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

$HIV\text{-}1_{IIIB}$ (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

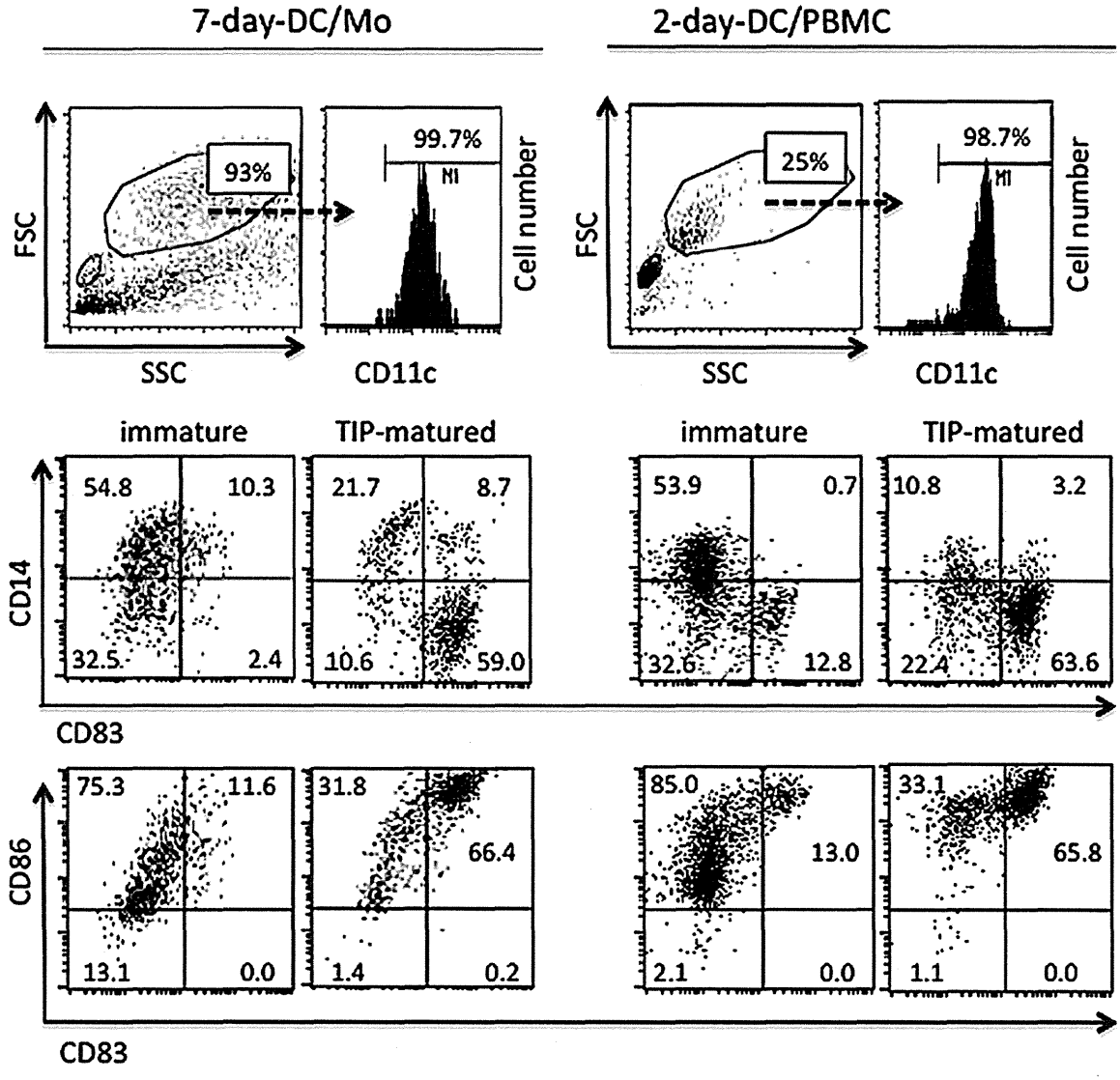

FIGURE 1 | Generation of functional human myeloid DCs directly from PBMCs *in vitro* within 2 days. MDDCs generated purified monocytes for 7 days (7-day-DC/Mo) or whole PBMCs cultured in medium containing GM-CSF, IL-4 and IFN-β (2-day-DC/PBMC), either non-treated or treated with the maturation cocktail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

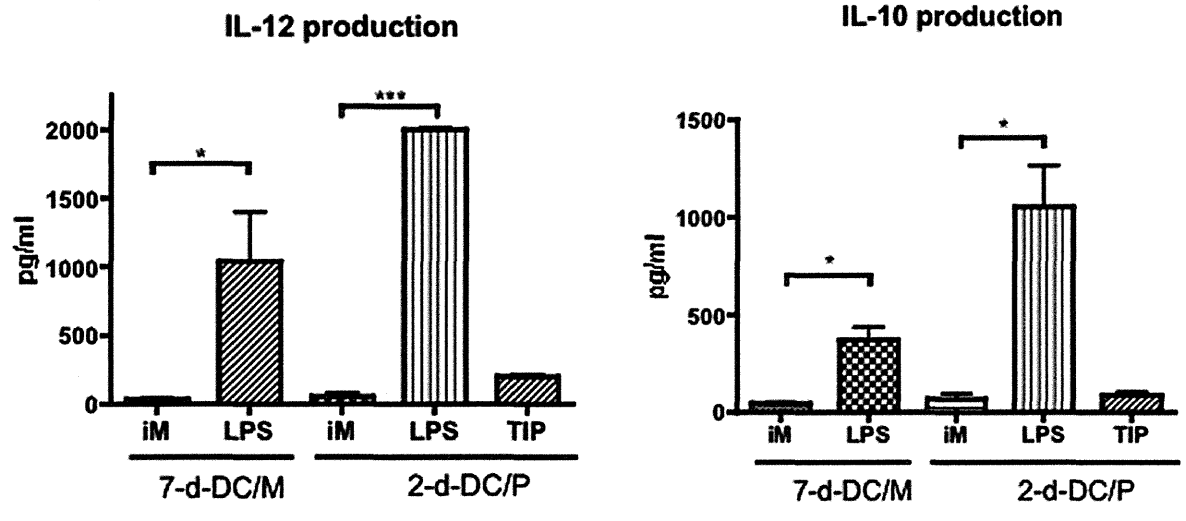

FIGURE 2 | Production of cytokines by the 2-day-DC/PBMC. The levels of IL-12 (bioactive p70 heterodimer) and IL-10 in the supernatants from MDDCs (7-d-DC/M) or 2-day-DC/PBMC (2-d-DC/P) either non-matured (iM) or matured with LPS or the TIP cocktail were determined by ELISA. Data are mean ± SD of triplicate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

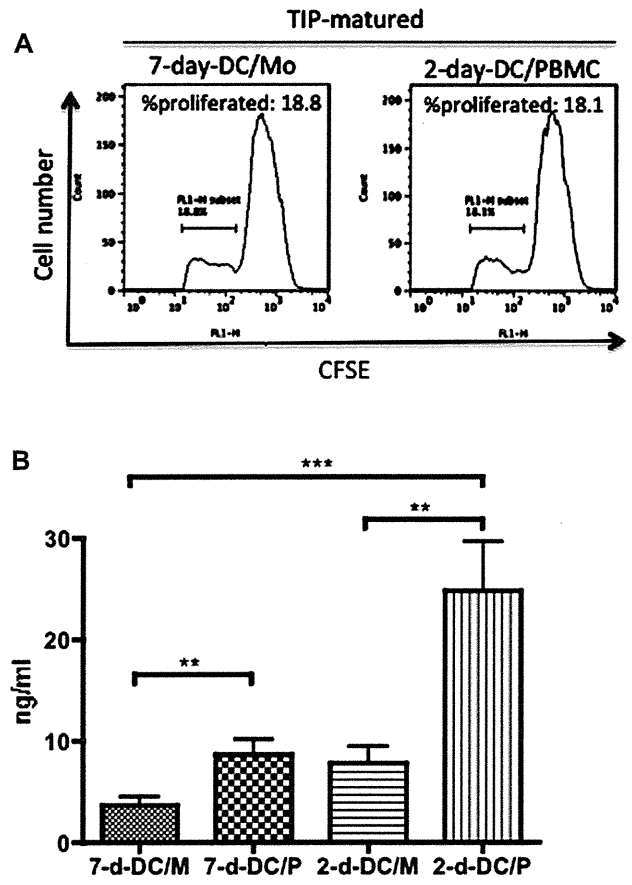

FIGURE 3 | High levels of production of IFN-γ by allogeneic T cells stimulated with 2-day-DCs generated from PBMCs. Matured MDDCs (7-day-DC/Mo) and 2-day-DCs generated from PBMCs (2-day-DC/PBMC) were co-cultured with CFSE-labeled allogeneic $CD4^+$ T cells at DC to T cell ratio of 1:50 for 4 days. The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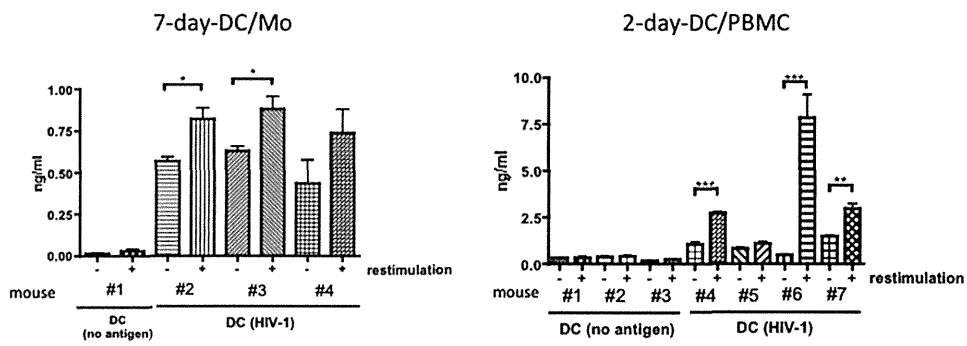

FIGURE 4 | Functional activity of the 2-days-DCs in hu-PBL-SCID mice. Fresh autologous PBMCs from normal human donors were transferred into the Rag2$^{-/-}$ mouse spleen together with autologous mature MDDCs (7-day-DC/Mo) or 2-days-PBMC-derived DCs (2-day-D/PBMC) pulsed with no antigen (no antigen) or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

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

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Abstract

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

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

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

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

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

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

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HTLV-1

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Glossary

Cytotoxic T cell A cytotoxic T cell belongs to a subgroup of T lymphocytes with CD8 receptor that are antigen- specific and capable of inducing the death of virus-infected somatic or tumor cells.

Gliosis Gliosis is the process of scarring in the central nervous system, caused by a proliferation of astrocytes.

Oligoclonal band Oligoclonal bands are bands of immunoglobulins that are seen when a blood serum (or plasma) or cerebrospinal fluid (CSF) is analyzed by protein electrophoresis. The presence of oligoclonal bands in CSF but not in blood serum (or plasma) means the production of immunoglobulins in central nervous system, that is, inflammation in the central nervous system.

Provirus A provirus is the form of the virus which is capable of being integrated into the chromosome of the host cell.

Spastic paraparesis Mild or moderate loss of motor function accompanied by spasticity in the extremities mainly caused by central nervous system (brain and spinal cord) diseases.

Human T-lymphotropic virus type-1 (HTLV-1) belongs to the *Deltaretrovirus* genus of the Orthoretrovirinae subfamily and infects 10–20 million people worldwide. HTLV-1 can be transmitted through sexual contact, intravenous drug use, and breastfeeding from mother to child. The infection is endemic in southwest Japan, the Caribbean, sub-Saharan Africa, South America, with smaller foci in Southeast Asia, South Africa, and northeastern Iran. 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. It was the first human retrovirus ever associated with a human cancer. Three years before the isolation of HTLV-1, a Japanese group reported adult T-cell leukemia (ATL), a rare form of leukemia endemic to southwest Japan, as a distinct clinical entity. In 1981, the same group demonstrated that ATL was caused by a new human retrovirus originally termed 'ATLV'. Later, 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 with a chronic progressive neurological disease, which was termed 'tropical spastic paraparesis (TSP)' in the Caribbean and 'HTLV-1 associated myelopathy (HAM)' in Japan. 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. HTLV-1 can cause other chronic inflammatory diseases such as uveitis, arthropathy, pulmonary lymphocytic alveolitis, polymyositis, Sjögren syndrome, and infective dermatitis. Only approximately 2–3% of infected persons develop ATL and another 0.25–4% develop chronic inflammatory diseases, while the majority of infected individuals remain lifelong asymptomatic carriers (ACs). Thus, the viral, host, and environmental risk factors, as well as the host immune response against HTLV-1 infection, appear to regulate in the development of HTLV-1-associated diseases. For over two decades, the investigation of HTLV-1-mediated pathogenesis has focused on Tax, an HTLV-1-encoded viral oncoprotein. Tax activates many cellular genes by binding to groups of transcription factors and coactivators and is necessary and sufficient for cellular transformation. However, recent reports have identified another regulatory protein, HTLV-1 basic leucine zipper factor (HBZ), that plays a critical role in the development of ATL and HAM/TSP.

HTLV-1-Associated Diseases

Adult T-cell leukemia

ATL is a fatal malignancy of mature CD4+ T cells. It arises in only a small proportion of HTLV-1-infected people (1–5% of infected individuals) after long latency periods following primary infection. ATL shows diverse clinical features, but can be divided into four clinical subtypes: smoldering, chronic, lymphoma, and acute. Each subtype is directly correlated with the prognosis of patients: the smoldering and chronic types are indolent, while the acute and lymphoma types are aggressive and characterized by resistance to chemotherapy and poor prognosis. Development of ATL is characterized by infiltration of various tissues with circulating ATL cells, called 'flower cells', which have conspicuous lobulated nuclei. These cells cause further symptoms including lymphadenopathy, lytic bone lesions, skin involvement, hepatosplenomegaly, and hypercalcemia. Laboratory findings of ATL patients typically reveal a marked leukocytosis, hypercalcemia, high serum levels of lactate dehydrogenase (LDH), and a soluble form of interleukin-2 receptor (IL-2R). In cohort studies of HTLV-1 carriers, the risk factors for ATL appeared to include vertical infection (mother to child transmission), male gender, older age, and increasing numbers of abnormal lymphocytes. Since ATL occurs mainly in vertically infected individuals, but not in those who become infected later in life, the impairment of HTLV-1-specific T-cell responses caused by vertical HTLV-1 infection has been suggested as a possible cause of disease development. The HTLV-1-specific cytotoxic T-cell (CTL) responses from ATL patients are significantly lower than that of HAM/TSP patients. However, insufficient HTLV-1-specific T-cell responses might also occur during and after the onset of ATL. Although ATL has a poor prognosis, recent advances in its treatment have led to significant gains in response rates and

survival. Accumulating evidence suggests that allogeneic bone marrow transplantation and allogeneic peripheral blood stem cell transplantation are potent therapies for aggressive ATL (i.e., the acute and lymphoma type). The combination of the antiretroviral agent zidovudine (AZT) and interferon-alfa (IFN-α) is also beneficial for overall survival in smoldering and chronic (i.e., indolent) ATL, although its efficacy has not yet been confirmed in well-designed prospective studies.

Since the discovery of HTLV-1, the viral transactivator Tax has been viewed as critical for leukemogenesis, due to its pleiotropic effects on both viral and many cellular genes responsible for cell proliferation, genetic instability, dysregulation of the cell cycle, and apoptosis. However, Tax expression is not detected in about 60% of freshly isolated samples from ATL cases. Recently, the expression of another regulatory protein, HBZ, has been reported in association with all ATL cases. This protein, which is encoded in the minus or antisense strand of the virus genome, promotes proliferation of ATL cells and induces T-cell lymphomas in CD4+ T cells by transgenic expression, indicating involvement of HBZ expression in the development of ATL. In addition, among the HTLV-1-encoded viral genes, only the HBZ gene sequence remains intact, unaffected by nonsense mutations and deletion. Thus, HBZ expression is indispensable for proliferation and survival of ATL cells and HTLV-1-infected cells, and Tax expression is not always necessary for the development of ATL.

HTLV-1-Associated Myelopathy/Tropical Spastic Paraparesis

HAM/TSP is a chronic progressive myelopathy characterized by spastic paraparesis, sphincter dysfunction, and mild sensory disturbance in the lower extremities. In addition to neurological symptoms, some HAM/TSP cases also exhibit autoimmune-like disorders, such as uveitis, arthritis, T-lymphocyte alveolitis, polymyositis, and Sjögren syndrome. To date, more than 3000 cases of HAM/TSP have been reported in HTLV-1-endemic areas. Sporadic cases have also been described in nonendemic areas such as the United States and Europe, mainly in immigrants from an HTLV-1-endemic area. The lifetime risk of developing HAM/TSP is different 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 vs. three cases/100 000 population), with a 2 to 3 times higher risk for women in both populations. 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 for ATL onset. HAM/TSP occurs both in vertically infected individuals and in those who become infected later in life (i.e., 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 other autoimmune diseases, the frequency of HAM/TSP is higher in women than in men (the male to female ratio of occurrence is 1:2.3).

The essential histopathological feature of HAM/TSP is a chronic progressive inflammation in the spinal cord, predominantly at the thoracic level. The loss of myelin sheaths and axons in the lateral, anterior, and posterior columns is associated with perivascular and parenchymal lymphocytic infiltration, reactive astrocytosis, and fibrillary gliosis. In addition to HTLV-1 antibody positivity, other laboratory findings of HAM/TSP include the presence of atypical lymphocytes (the so-called flower cells) in peripheral blood and cerebrospinal fluid (CSF), a moderate pleocytosis, and raised protein content in CSF. Oligoclonal bands, raised concentrations of inflammatory markers such as neopterin, tumor necrosis factor (TNF)-α, IL-6 and IFN-γ, and an increased intrathecal antibody synthesis specific for HTLV-1 antigens have also been described in CSF of HAM/TSP patients.

A previous population association study in HTLV-1 endemic in southwest Japan revealed that one of the major risk factors is the HTLV-1 proviral load (PVL), as the PVL is significantly higher in HAM/TSP patients than in ACs. A high PVL was also associated with an increased risk of progression to disease. Higher PVL in HAM/TSP patients than in ACs was also observed in other endemic areas such as the Caribbean, South America, and the Middle East. In southwest Japan, an association was suggested between possession of the HLA-class I genes HLA-A*02 and Cw*08 and a statistically significant reduction in both PVL and the risk of HAM/TSP. By contrast, possession of HLA-class I HLA-B*5401 and class II HLA-DRB1*0101 predisposed patients in the same population to HAM/TSP. Since 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 HTLV-1 PVL and the risk of HAM/TSP.

To date, no generally agreed standard treatment regimen has been established for HAM/TSP, as no treatment for HAM/TSP has proven to be consistently effective and long term. Therefore, current clinical practice for treatment of HAM/TSP is based on case series and open, nonrandomized uncontrolled studies. Although mild to moderate beneficial effects have been reported with corticosteroids, immunosuppressants, high-dose intravenous gammaglobulin, antibiotics (erythromycin and fosfomycin), and vitamin C, the clinical benefits are only transient and limited. The complications of steroid use limit their use particularly in postmenopausal females, who are at higher risk of developing HAM/TSP. Only three randomized placebo-controlled trials have been conducted for HAM/TSP treatment. These studies indicate that IFN-α is an effective therapy, with an acceptable side-effects profile. By contrast, no evidence yet exists of any benefit of zidovudine plus lamivudine for treating HAM/TSP. More clinical trials with adequate power are needed in the future.

Other HTLV-1-Associated Diseases

HTLV-1 has been implicated in the pathogenesis of other inflammatory disorders such as uveitis, arthropathy, infective dermatitis, pulmonary lymphocytic alveolitis, polymyositis, Sjögren syndrome, and autoimmune thyroid diseases, based on the higher HTLV-1 PVL and the higher seroprevalence in patients than in ACs. However, direct evidence for an association between these disorders and HTLV-1 infection is still lacking. Nonetheless, HTLV-1 may be a significant trigger for the development of these autoimmune disorders.

See also: Retroviruses

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TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Protective Roles of Epithelial Cells in the Survival of Adult T-Cell Leukemia/Lymphoma Cells

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Adult T-cell leukemia/lymphoma (ATL) is a highly invasive and intractable T-cell malignancy caused by human T-cell leukemia virus-1 infection. We demonstrate herein that normal tissue–derived epithelial cells (NECs) exert protective effects on the survival of leukemic cells, which may partially account for high resistance to antileukemic therapies in patients with ATL. Viral gene–silenced, ATL-derived cell lines (ATL cells) dramatically escaped from histone deacetylase inhibitor–induced apoptosis by direct co-culture with NECs. Adhesions to NECs suppressed $p21^{Cip1}$ expression and increased a proportion of resting G0/G1 phase cells in trichostatin A (TSA)–treated ATL cells. ATL cells adhering to NECs down-regulated CD25 expression and enhanced vimentin expression, suggesting that most ATL cells acquired a quiescent state by cell-cell interactions with NECs. ATL cells adhering to NECs displayed highly elevated expression of the cancer stem cell marker CD44. Blockade of CD44 signaling diminished the NEC-conferred resistance of ATL cells to TSA-induced apoptosis. Co-culture with NECs also suppressed the expression of NKG2D ligands on TSA-treated ATL cells, resulting in decreased natural killer cell–mediated cytotoxicity. Combined evidence suggests that interactions with normal epithelial cells augment the resistance of ATL cells to TSA-induced apoptosis and facilitate immune evasion by ATL cells. *(Am J Pathol 2013, 182: 1832–1842; http://dx.doi.org/10.1016/j.ajpath.2013.01.015)*

Adult T-cell leukemia/lymphoma (ATL) is an intractable and fatal T-cell malignancy caused by human T-cell leukemia virus type 1 (HTLV-1).[1] After more than two decades of long-term latency, approximately 4% of HTLV-1 carriers develop ATL.[2,3] A striking feature of ATL is aggressive invasion of leukemia cells into the skin and epithelial linings of the gastrointestinal tract and lung.[4,5] Leukemia cells that have invaded the tissues are resistant to chemotherapy, presenting a major obstacle to the effective treatment of ATL.[6] Therefore, understanding the mechanisms by which tissue-infiltrating leukemia cells acquire resistance to chemotherapy is key to developing new promising treatments for patients with ATL.

HTLV-1 proteins are generally undetectable in HTLV-1–infected cells isolated from HTLV-1 carriers because of viral gene silencing. Such silencing is observed not only in asymptomatic carriers but also in patients with ATL, indicating that it allows ATL cells to evade the host immune response *in vivo*.[7,8] However, the mechanisms leading to viral gene silencing are poorly understood. Freshly isolated HTLV-1–infected cells begin to express viral genes after overnight culture *in vitro*, implying that an unknown mechanism exists to suppress viral genes *in vivo*.[9] Recently, primary ATL cells were shown to be well maintained *in vitro* by co-culture in direct contact with stromal cells.[10] Furthermore, type I interferon–induced HTLV-1 *gag* expression was suppressed in an ATL cell line when it was co-cultured with stromal cells.[11] These observations suggest that viral gene silencing occurs in ATL cells by interactions with the host microenvironment.

Epigenetic regulations, such as histone acetylation, are also assumed to be involved in viral gene silencing in ATL

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cells.[12] Thus, the *trans*-activity of the HTLV-1 long terminal repeats (LTRs) is induced by histone deacetylase (HDAC) inhibitors in HTLV-1–infected MT4 cells.[13] Also, HTLV-1 Tax expression was doubled when primary ATL cells were treated with HDAC inhibitors.[14] These observations indicate that in ATL cells cultured *in vitro*, viral gene expression is suppressed by epigenetic regulation and that HDAC inhibitors can reactivate silenced viral genes. Taken together, available evidence indicates that viral gene expression is suppressed in ATL cells by the dual action of the microenvironment of the host-tumor interface and epigenetic regulation in host cells.

Stromal fibroblasts constituting the bone marrow microenvironment prevent chemotherapy-induced apoptosis in acute myeloid leukemia and chronic lymphocytic leukemia cells.[15,16] Recently, stromal cell–derived factor-1α and its cognate receptor CXCR4 have emerged as critical mediators of leukemic/stromal cell interactions.[17] Blockade of the stromal cell–derived factor-1α–CXCR4 axis by the CXCR4 antagonist AMD3100 suppresses the migration of cultured ATL cells,[18] suggesting that this axis may also be involved in formation of the stromal niche environment in ATL.

The present study was undertaken to determine whether epithelial cells, another major component constituting the microenvironment of the host-tumor interface, affect the survival and phenotype of ATL cells. We show that co-culture with normal tissue–derived epithelial cells (NECs) increases a proportion of ATL cells in G0/G1 phase and rescues ATL cells from HDAC inhibitor–induced apoptosis. Adhesions to NECs induced prominent surface expression of CD44 on ATL cells; they also induced internalization of CD44 and nuclear translocation of cyclin D1 in a fraction of HDAC inhibitor–treated ATL cells, thus enabling such ATL cells to resume cell-cycle progression and leading to the ultimate survival of ATL cells. ATL cells co-cultured with NECs down-regulated the expression of NKG2D ligands, suggesting that this interaction also facilitates immune evasion by tumor cells.

Materials and Methods

Cells

The HTLV-1–positive ATL cell lines ATL-CR and ATL-TH were obtained from the Reference Center for HTLV Infection (Rio de Janeiro, Brazil). These cell lines were established from Brazilian patients with ATL after they provided informed consent. Briefly, peripheral blood mononuclear cells (PBMCs) isolated from patients with ATL were cultured in the presence of recombinant IL-2. After long-term culture, they acquired IL-2 independence, resulting in establishment of the ATL-CR and ATL-TH cell lines. Jurkat and HUT78, a T-cell lymphoma line derived from a patient with Sezary syndrome,[19] were used as HTLV-1–negative T-cell lymphoma cells. ATL-CR, ATL-TH, Jurkat, and HUT78 were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. HEK293 and HEK293T, human embryo kidney epithelial-like cell lines, and primary normal human dermal fibroblasts were maintained in Dulbecco's minimal essential medium supplemented with 10% FBS and penicillin/streptomycin. 1C3IKEI, a primary normal human embryonic pancreas–derived epithelial-like cell line, was purchased from the RIKEN BioResource Center (Tsukuba, Japan) and maintained in Dulbecco's minimal essential medium supplemented with 15% FBS. KHYG-1, an NKG2D$^+$ natural killer (NK) cell line,[20] was purchased from the Health Science Research Resources Bank (Osaka, Japan) and was maintained in Dulbecco's minimal essential medium supplemented with 100 U of recombinant human IL-2 (Shionogi, Osaka, Japan), 10% FBS, and penicillin/streptomycin. Fresh ATL cells were obtained from patients with chronic or acute ATL after informed consent was provided. Briefly, PBMCs isolated from heparinized peripheral blood by Ficoll-Paque PLUS (GE Healthcare Life Sciences, Little Chalfont, UK) were resuspended in RPMI 1640 medium supplemented with 10% FBS and penicillin/streptomycin and were subjected to experiments immediately.

Antibodies, Plasmids, and Reagents

Rat anti-human/mouse CD44 antibody (clone IM7; eBioscience Inc., San Diego, CA) was used for flow cytometry (FCM) and immunofluorescence staining. Mouse anti-human MICA/B antibody (clone 6D4; eBioscience) was used for FCM. Mouse anti-human CD25 (clone BC96; eBioscience), polyclonal rabbit anti-human fibronectin (Dako, Glostrup, Denmark), mouse anti-human vimentin (clone V9; Dako), polyclonal rabbit anti-human p21^{Cip1} (eBioscience), and mouse anti-human Ki-67 (clone MIB-1; Dako) antibodies were used for immunofluorescence staining. HTLV-1 LTR luciferase plasmids have been described previously.[21] HDAC inhibitors, such as trichostatin A (TSA), valproic acid sodium salt (VPA), and sodium butyrate (NaB), were purchased from Sigma-Aldrich (St. Louis, MO).

Co-Culture

ATL cells were co-cultured with NECs using a direct co-culture system that allowed for cell-cell contact. NECs were labeled with 1 μmol/L of 5- (and 6-) carboxyfluorescein diacetate succinimidyl ester (CFSE) at a concentration of 1×10^6 cells/mL in PBS for 20 minutes at 37°C and then washed three times in PBS. CFSE-labeled NECs were used immediately for each experiment. ATL cells, 5×10^5, were directly co-cultured with CFSE-labeled NECs growing as monolayers in 24-well plates. Cell culture inserts (pore size, 0.4 μm; Invitrogen, Camarillo, CA) were used for an indirect co-culture system. ATL cells were co-cultured with CFSE-labeled NECs using the indicated concentrations of TSA. After the indicated co-culture time, whole cells in co-culture were harvested for subsequent experiments. In some experiments, nonadhering ATL cells in the supernatant were collected separately from the ATL cells adhering to the

monolayer of labeled NECs, and then the adhering ATL cells with labeled NECs were collected after rinsing twice gently with RPMI 1640 medium to remove nonadhering ATL cells. For the proliferation assay, ATL-CR cells were labeled with CFSE and then co-cultured with unlabeled HEK293T cells for 72 hours.[22]

FCM Analysis

Cell-cycle distribution of ATL cells was analyzed by DNA content using the propidium iodide (PI) staining method. Co-cultured cells were harvested after the indicated durations of TSA treatment. After fixation in 70% ethanol at −20°C overnight, cells were incubated in a PI/RNase A staining buffer (50 μg/mL of PI and 20 μg/mL of RNase A in PBS). Cells were then analyzed by FCM using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ). CFSE and PI were detected in the FL-1 and FL-2 channels, respectively. Co-cultured ATL cells gated on CFSE-negative cells were acquired in 10,000 events. Expression of cell-surface molecules was analyzed using FCM. Analysis was performed using CellQuest Pro software version 6.0 (Becton Dickinson Immunocytometry Systems, San Jose, CA). Sigmoid-like dose-response curves were drawn using the logistic curve-fitting software ImageJ, version 1.46 (NIH, Bethesda, MD).[23]

Immunofluorescence Staining

Cells cultured on chamber slides were fixed with 4% paraformaldehyde for 15 minutes. For intracellular staining, cells were treated with PBS containing 0.1% Triton X-100 (Roche Diagnostics GmbH, Mannheim, Germany) for 4 minutes and then fixed with ice-cold 70% methanol for 4 minutes. Nonspecific binding was blocked with 0.05% Tween 20 in PBS containing 0.1% goat serum for 10 minutes. After incubation with primary antibodies for each targeted protein, Alexa Fluor–conjugated goat polyclonal antibody was used as a secondary antibody. Images were acquired using an Olympus DP70 camera with its own Olympus DP controller software version 1.2.1.108 (Olympus, Tokyo, Japan). Three-dimensional analyses were reconstructed by images acquired by confocal microscopy using a laser scanning confocal microscope (FV-300; Olympus, Tokyo, Japan).

Functional Assays

Reporter genes were introduced into ATL cells directly using FuGENE transfection reagents (Roche Diagnostics, Branchburg, NJ) according to the manufacturer's instructions. Briefly, 5×10^5 ATL-CR cells were cultured in 60-mm dishes and cotransfected with 1 μg of HTLV-1 LTR firefly luciferase reporter plasmid together with 50 ng of the *Renilla* luciferase reporter pRL-TK. Eighteen hours after transfection, ATL-CR cells were washed five times in PBS and then were co-cultured with HEK293T cells in the presence of TSA in triplicate wells of 24-well plates (2×10^5 cells per well). Reporter activities were measured using the Dual-Luciferase reporter assay system (Promega, San Luis Obispo, CA). Briefly, cells were lysed in 1× passive lysis buffer, and firefly and *Renilla* luciferase activities were measured using a Turner 20/20 luminometer (Promega). Reporter activities were normalized using *Renilla* luciferase values.

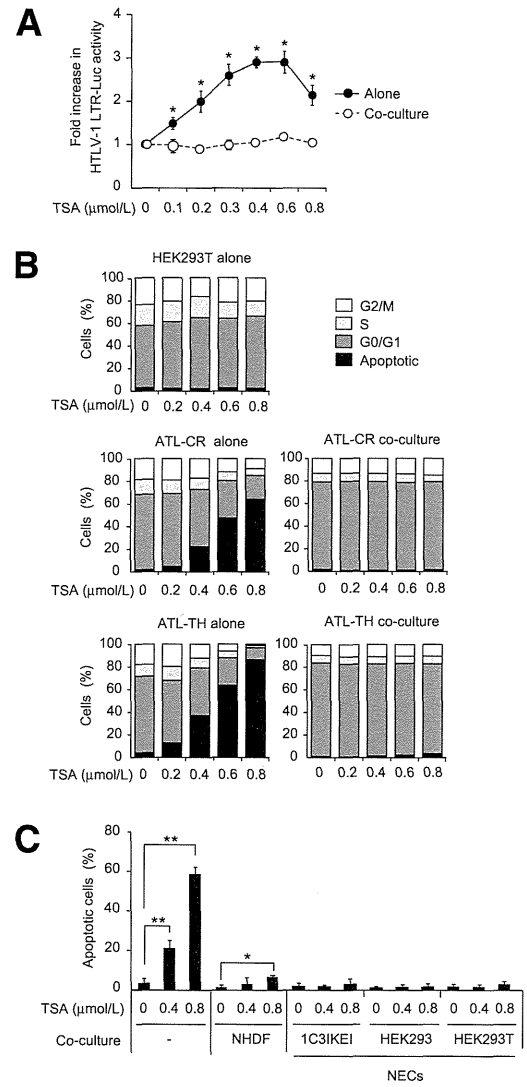

Figure 1 Direct co-culture with NECs rescued ATL cells from TSA-induced apoptosis. **A**: Transcriptional activity of HTLV-1 LTRs in TSA-treated ATL-CR cells. ATL-CR cells were cotransfected with 1 μg of HTLV-1 LTR firefly luciferase reporter plasmid together with 50 ng of pRL-TK 1 day before co-culture with HEK293T cells. After 18 hours of co-culture with the indicated concentrations of TSA, reporter activities were measured and normalized to *Renilla* luciferase values. The values indicate the means ± SD fold increases obtained by three independent experiments normalized to the untreated control sample. **B**: Cell-cycle analysis in ATL-CR and ATL-TH cells. ATL cells, 5×10^5 per well, were cultured alone or with HEK293T cells in the presence of the indicated concentrations of TSA for 48 hours. HEK293T cells were labeled with CFSE 1 day before co-culture. After the whole co-cultured cells were harvested, cell-cycle analysis was performed by FCM. **C**: Percentage of apoptotic ATL-CR cells co-cultured with normal human dermal fibroblasts (NHDFs), 1C3IKEI cells, HEK293 cells, and HEK293T cells. The data are presented as the means ± SD of three independent experiments. $*P < 0.05$, $**P < 0.01$.

Cytotoxicity Assays

Cytotoxicity assays used a combination of two dyes: CFSE was used to label effector cells (KHYG-1 cells) and HEK293T cells and PI was used to stain target cells (ATL-CR cells). CFSE-labeled effector cells were incubated with nonlabeled target cells cultured alone or co-cultured with CFSE-labeled HEK293T cells at effector to target cell ratios of 3:1, 6:1, or 9:1 in triplicate wells of 24-well plates for 18 hours. Apoptotic ATL-CR cells were evaluated by the percentage of sub G0/G1 phase cells in CFSE-negative cells as determined by FCM. Mean values were calculated from three independent experiments.

Statistical Analysis

Data were analyzed using either the Student's *t*-test or repeated-measures analysis of variance. A $P < 0.05$ was considered statistically significant.

Results

Direct Co-Culture with NECs Rescues ATL Cells from TSA-Induced Apoptosis

HTLV-1 gene expression is hardly detectable in ATL cells because of gene silencing. With the aim of eliminating viral gene silencing, we treated the IL-2–independent ATL-derived cell line ATL-CR with TSA. This treatment increased the amount of HTLV-1 p19 gag protein in the supernatants and lysates prepared from cultured ATL-CR cells (data not shown) and enhanced the transcriptional activity of HTLV-1 LTRs in a dose-dependent manner (Figure 1A), indicating that TSA effectively eliminates viral gene silencing. However, when ATL-CR cells were co-cultured with HEK293T cells (used as NECs), treatment with TSA did not increase the transcriptional activity of HTLV-1 LTRs, indicating that co-culture with NECs blocked TSA-induced viral gene reactivation (Figure 1A).

Next, we performed cell-cycle analysis to determine whether HEK293T cells affect the fate of TSA-treated ATL-CR and ATL-TH cells (Figure 1B). When ATL-CR cells were cultured alone in the presence of TSA, they displayed a dose-dependent increase in the percentage of apoptotic cells and a decrease in the percentage of G0/G1 phase cells; similar changes were observed in TSA-treated ATL-TH cells but not in TSA-treated HEK293T cells. ATL-CR and ATL-TH cells co-cultured with HEK293T cells showed no increase in the percentage of apoptotic cells; instead, the percentage of G0/G1 phase cells was increased. The ability of HEK293T cells to prevent apoptosis was evident even at an ATL-CR to HEK293T cell ratio of 25:1 (data not shown). Similar apoptosis-protective effects were also observed when ATL-CR cells were co-cultured with primary epithelial cells 1C3IKEI or primary dermal fibroblasts (Figure 1C). These results indicate that co-culture with NECs generally prevents ATL cells from TSA-induced apoptosis.

Cell-Cell Contact–Dependent Interactions with NECs Are Required to Rescue ATL Cells from HDAC Inhibitor–Induced Apoptosis

To examine whether cell-cell contact with NECs is required to rescue ATL cells from TSA-induced apoptosis, we co-cultured ATL-CR cells in direct or indirect contact with HEK293T cells in the presence of TSA. When ATL-CR cells were separated by 0.4-μm membranes from HEK293T cells, the ratio of apoptotic ATL-CR cells was lower than when they were cultured alone but showed a marked increase compared with when ATL-CR cells were cultured in direct

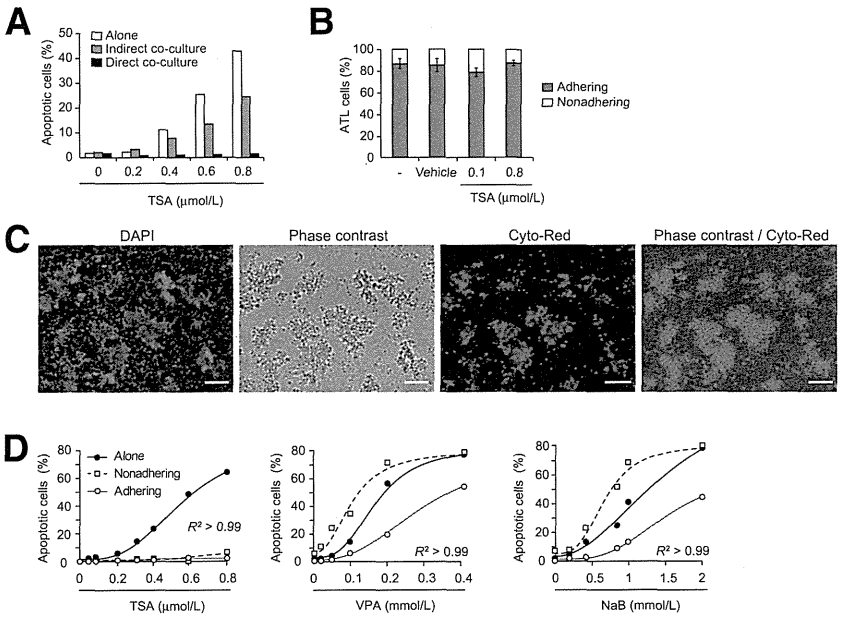

Figure 2 Cell-cell contact–dependent interactions with NECs are required to rescue ATL cells from TSA-induced apoptosis. **A:** Percentage of apoptotic ATL-CR cells. ATL-CR cells were directly co-cultured with HEK293T cells or indirectly co-cultured with HEK293T cells using cell culture inserts (pore size, 0.4 μm) in the presence of the indicated concentrations of TSA for 48 hours. **B:** Means ± SD percentage of ATL-CR cells adhering to HEK293T cells after co-culture for 24 hours. **C:** Most of the ATL-CR cells (red) showed adhesion-dependent growth on a monolayer of HEK293T cells after 24 hours of direct co-culture. Scale bars: 50 μm. **D:** Percentage of apoptotic ATL-CR cells. ATL-CR cells were co-cultured with HEK293T cells in the presence of the indicated concentrations of TSA, VPA, and NaB. After co-culture, nonadhering ATL-CR cells in the supernatant were collected separately from the ATL-CR cells adhering to the monolayer of CFSE-labeled HEK293T cells. The data are presented as the means ± SD of three independent experiments.

contact with HEK293T cells (Figure 2A), indicating that efficient suppression of apoptosis requires cell-cell contact. Approximately 84% of ATL-CR cells adhered to a monolayer of HEK293T cells by 24 hours after co-culture with HEK293T cells (Figure 2, B and C). There was no correlation between the dose of TSA and the adhesiveness of ATL-CR cells (Figure 2B), suggesting that TSA treatment did not augment the adhesive activity of ATL cells. To examine whether the extracellular matrix can substitute for NECs, ATL-CR cells were cultured in an extracellular matrix (BD Matrigel Matrix; Becton Dickinson)—coated dish in the presence of TSA. These experiments showed that extracellular matrix alone was completely ineffective in reducing TSA-induced apoptosis in ATL cells (data not shown).

We next examined whether ATL-CR cells co-cultured with HEK293T cells acquire apoptosis resistance to treatment with other HDAC inhibitors, such as VPA and NaB (Figure 2D). TSA-treated ATL-CR cells directly co-cultured with HEK293T cells acquired dramatic apoptosis resistance regardless of whether they adhered to HEK293T cells. In contrast, apoptosis resistance to VPA or NaB was acquired only in adhering ATL-CR cells, and the extent of acquired resistance was diminished. These results indicate that cell-cell contact—dependent interactions with NECs make ATL-CR cells more or less resistant to apoptosis induced by HDAC inhibitors.

Co-Culture with NECs Induces a Quiescent State in TSA-Treated ATL Cells

To examine the cellular changes that follow co-culture with NECs more closely, we analyzed cell-cycle distribution in ATL cells cultured alone, co-cultured with NECs, or co-cultured with NECs in the presence of TSA (Figure 3A). Co-culture with NECs decreased the proportion of S and G2/M phase cells and increased the proportion of G0/G1 phase cells. Co-culture in the presence of 0.8 μmol/L TSA further strengthened these changes. In contrast, TSA-treated ATL-CR cells cultured alone did not show such cell-cycle changes and underwent apoptosis (Figure 3B). Furthermore, when CFSE-labeled ATL-CR cells were cultured alone or with HEK293T cells for 72 hours, co-cultured ATL-CR cells showed less proliferation than those cultured alone, in the presence and absence of 0.8 μmol/L TSA (Figure 3C). This is consistent with the observation that co-culture with NECs induces G0/G1 accumulation in ATL-CR cells and allows them to escape from TSA-induced apoptosis (Figure 3A).

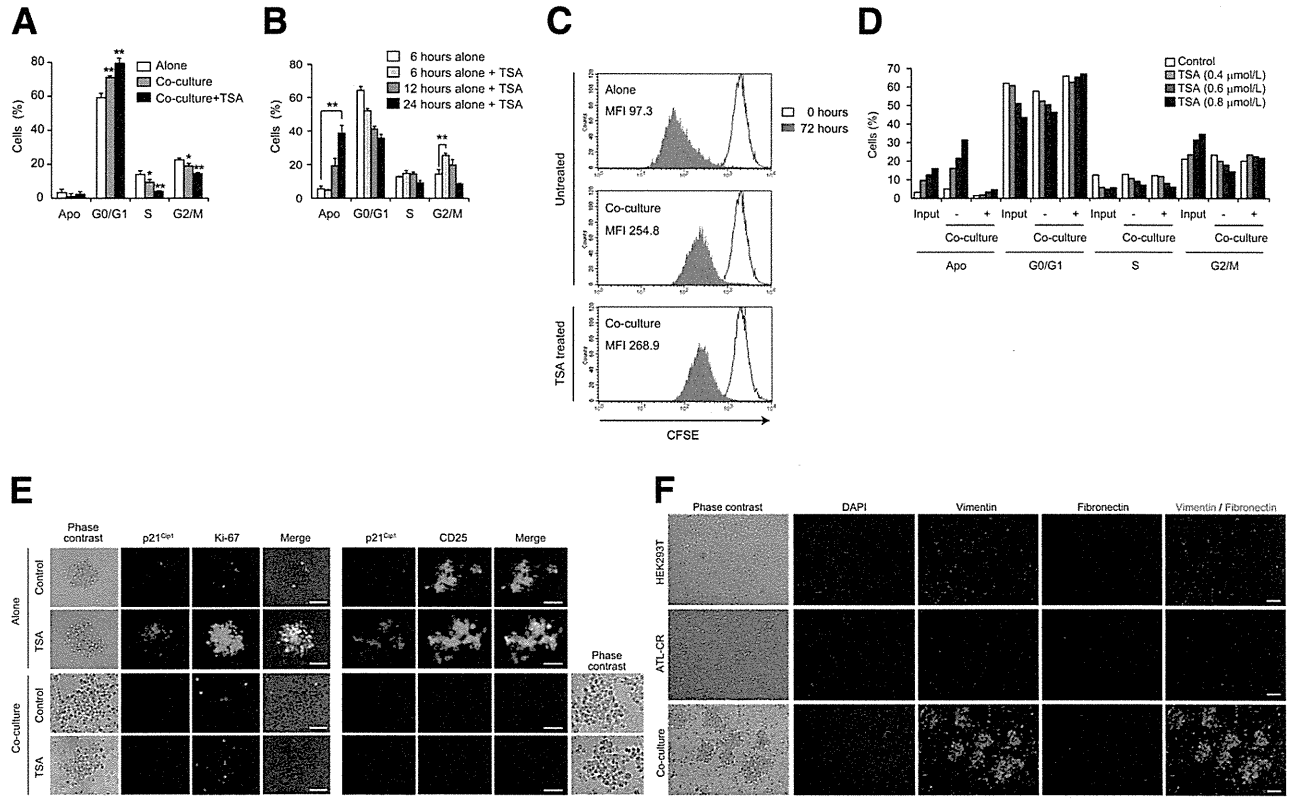

Figure 3 Co-culture with NECs induced a quiescent state in TSA-treated ATL-CR cells. **A:** Cell-cycle analysis in ATL-CR cells. ATL-CR cells co-cultured with HEK293T cells were treated with 0.8 μmol/L TSA for 24 hours. **B:** Changes in cell-cycle distribution were followed for 24 hours in ATL cells cultured alone in the presence or absence of TSA. **C:** Proliferation analysis in ATL-CR cells. CFSE-labeled ATL-CR cells were cultured alone or with HEK293T cells in the presence or absence of TSA for 72 hours. CFSE intensity was analyzed after gating on live cells. **D:** Recovery of TSA-pretreated ATL-CR cells by direct co-culture with NECs. ATL-CR cells were pretreated with the indicated concentration of TSA for 18 hours and then were cultured alone or with HEK293T cells in fresh RPMI 1640 medium for 24 hours. ATL-CR alone (input) indicates TSA-pretreated ATL-CR cells cultured alone. **E:** $p21^{Cip1}$, Ki-67, and CD25 expression in ATL-CR cells. ATL-CR cells were cultured alone or with HEK293T cells in the presence of 0.8 μmol/L TSA. **F:** Expression of vimentin and fibronectin in ATL-CR cells co-cultured with HEK293T cells. Apo, apoptotic cells. The data are presented as means ± SD. $*P < 0.05$, $**P < 0.01$. Scale bars: 50 μm (**E** and **F**).

Next, to examine whether NECs have a protective role on ATL-CR cells pretreated with TSA, ATL-CR cells were incubated with TSA for 18 hours and then were transferred to the fresh medium free of TSA. Pretreatment of ATL-CR cells with TSA increased the proportion of G2/M phase cells in a dose-dependent manner (Figure 3D). These TSA-pretreated cells (shown as input cells) were transferred to the fresh TSA-free medium and were cultured for an additional 24 hours in the presence or absence of HEK293T cells. In the absence of HEK293T cells, the proportion of cells in the G0/G1, S, and G2/M phases continued to decline, and ATL-CR cells continued to undergo apoptosis. In contrast, the ratio of apoptotic cells did not increase appreciably in the presence of HEK293T cells. These results suggest that co-culture with HEK293T cells induced ATL-CR cells to enter the G0/G1 resting phase.

Ki-67 antigen is present during all active phases of the cell cycle (G1, S, G2, and M) but is absent from cells in resting G0 phase. When ATL-CR cells were stained with anti–Ki-67 antibody, most cells were not stained; however, when they were treated with TSA, 44.2% of the cells became positive with Ki-67 and entered the cell cycle (data not shown). TSA increases $p21^{Cip1}$ levels in cancer cells, leading to cell-cycle arrest.[24] When ATL-CR cells cultured alone were treated with TSA, they exhibited markedly induced expression of $p21^{Cip1}$ and Ki-67 and retained high expression of CD25 (Figure 3E). In contrast, when ATL-CR cells were treated with TSA and co-cultured with NECs, expression of $p21^{Cip1}$ and Ki-67 remained low, and expression of CD25 was strongly diminished (Figure 3E).

Vimentin is a mesenchymal intermediate filament supporting the structural integrity of quiescent cells while participating in adhesion, survival, growth regulation, and cell signaling processes.[25] It is a growth-regulated protein whose expression is induced in quiescent cells $\leq$2 hours after mitogenic stimulation, even when protein synthesis is inhibited.[26] We, therefore, examined the expression of vimentin and fibronectin, another mesenchymal marker, in ATL cells adhering to NECs. Expression of vimentin, but not fibronectin, was markedly elevated in ATL-CR cells co-cultured with HEK293T cells (Figure 3F). These results are consistent with the idea that adhesion to NECs induced cellular quiescence and resistance to TSA-induced apoptosis in ATL cells through vimentin expression.

ATL Cells Adhering to NECs Augment CD44 Expression

Cancer stem cell (CSC)–like cells often exhibit markers of epithelial-mesenchymal transition, such as vimentin.[25] Recent evidence indicates that CD44 directly reprograms stem cell properties in colon cancer cells.[27] We, therefore, examined whether the cellular quiescence achieved by co-culture with NECs was accompanied by elevated expression of CD44, a hyaluronan receptor known to be involved in cellular quiescence and now recognized as a marker of CSC-like cells (Figure 4A). When we compared cell surface expression of CD44 on ATL cells co-cultured with NECs and cultured alone in the presence or absence of TSA, CD44 expression was markedly increased in ATL-CR cells co-cultured with HEK293T cells compared with ATL cells cultured alone regardless of the presence or absence of TSA. Staining for CD44 was more pronounced in ATL-CR cells located at the upper and central regions of colonies, whereas vimentin tended to be evenly expressed on all ATL-CR cells (Figure 4, B and C). These observations suggest the involvement of CD44-mediated interactions in the induction of cellular quiescence in co-cultured ATL cells.

ATL Cells that Form Colonies on the Monolayer of NECs Show Intracellular Expression of CD44

ATL cells that formed colonies on the monolayer of NECs were more strongly positive for CD44 than were those that did not form colonies and floated in the medium (Figure 5A). To

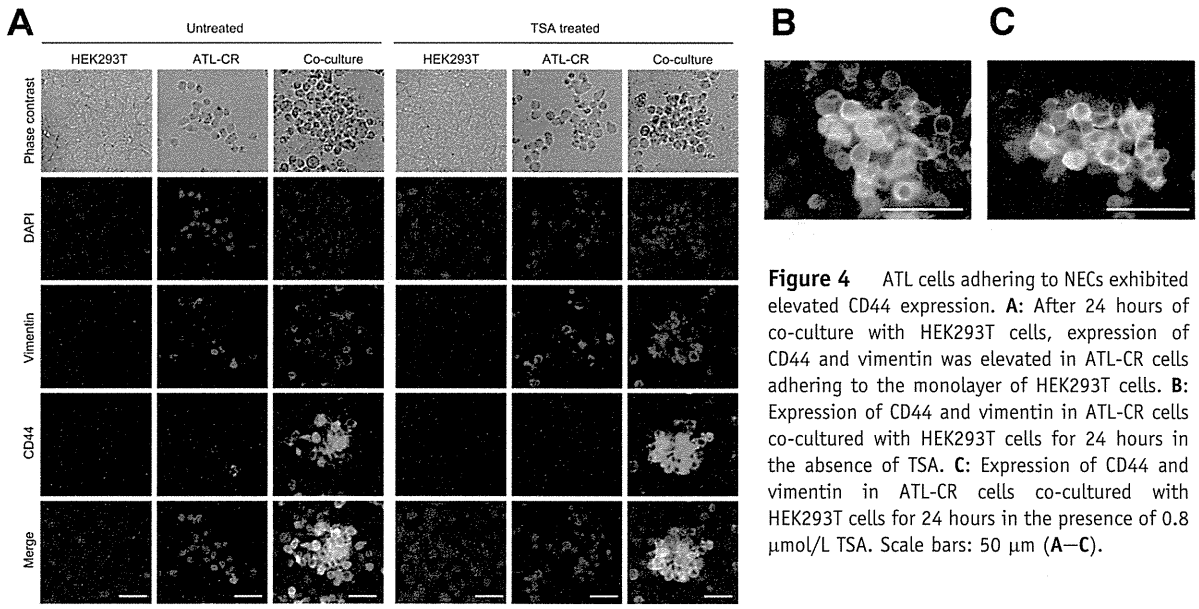

Figure 4 ATL cells adhering to NECs exhibited elevated CD44 expression. **A:** After 24 hours of co-culture with HEK293T cells, expression of CD44 and vimentin was elevated in ATL-CR cells adhering to the monolayer of HEK293T cells. **B:** Expression of CD44 and vimentin in ATL-CR cells co-cultured with HEK293T cells for 24 hours in the absence of TSA. **C:** Expression of CD44 and vimentin in ATL-CR cells co-cultured with HEK293T cells for 24 hours in the presence of 0.8 μmol/L TSA. Scale bars: 50 μm (**A–C**).