macrophages, reactive astrocytosis, and fibrillary gliosis, predominantly at the thoracic level (Iwasaki 1990; Izumo et al. 2000; Yoshioka et al. 1993), suggesting that the immune response against HTLV-1 causes the inflammatory spinal cord damage seen in patients with HAM/TSP (Bangham 2000). In patients with active chronic lesions in the spinal cord, perivascular inflammatory infiltration with similar composition of cell subsets was also seen in the brain (Aye et al. 2000). The peripheral nerve pathology of patients with HAM/TSP with sensory disturbance showed varying degrees of demyelination, remyelination, axonal degeneration, regeneration, and perineural fibrosis (Bhigjee et al. 1993; Kiwaki et al. 2003).

Treatment of HAM/TSP

To date, no generally agreed standard treatment regimen has been established for HAM/TSP, and no treatment has proven to be consistently effective on a long-term basis. Therefore, clinical practice for treatment of patients with HAM/TSP is based on case series and open, nonrandomized, uncontrolled studies. Mild to moderate beneficial effects have been reported for a number of agents in open-label studies including corticosteroids (Nakagawa et al. 1996), danazol (Harrington et al. 1991), pentoxifylline (Shirabe et al. 1997), immunosuppressants such as ciclosporin A (Martin et al. 2012), high-dose intravenous gamma globulin (Kuroda et al. 1991), plasmapheresis (Matsuo et al. 1988), antibiotics (erythromycin and fosfomycin), and vitamin C (Nakagawa et al. 1996). It should be noteworthy that oral prednisolone was effective in 81.7 %

of 131 patients in a large-scale case series study (Nakagawa et al. 1996). However, the complications of corticosteroids limit their use, particularly in post-menopausal women, who are at higher risk for developing HAM/TSP. Multicenter double-blind randomized placebo-controlled trials for the IFN-α treatment indicate that IFN-α is an effective therapy with an acceptable side effects profile (Izumo et al. 1996), although the benefit of long-term IFN-α therapy has not been well studied. In regard to oral antiviral drugs zidovudine plus lamivudine, no evidence of significant benefit yet exists from randomized placebo-controlled trials (Taylor et al. 2006). Recently, oral administration of histone deacetylase inhibitor valproic acid (VPA) has been conducted as a single-center, open-label trial (Olindo et al. 2011). Although administration of VPA induced a transient increase of HTLV-1 expression to expose virus-positive cells to the host immune response, clinical measures and PVL were stable overall. It has also been reported that the antibiotic minocycline significantly inhibited spontaneous lymphocyte proliferation and degranulation/IFN-γ expression in $CD8^{+}$ T cells of patients with HAM/TSP, suggesting its potential for treatment (Enose-Akahata et al. 2012). Overall, more clinical trials with adequate power are needed in the future.

Risk factors for developing HAM/TSP

It is well-known that HAM/TSP occurs in only a minority of HTLV-1-infected individuals. A previous population association study in HTLV-1 endemic southwest Japan revealed that one of the major risk factors is the HTLV-1 PVL, because the PVL is significantly higher in patients with HAM/TSP than in ACs (Nagai et al. 1998). A higher PVL in patients with HAM/TSP than in ACs was also observed in other endemic areas such as the Caribbean (Manns et al. 1999), South America (Adaui et al. 2006), and the Middle East (Sabouri et al. 2005). In southwest Japan, it was suggested that genetic factors such as the human leukocyte antigen (HLA) genotype are related to the high PVL in patients with HAM/TSP and genetic relatives. Namely, possession of the HLA class I genes HLA-A*02 and Cw*08 was associated with a statistically significant reduction in both HTLV-1 PVL and the risk of HAM/TSP, whereas possession of HLA class I HLA-B*5401 and class II HLA-DRB1*0101 predispose to HAM/TSP in the same population (Jeffery et al. 2000; Jeffery et al. 1999). Because the function of class I HLA proteins is to present antigenic peptides to CTL, these results imply that individuals with HLA-A*02 or HLA-Cw*08 mount a particularly efficient CTL response against HTLV-1, which may be an important determinant of PVL and the risk of HAM/TSP. In accordance with this observation, it has been reported that CTL spontaneously kill autologous HTLV-1-infected cells ex vivo (Hanon et al. 2000), granzymes and perforin are more highly expressed in individuals with a low PVL (Vine et al. 2004), and the lytic efficiency of the CD8+ T cell response (i.e., the fraction of autologous HTLV-1-expressing cells eliminated per CD8+ T cell per day) was inversely correlated with both PVL and the rate of spontaneous proviral expression (Kattan et al. 2009). Furthermore, the major histocompatibility complex (MHC) class I tetramer analysis of lymphocytes isolated from the CSF of patients with HAM/TSP showed even higher frequencies of HTLV-1 Tax11-19-specific, HLA-A*02-restricted CD8+ lymphocytes compared with PBMCs (Nagai et al. 2001b). These findings indicate that an increased proliferation or migration of HTLV-1-infected and/or HTLV-1-specific lymphocytes to the central nervous system might be closely associated with the pathogenesis of HAM/TSP (Hayashi et al. 2008a), and the CTLs against HTLV-1 reduce both PVL and the risk of HAM/TSP. Recently, using a combination of computational and experimental approaches, MacNamara et al. reported that a CTL response against HBZ restricted by protective HLA alleles such as HLA-A*02 or Cw*08, but not a response to the immunodominant protein Tax, also determines the outcome of HTLV-1 infection (Macnamara et al. 2010).

Meanwhile, analysis of non-HLA host genetic factors by candidate gene approaches revealed that non-HLA gene polymorphisms also affect the risk of developing HAM/TSP. Namely, the TNF-α promoter −863 A allele (Vine et al. 2002) and the longer CA repeat alleles of matrix metalloproteinase 9 promoter (Kodama et al. 2004) predisposed to HAM/TSP, whereas IL-10 −592 A (Sabouri et al. 2004), stromal-derived factor 1 +801A (Vine et al. 2002), and IL-15 +191 C alleles (Vine et al. 2002) conferred protection against HAM/TSP. The polymorphisms in the matrix metalloproteinase 9 and IL-10 promoters were each associated with differences in the HTLV-1 Tax-mediated transcriptional activity of the respective gene (Kodama et al. 2004; Sabouri et al. 2004). However, the contributions of these non-HLA genes to the pathogenesis of HAM/TSP are largely unknown and these data have not yet been reproduced in different populations. Further candidate gene studies together with genome-wide association studies in different ethnic populations in a larger sample size may provide evidence for the association of non-HLA genes with the pathogenesis of HAM/TSP.

It has been reported that the lifetime risk of developing HAM/TSP differs among ethnic groups, ranging between 0.25 and 4 %. The annual incidence of HAM/TSP is higher among Jamaican subjects than among Japanese subjects (20 versus 3 cases/100,000 population), with a two to three times greater risk for women in both populations (Hisada et al. 2004; Kramer et al. 1995; Nakagawa et al. 1995; Osame et al. 1990). Although most studies of HTLV-1 genotype have reported no association between variants of HTLV-1 and the risk of HAM/TSP, Furukawa et al. reported the association between HTLV-1 Tax gene variation and the risk of HAM/TSP (Furukawa et al. 2000). Tax subgroup A, which belongs to cosmopolitan

subtype A, was more frequently observed in patients with HAM/TSP, and this association was independent of the protective effect of HLA-A*02. Interestingly, HLA-A*02 appeared to give protection against only one of the two prevalent sequence variants of HTLV-1, Tax subgroup B, which belongs to cosmopolitan subtype B, but not against Tax subgroup A in the Japanese population (Furukawa et al. 2000). Jamaican subjects, who had a higher annual incidence of HAM/TSP, also have cosmopolitan subtype A, whereas approximately 80 % of Japanese subjects, who had a lower annual incidence of HAM/TSP, have cosmopolitan subtype B. Interestingly, HLA-A*02 did not appear to provide protection against HAM/TSP development with cosmopolitan subtype A in a population in Iran (Sabouri et al. 2005).

To test whether the genomic integration site determines the abundance and the pathogenic potential of an HTLV-1-positive T cell clone, Gillet et al. recently reported the results of high-throughput mapping and quantification of HTLV-1 proviral integration in the host genome (Gillet et al. 2011). They mapped >91,000 unique insertion sites (UISs) of the provirus in primary PBMCs from 61 HTLV-1-infected individuals and showed that a typical HTLV-1-infected host carries between 500 and 5,000 UISs in 10 μg of PBMC genomic DNA. They calculated an oligoclonality index to quantify the clonality of HTLV-1-infected cells in vivo and found that the oligoclonality index did not distinguish between ACs and patients with HAM/TSP and that there was no correlation between the oligoclonality index and HTLV-1 PVL in either ACs or patients with HAM/TSP. These results indicate that the higher PVL observed in patients with HAM/TSP was attributable to a larger number of UISs but not, as previously thought, to a difference in clonality. They also obtained evidence that the abundance of established HTLV-1 clones is determined by genomic features of the host DNA flanking the provirus. Namely, HTLV-1 clonal expansion in vivo is favored by a proviral integration site near a region of host chromatin undergoing active transcription or same-sense transcriptional orientation of the provirus. In contrast, negative selection of infected clones, probably by CTLs during chronic infection, favors establishment of proviruses integrated in transcriptionally silenced DNA, and this selection is more efficient in ACs than in HAM/TSP, indicating the selection of HTLV-1-infected T cell clones with low pathogenic potential. More recent reports indicate that circulating HTLV-1-positive cells each contain a single integrated proviral copy (Cook et al. 2012), and cells expressing HTLV-1 Tax protein (i.e., viral protein expression) were significantly more frequent in clones of low abundance in vivo, whereas certain transcription start sites immediately upstream of the viral integration site were associated with virus latency (i.e., no viral protein expression). In particular, Tax-expressing, more "pathogenic" clones were efficiently controlled by the immune response, especially CTLs, whereas non-Tax-expressing "invisible" infected clones were associated with mitotic clonal expansion in vivo (Melamed et al. 2013).

The innate immune response in HAM/TSP

Type I IFN is a key innate immune cytokine produced by cells in response to viral infection. The type I IFN response protects cells against invading viruses by inducing the expression of IFN-stimulated genes, which execute the antiviral effects of IFN (Samuel 2001). The IFN-stimulated genes then generate soluble factors including cytokines that activate adaptive immunity or directly inhibit the virus itself (Liu et al. 2011). In PBMCs of HTLV-1-infected individuals, the level of HTLV-1 messenger RNA is very low and viral protein is not detectable, but these molecules are rapidly expressed after a short time in culture in vitro (Hanon et al. 2000). However, the mechanisms of this phenomenon are largely unknown. Recently, it has been reported that HTLV-1 expression in HTLV-1-infected T cells is suppressed by stromal cells (i.e., epithelial cells and fibroblasts) in culture through type I IFNs (Kinpara et al. 2009). Namely, HTLV-1 Gag protein expression was suppressed when contacted with stromal cells and restored when separated from the stromal cells. Although neutralizing antibodies against human IFN-α/β receptor only partly abrogated this phenomenon, the results indicate that the innate immune system suppresses HTLV-1 expression in vitro and in vivo, at least through type I IFN. More recently, it has been reported that IFN-stimulated genes were overexpressed in circulating leukocytes and the expression correlated with the clinical severity of HAM/TSP (Tattermusch et al. 2012).

Previous reports indicated that patients with HAM/TSP had both a lower frequency and a lower activity of natural killer (NK) cells (especially the CD3+ CD16+ subset) than ACs, although the results were not normalized with respect to the PVL (Yu et al. 1991). Because an important mechanism of induction of NK cell-mediated killing is recognition by the NK cell of a complex of the non-polymorphic MHC molecule HLA-E bound to a peptide derived from the signal sequence of some other MHC class I molecules, the synthetic tetramers of HLA-E with the HLA-G signal sequence peptide were used to identify NK cells in patients with HAM/TSP (Saito et al. 2003). The results clearly showed a lower frequency of HLA-E tetramer-binding cells in patients with HAM/TSP than in ACs; as in the earlier studies (Yu et al. 1991), this reduction in frequency was particularly notable in the CD3+ cells, whereas there was no significant difference in the frequency of HLA-E tetramer-binding CD3− cells between patients with HAM/TSP and ACs (Saito et al. 2003). Recent reports also suggest that the frequency of invariant natural killer T (NKT) cells in the peripheral blood of patients with HAM/TSP is significantly decreased when compared with that in healthy subjects and/or ACs (Azakami et al. 2009; Ndhlovu et al. 2009). These findings indicated that the activity of the NK or NKT cell response was associated with the presence or absence of HAM/TSP. Interestingly, a previous uncontrolled preliminary trial of viable *Lactobacillus casei* strain Shirota-containing