

about the efficacy of abrupt discontinuation of immunosuppression for a GVL effect [25-27], and the generation of cytotoxic T lymphocytes against some epitopes of ATL cells might be induced [28-30]. In our study, 4 (66.7%) of the 6 patients in whom the dose of a calcineurin inhibitor was reduced and administration was abruptly discontinued achieved CR or PR, suggesting that these are effective for induction of a GVL effect. However, discontinuation of the administration of a calcineurin inhibitor was not effective for 2 patients (Cases 5 and 8) in whom disease progression developed rapidly after allo-HSCT. Thus, it is thought that a GVL effect induced by abrupt discontinuation of immunosuppression may not be sufficient for disease control at the stage of aggressive disease progression. It is possible that the magnitude of a GVL effect varies with the individual, but this is not clear in the present study. Further studies are needed to clarify the mechanism of a GVL effect. On the other hand, most of the cases in which disease control was achieved by abrupt discontinuation of administration of a calcineurin inhibitor developed severe GVHD, suggesting that a balance between GVHD and GVL effect is a critical point in clinical management. However, immunosuppression (calcineurin inhibitor and/or steroid) was restarted in all cases with severe GVHD. Because no cases showed regrowth of ATL or reappearance of recipient-type cells in chimerism analysis for the restart of immunosuppression, a GVL effect induced by abrupt discontinuation of calcineurin inhibitor may continue for a long time even after the restart of immunosuppression.

In this study, HTLV-1 proviral DNA became undetectable more than 1 time after allo-HSCT in all patients with positive HTLV-1 proviral DNA before allo-HSCT. However, in 5 patients, HTLV-1 proviral DNA became detectable again, and only 1 patient relapsed. Because complete chimerism was maintained in peripheral blood in the other 4 patients, donor-derived cells appeared to be infected with HTLV-1 in the host after allo-HSCT according to the results of a past study [31]. Further studies are needed to clarify the association between relapse and reappearance of HTLV-1 proviral DNA after allo-HSCT.

The risk factors before allo-HSCT, particularly the disease status in allo-HSCT for ATL, are still unclear. In this study, the patient who had PD before allo-HSCT developed disease progression at an early stage after allo-HSCT. On the other hand, in the patients who had been in PR before allo-HSCT, only 1 patient relapsed after allo-HSCT and the other 4 patients have been in CR, an outcome that is not inferior to the outcome of patients who had been in CR before allo-HSCT. Further studies are needed to establish the of risk classification in allo-HSCT with ATL.

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Plasmacytoid dendritic cells prime alloreactive T cells to mediate graft-versus-host disease as antigen-presenting cells

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Dendritic cells (DCs) can be classified into 2 distinct subsets: conventional DCs (cDCs) and plasmacytoid DCs (pDCs). cDCs can prime antigen-specific T-cell immunity, whereas in vivo function of pDCs as antigen-presenting cells remains controversial. We evaluated the contribution of pDCs to allogeneic T-cell responses in vivo in mouse models of graft-versus-host disease (GVHD) after allogeneic hematopoietic stem cell trans-

plantation by an add-back study of MHC-expressing pDCs into major histocompatibility complex-deficient mice that were resistant to GVHD. Alloantigen expression on pDCs alone was sufficient to prime alloreactive T cells and cause GVHD. An inflammatory environment created by host irradiation has the decisive role in maturing pDCs for T-cell priming but this process does not require Toll-like receptor signaling. Thus, functional out-

comes of pDC–T-cell interactions depend on the immunologic context of encounter. To our knowledge, these results are the first to directly demonstrate an in vivo pathogenic role of pDCs as antigen-presenting cells in an antigen-specific T cell–mediated disease in the absence of other DC subsets and to provide important insight into developing strategies for tolerance induction in transplantation. (Blood. 2009;113:2088-2095)

Introduction

The interaction of naive T cells and dendritic cells (DCs) is essential for initiating primary immune responses. DCs can be divided into 2 distinct subsets: conventional DCs (cDCs) and plasmacytoid DCs (pDCs) according to their immunophenotype and functional properties.¹⁻³ pDCs represent a CD11c^{int} B220⁺ DC subset that differs from the CD11c^{high} B220⁻ major histocompatibility complex (MHC) class II^{high} cDCs, commonly viewed as the classic stimulators of naive T cells. One distinctive feature of pDCs is their capacity to rapidly produce high levels of type I interferon (IFN) in response to viral and bacterial stimuli, highlighting the importance of pDCs in innate immune responses.²⁻⁸ pDCs express low levels of surface MHC and classical costimulatory molecules; therefore, they are poor T-cell stimulators.⁵⁻¹¹ In contrast, pDCs matured with CD40 ligands or Toll-like receptor (TLR) ligands are potent antigen-presenting cells (APCs), capable of stimulating naive T-cell proliferation and differentiation to helper, killer, memory, and regulatory T cells in vitro.^{7,12,13} In vivo, injection of pDCs activated by synthetic oligodeoxynucleotides containing unmethylated cytosine-guanine motifs (CpG), but not immature pDCs, is capable of eliciting antigen-specific CD8⁺ T-cell responses.^{10,14} On the other hand, OVA-pulsed pDCs protected mice against OVA-induced asthma development.¹⁵ pDCs in the tumor-draining lymph nodes express indole 2, 3-dioxygenase, and suppress antitumor T-cell responses.¹⁶ In patients with ovarian cancer, large numbers of pDCs, which induced interleukin 10 (IL10)-producing regulatory T cells, were found in ascites.¹⁷ pDCs mediate tolerance and prolong survival of cardiac allografts.^{11,18,19}

Several recent clinical observations also suggest that pDCs play important regulatory roles in transplant outcome. An increased ratio of pDCs/cDCs is associated with the successful withdrawal of immunosuppressants after liver transplants.²⁰ In allogeneic hematopoietic stem cell transplantation (HSCT), low pDC count in the peripheral blood is a risk for graft-versus-host disease (GVHD),²¹ while larger numbers of pDCs in donor bone marrow (BM) are associated with increased relapse.²² Collectively, accumulating data suggest that pDCs are mostly tolerogenic in vivo. However, it remains unclear whether pDCs as APCs have a causative role in antigen-specific T cell–mediated diseases in vivo, although pDCs are involved in the pathogenesis of systemic lupus erythematosus (SLE) and psoriasis through IFN- α production.^{23,24}

GVHD, the major obstacle to successful outcome after allogeneic HSCT, is mediated by donor T cells stimulated by recipient DCs.²⁵⁻²⁷ MHC class I- or II-deficient (H2-Ab1^{-/-}) mice are resistant to CD8- and CD4-dependent GVHD, respectively.²⁶⁻²⁸ When H2-Ab1^{-/-} mice are repopulated with syngeneic MHC class II-expressing DCs, these mice succumb to acute GVHD.^{26,28} Thus, recognition of MHC class II alloantigens on host-derived DCs, alone, is sufficient to prime donor CD4⁺ T cells and cause lethal acute GVHD. CD4-mediated GVHD can develop even in the absence of MHC class II alloantigen expression on GVHD target cells, such as epithelium, endothelium, and parenchyma.^{26,28} Thus, this GVHD model system using H2-Ab1^{-/-} mice presents a stringent test of the allostimulatory capacity of a DC subset when the donor and recipient differ at only MHC class II loci. Using this

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model system with modification, we addressed whether pDCs as APCs have a causative role in antigen-specific T cell-mediated diseases, such as GVHD, or induce tolerance.

Methods

Mice

Female C57BL/6 (B6: H-2^b, CD45.2⁺), B6D2F1 (H-2^{b/d}), and BALB/c (H-2^d) mice were purchased from Charles River Japan (Yokohama, Japan). C3H/HeJ (C3H: H-2^k) and AKR/J (AKR: H-2^k) mice were purchased from Japan SLC (Shizuoka, Japan). B6-Ly5a (H-2^b, CD45.1⁺), and C3H-background β_2m -deficient ($\beta_2m^{-/-}$: C3.129P2(B6)- $B2m^{tm1Uhc}/Dcr$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6-background MHC class II^{-/-} mice ($H2-Ab1^{-/-}$: B6.129- Abb^{tm1} N12) were from Taconic (Germantown, NY). B6-background Toll-IL-1 receptor domain-containing adaptor inducing IFN- β /myeloid differentiation factor 88 double-deficient (TRIF/MyD88 DKO) mice²⁹ were kindly provided by Dr Kiyoshi Takeda at Kyushu University (Fukuoka, Japan). The age of the mice ranged between 9 and 16 weeks. Mice were maintained in a specific pathogen-free condition and received normal chow and hyperchlorinated drinking water for the first 3 weeks after transplantation. All animal experiments were performed under the auspices of the Institutional Animal Care and Research Advisory Committee at the Department of Animal Resources at Kyushu University.

Cell isolation

To expand DCs, we injected mice subcutaneously once daily with 10 μ g recombinant human fms-like tyrosine kinase 3 ligand (FL; Amgen, Seattle, WA) for 10 consecutive days, and cDCs, pDCs, and B cells were isolated, as previously described, with a modification.^{28,30} Briefly, cDCs were enriched from splenocytes using CD11c microbeads and the AutoMACS system (Miltenyi Biotec, Bergisch Gladbach, Germany), followed by cell sorting of CD11c^{high} B220⁻ cells using a BD FACSAria (BD Biosciences, San Jose, CA). B cells were enriched from splenocytes with B220 microbeads and the AutoMACS, and sorted as CD11c⁻ B220⁺ cells. pDCs were enriched from BM by depleting CD3⁺, CD19⁺, CD11b⁺, CD49b⁺, and Ly-76⁺ cells using a cocktail of biotin-conjugated mAbs, streptavidin microbeads, and the AutoMACS system, followed by a FACS sorting of CD11c^{int} B220⁺ cells. CD4⁺ T cells were negatively selected from splenocytes by depleting CD8⁺, CD49b⁺, CD11b⁺, Ly-76⁺, and B220⁺ cells. CD8⁺ T cells were negatively selected from splenocytes by depleting CD4⁺, CD49b⁺, CD11b⁺, Ly-76⁺, and B220⁺ cells, using the AutoMACS system, followed by a FACS sorting of CD4⁻CD8⁺ cells. T-cell depletion (TCD) of donor BM cells was performed using CD90 microbeads and the AutoMACS system.

Induction and assessment of GVHD

GVHD was induced as previously described.²⁸ In brief, mice received 11 Gy total body irradiation (TBI), split into 2 doses separated by 4 hours to minimize gastrointestinal toxicity, and injected intravenously with 2×10^6 each APC subset on day -1. On day 0, mice were injected intravenously with 2×10^6 CD4⁺ or CD8⁺ T cells with or without 5×10^6 TCD-BM. Survival after BMT was monitored daily, and the degree of clinical GVHD was assessed weekly using a scoring system that evaluated changes in 5 clinical parameters: weight loss, posture, activity, fur texture, and skin integrity (maximum index = 10).³¹ Acute GVHD was also assessed by detailed histopathologic analysis of the liver and intestine. Slides stained with hematoxylin and eosin were examined systematically using a semiquantitative scoring system.³² Pictures from tissue sections were taken at room temperature using a ProgRes 3012 mF digital camera (Jenoptik Laser Optik Systeme, Jena, Germany) mounted on an Olympus BX51 microscope (Olympus, Tokyo, Japan) and analyzed using a ProgRes Plugin for PCI software version 5.0 (Jenoptik Laser Optik Systeme). Images were acquired using an UPlan Apochromat 10 \times /0.40 numeric aperture (NA) or a Plan

Apochromat 40 \times /0.90 NA WLSM objective, depending on the desired magnification.

Cell culture and enzyme-linked immunosorbent assay

All culture media and incubation conditions have been described previously.³³ CD4⁺ T cells were cultured at a concentration of 2×10^5 cells/well with 10⁴ irradiated (20 Gy) APCs. After culturing for 3 days, supernatants were harvested for cytokine measurements, and cells were pulsed with ³H-thymidine (1 μ Ci per well) for further 16 hours. Proliferation was determined using a Topcount NXT (Packard Instruments, Meriden, CT). In some experiments, 1 μ M CpG 1668 (Sigma-Aldrich Japan, Ishikari, Japan) or 100 μ g/mL lipopolysaccharide (LPS; Sigma-Aldrich Japan) was added to the culture. To determine secretion of IFN- α and IL-12 p70 from APCs, 10⁵ APCs were incubated with 1 μ M CpG 2216 (Sigma-Aldrich Japan) for 16 hours. Enzyme-linked immunosorbent assay (ELISA) for IFN- γ (BD Biosciences), IFN- α (PBL Biomedical Laboratories, Piscataway, NJ), and IL-12 p70 (R&D Systems, Minneapolis, MN) was performed according to the manufacturer's protocols with the sensitivity of 31.3 pg/mL, 12.5 pg/mL, and 2.5 pg/mL, respectively.

Flow cytometric analysis

The mAbs used were fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, or allophycocyanin-conjugated anti-mouse TCR β , CD4, CD8 α , CD11c, CD11b, CD19, CD49b, CD45.1, CD45.2, CD90.2, CD86, B220 (CD45R), H-2K^b, H-2K^d, H-2K^k, I-A^b, Ly6C (BD Biosciences), mPDCA-1 (Miltenyi Biotec), and Foxp3 (eBioscience, San Diego, CA). Cells were stained as previously described.³³ Irrelevant IgG_{2a/b} mAbs were used as a negative control. For intracellular IFN- γ staining, splenocytes were incubated for 4 hours with leukocyte activation cocktail and BD GolgiPlug (BD Biosciences) at 37°C. Then, the cells underwent permeabilization with BD Cytofix/Cytoperm solution (BD Biosciences) and were stained with FITC-conjugated anti-IFN- γ mAbs. For carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling, CD4⁺ T cells were stained in phosphate-buffered saline (PBS) with 1 μ M CFSE (Molecular Probes, Invitrogen, Eugene, OR), as described earlier.³⁴ At least 5000 live samples were acquired for analysis. Dead cells were identified as 7-amino-actinomycin D (BD Biosciences) or 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes) positive cells. The cells were analyzed using a FACSCalibur or a FACSAria flow cytometer (BD Biosciences), and the data were analyzed using FlowJo software (TreeStar, San Carlos, CA).

Statistical analysis

Mann-Whitney U tests were used to analyze cell counts and clinical scores. We used the Kaplan-Meier product limit method to obtain the survival probability and the log-rank test was applied to compare the survival curves. We defined *P* values of less than .05 as statistically significant.

Results

Isolation and characterization of pDCs

Low frequency of pDCs in vivo has hampered the study of this cell population. To expand DCs in vivo, mice were injected with 10 μ g FL for 10 days.^{6,28,30} As previously reported,^{35,36} FL treatment resulted in an increase in frequency of CD11c⁺B220⁺ pDCs to 6.7% in spleen and 18.6% in BM (Figure 1A). pDCs, cDCs, and B cells were then isolated as described in the methods section. The purity of each population was greater than 98% with less than 0.1% contamination by the others (Figure 1A). pDCs appeared plasmacytoid round shaped with excentered nuclei and basophilic cytoplasm (Figure 1B). They expressed low levels of CD86 and MHC class II but high levels of Ly6C and mPDCA-1 (Figure 1C). These cells did not express CD19, CD49b, or CD11b, thus ruling out contamination of B cells, natural killer (NK) cells, and IFN-producing killer

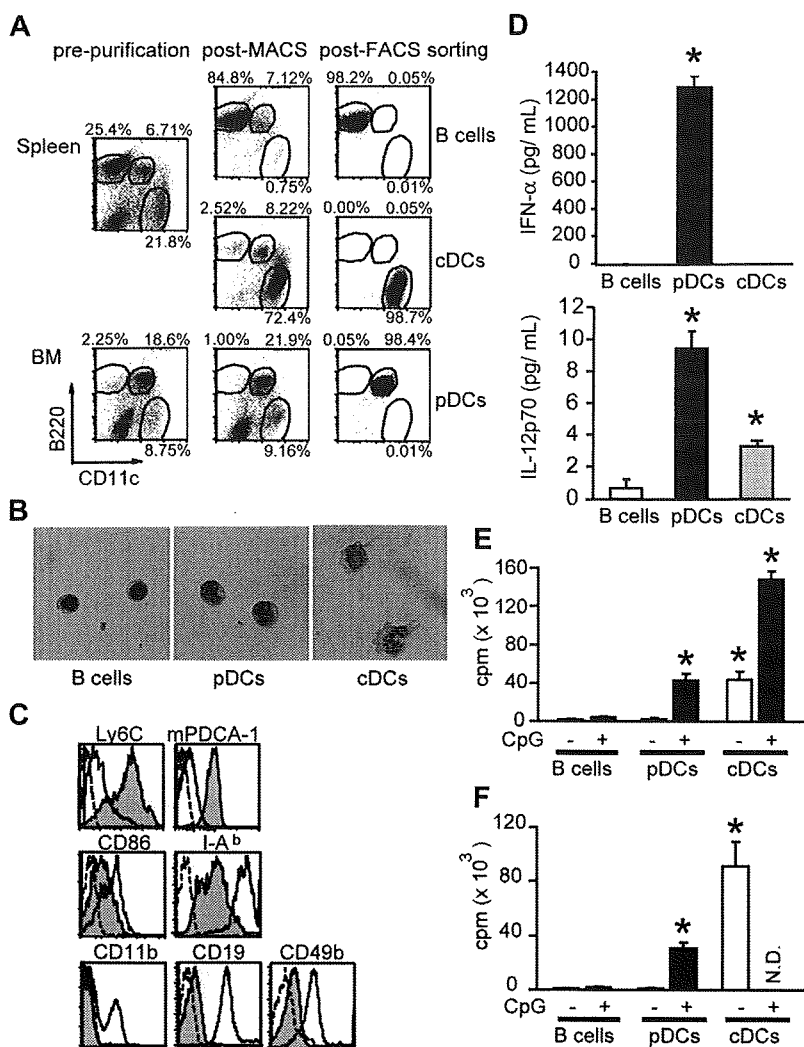


Figure 1. Isolation of pDCs and cDCs. B cells ($CD11c^- B220^+$), pDCs ($CD11c^{int} B220^+$), and cDCs ($CD11c^{high} B220^-$) were isolated from the spleen or BM of FL-treated mice. (A) Two-dimensional counter plots of B220 and CD11c staining. Percentages of cells are shown enclosed in circles. (B) Cell morphology stained with May-Giemsa (magnification $\times 400$). (C) Immunophenotyping. Filled histograms; pDCs, broken-lined open histograms; pDCs stained with isotype controls, solid-lined open histograms for Ly6C, mPDCA-1, CD86, I-A^b, and CD11b; cDCs, those for CD19 and CD49b; B cells and enriched CD49b⁺ cells as positive controls, respectively. (D) Production of IFN- α (top) and IL-12p70 (bottom) after incubation of cells with CpG 2216 for 16 hours (mean \pm SD). (E, F) Aliquots of 2×10^5 BALB/c CD4⁺ T cells were cultured with 10^4 cells from each APC subset isolated from FL-treated (E) and FL-untreated mice (F), with or without $1 \mu M$ CpG 1668, and their proliferation 3 days later was shown as mean plus or minus SD. N.D. indicates not done. Data are representative of at least 2 similar experiments. * $P < .05$ compared with B cells.

DCs^{37,38} in the pDC fraction. After stimulation with CpG 2216, pDCs secreted high levels of IFN- α and IL-12 p70, whereas cDCs secreted moderate levels of IL-12 p70, but not IFN- α , as has been described³⁹(Figure 1D).

Next, we examined the allostimulatory capacity of pDCs in mixed lymphocyte reaction. Freshly isolated pDCs were poor inducers of allogeneic CD4⁺ T-cell proliferation as previously reported³ (Figure 1E). However, pDCs matured with CpG 1668 were capable of priming T cells nearly as effectively as cDCs (Figure 1E). Similar results were obtained when pDCs were isolated from mice without treatment with FL (Figure 1F).

MHC class II-expressing pDCs alone sufficiently stimulate donor CD4⁺ T cells to cause GVHD in MHC class II-deficient mice

H2-Ab1^{-/-} B6 mice are resistant to CD4-dependent GVHD.^{26,28} We studied whether the add-back of MHC class II^{+/+} pDCs could prime alloreactive T cells using this system with modification. In our previous studies, we used a bm12 \rightarrow B6 model across MHC class II disparity alone. In contrast, the B6 and BALB/c strain combination used in the current study differs at both MHC class I and class II loci. To avoid the confounding effects of MHC class I and CD8⁺ T cells, therefore, purified CD4⁺ T cells were used to induce GVHD, while whole T cells were used in the previous studies. Contamination of CD8⁺ T cells in the CD4⁺ cell fraction

was $< 0.1\%$. H2-Ab1^{-/-} B6 (H-2^b) mice were irradiated with 11 Gy TBI and injected with 2×10^6 pDCs, cDCs, or B cells isolated from wild-type (WT) B6 mice on day -1. Mice were then injected with 2×10^6 CD4⁺ T cells from BALB/c (H-2^d) donors that differ at MHC and multiple minor histocompatibility antigens (miHAs) from B6 mice on day 0. On day 0, we confirmed homing of the injected cDCs and pDCs to spleen and lymph nodes (LNs), where mature DCs form long-lived contacts with T cells (data not shown).⁴⁰ Flow cytometric analysis of the mesenteric LNs (mLNs) and spleen on day +6 demonstrated the significant expansion of donor CD4⁺ T cells (H-2K^dCD4⁺) in animals that had been repopulated with cDCs or pDCs compared with those with B cells (Figure 2A). No CD8⁺ T-cell expansion was observed ($< 0.1\%$), confirming the stimulation of only CD4⁺ donor T cells in this system. Similarly, CFSE-labeled donor CD4⁺ T cells showed robust cell division in animals preinjected with cDCs or pDCs, while those underwent some homeostatic divisions in animals preinjected with B cells (Figure 2B). Donor CD4 expansion in these recipients was associated with increased expression of IFN- γ (data not shown). Evaluation of Foxp3 expression on CD4⁺ T cells ruled out the possibility of expansion of Foxp3⁺ regulatory T cells in response to pDCs (data not shown). We then investigated the effects of delayed add-back of pDCs on GVHD. H2-Ab1^{-/-} mice were irradiated on day -4 and injected with pDCs on day -1, followed by the injection of CFSE-labeled BALB/c CD4⁺ T cells

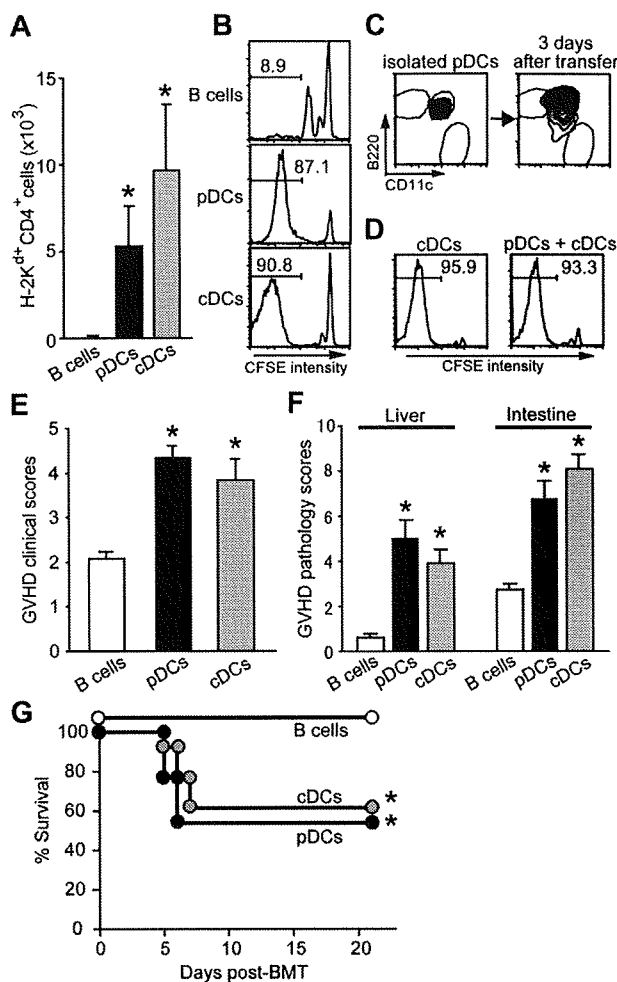


Figure 2. Host pDCs or cDCs alone are sufficient to stimulate alloreactive CD4⁺ T cells and induce GVHD. (A,B) Totals of 2×10^6 BALB/c (H-2K^{d+}) CD4⁺ T cells were transferred to irradiated H2-Ab1^{-/-} mice preinjected with 2×10^6 WT B cells, pDCs, or cDCs. Expansion of H-2K^{d+} donor CD4⁺ T cells in the mLN (mean \pm SD; A) and cell division of CFSE-labeled donor CD4⁺ T cells in the spleens (B). (C) pDCs were injected to irradiated H2-Ab1^{-/-} mice. Expression levels of B220 and CD11c on I-A^{b+} cells were analyzed 3 days after transfer and compared with those before transfer. (D) Aliquots of 2×10^6 CFSE-labeled BALB/c CD4⁺ T cells were transferred to irradiated H2-Ab1^{-/-} mice preinjected with 10^6 pDCs plus 10^6 cDCs or 2×10^6 cDCs from WT B6 mice. Cell divisions of donor CD4⁺ T cells in the spleens on day 7 are shown. (E,F) BALB/c CD4⁺ T cells were transferred as above and GVHD clinical scores (E), and pathology scores in the liver and intestine (F) on day 6 are shown as mean plus or minus SEM. (G) Lethally irradiated H2-Ab1^{-/-} mice preinjected with pDCs, cDCs, or B cells were injected with 2×10^6 CD4⁺ T cells and 5×10^6 TCD-BM from BALB/c mice. Survival after BMT is shown. Results from 2 similar experiments were combined. * $P < .05$ compared with B cells.

on day 0. Flow cytometric analysis of the spleen showed robust division of donor CD4⁺ T cells, thus suggesting that TBI-mediated inflammation persist at least for 3-4 days after TBI (data not shown). In these experiments, we did not have syngeneic controls, since it was apparent in a previous study of DC add-back²⁸ that there was no expansion of donor T cells in syngeneic controls, where H2-Ab1^{-/-} B6 recipients that had been repopulated with WT B6 DCs were injected with CD4⁺ T cells from congenic B6-Ly5a mice.

To rule out the possibility that allostimulatory capacity of pDCs is due to FL treatment, pDCs were isolated from mice without treatment with FL, and their allostimulatory capacity was evaluated similarly. Again, CD4⁺ T cells primed by pDCs underwent robust cell divisions (Figure S1A, available on the *Blood* website; see the Supplemental Materials link at the top of the online article) and

differentiated into effectors secreting high levels of IFN- γ (Figure S1B) in H2-Ab1^{-/-} mice 7 days after transfer, indicating that the ability of pDCs to prime T cells is not due to FL treatment. Therefore, we restricted our subsequent analysis to FL-expanded pDCs.

There was still a possibility that pDCs differentiate into cDCs fully endowed with APC function after transfer to H2-Ab1^{-/-} mice.⁴¹ To examine this possibility, we isolated and injected pDCs into irradiated H2-Ab1^{-/-} mice. Flow cytometric analysis of the spleens, 3 days later, showed that pDCs still maintained the phenotype of pDCs (Figure 2C), thus ruling out this possibility. Next, we evaluated whether pDCs were capable of down-regulating T-cell activation induced by cDCs. To investigate this, CFSE-labeled donor CD4⁺ T cells were transferred to H2-Ab1^{-/-} mice preinjected with cDCs alone or with cDCs and pDCs. However, flow cytometric analysis of the spleen 7 days after transfer showed that the addition of pDCs did not suppress donor CD4 expansion mediated by cDCs (Figure 2D).

We then tested the hypothesis that alloantigen expression on pDCs alone is sufficient for the induction of GVHD target organ damage. H2-Ab1^{-/-} mice were irradiated and injected with 2×10^6 WT pDCs, cDCs, or B cells on day -1. Mice were then injected with 2×10^6 BALB/c CD4⁺ T cells. Mice preinjected with pDCs or cDCs developed significant GVHD, as assessed by the clinical scores³¹ (Figure 2E) and pathology scores on day +6³² (Figure 2F), compared with those with B cells. Liver histology of mice preinjected with pDCs showed standard histologic features of acute GVHD, including mononuclear cell infiltration in bile ducts and portal triads, and hepatocellular damage with acidophilic bodies (Figure 3B). Histopathology of the small and large intestine also showed significant changes in these mice, including villous atrophy with epithelial apoptosis (Figure 3E), as well as lymphocytic infiltration and granulation tissue formation (Figure 3H). To note, these pathologic features were similar to those observed in recipients preinjected with cDCs (Figure 3C, F, and I), whereas animals preinjected with B cells showed no significant pathologic signs of GVHD, as previously described²⁸ (Figure 3A,D,G). These results demonstrated that pDCs alone are sufficient to activate donor CD4⁺ T cells to trigger GVHD as effectively as cDCs. Finally, we evaluated whether pDC-mediated T-cell activation induces GVHD mortality. Lethally irradiated H2-Ab1^{-/-} mice preinjected with 2×10^6 WT pDCs, cDCs, or B cells on day -1 were injected with 2×10^6 CD4⁺ T cells and 5×10^6 TCD-BM from BALB/c donors on day 0. Mice preinjected with pDCs or cDCs developed lethal GVHD (Figure 2G).

pDCs are solely sufficient to activate alloreactive CD8⁺ T cells in β_2m -deficient mice

We examined whether presence of allogeneic pDCs could also stimulate donor CD8⁺ T cells in β_2m ^{-/-} mice with impaired cellular expression of functional MHC class I. Irradiated β_2m ^{-/-} C3H mice (CD90.2⁺) were injected with WT pDCs, cDCs, or B cells on day -1, followed by the injection with 2×10^6 CD8⁺ T cells from MHC-matched, miHA-mismatched AKR mice (CD90.1⁺) on day 0. Flow cytometric analysis of the mLN and spleen on day +6 showed significantly greater expansion (Figure 4A) and IFN- γ production (Figure 4B) of donor CD8⁺ T cells (CD90.2⁻ CD8⁺) in mice preinjected with pDCs or cDCs than in those with B cells. Thus, alloantigen expression on pDCs can solely prime alloreactive CD8⁺ T cells in vivo as potently as cDCs, although we were unable to examine whether these CD8⁺ T cells could cause GVHD target organ injury because miHA expression

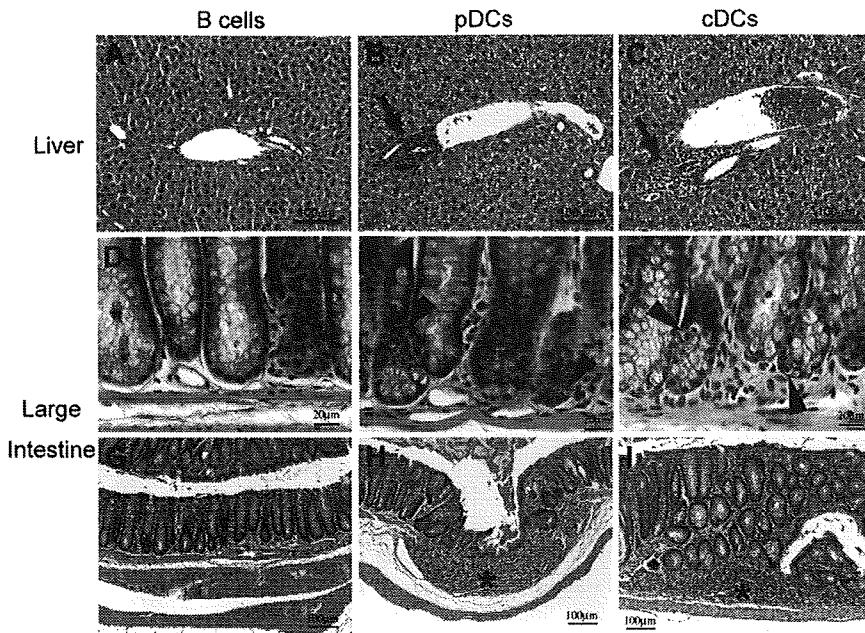


Figure 3. pDCs or cDCs alone mediate standard acute GVHD. Histologic findings of the liver (A-C) and large intestine (D-I). Periportal mononuclear infiltrates in the liver (panels B,C), and crypt cell apoptosis (panels E,F) and granulation tissue (* in panels H,I) in the large intestine are shown.

on target epithelium was required to induce GVHD in MHC-matched, miHA mismatched HSCT.^{27,42}

Cognate interaction between pDCs and T cells is required for pDCs to prime alloreactive T cells

pDCs have the ability to produce large amounts of cytokines; therefore, they can be pathogenic through cytokine production as in SLE.²³ To confirm the pathogenic role of pDCs functioning as APCs in GVHD, CFSE-labeled BALB/c CD4⁺ T cells were adoptively transferred to irradiated H2-Ab1^{-/-} mice preinjected with H2-Ab1^{-/-} pDCs. Flow cytometric analysis of the spleens 6 days after transfer showed that almost 90% of CFSE-labeled T cells had progressed through at least 3 cell divisions in animals preinjected with WT pDCs or cDCs, whereas only a small population did so with some homeostatic divisions in mice with H2-Ab1^{-/-} pDCs or cDCs (Figure 5). These results demonstrate that cognate interaction between pDCs and T cells is required for pDCs to prime alloreactive T cells.

Irradiation is critical for pDCs to prime alloreactive T cells

Activation of pDCs is critical to prime alloreactive T cells as shown in Figure 1. Since it has been shown that irradiation

induces maturation of cDCs in vivo,²⁷ we hypothesized that pretransplantation TBI also plays an important role in activating pDCs. To test this hypothesis, we irradiated mice with 11Gy TBI and performed a flow cytometric analysis of the spleens and mLNs 10 hours after TBI. Expression of CD86 and MHC class II was up-regulated on both pDCs and cDCs isolated from irradiated mice, compared with those from unirradiated mice (Figure 6A). Next, we investigated whether maturation of pDCs are mediated by direct effects of irradiation on pDCs or by effects of irradiation on host tissues. To examine this, 80 × 10⁶ BM cells and splenocytes collected from B6 (CD45.2⁺) mice were transferred to congenic B6-Ly5a (CD45.1⁺) mice that had been irradiated 1 hour before transfer. Flow cytometric analysis of the mLNs isolated 10 hours later showed up-regulated MHC class II expression on CD45.2⁺ pDCs in irradiated mice compared with those isolated from unirradiated mice (Figure 6B). Collectively, irradiation, likely from the inflammation, is responsible for maturation of pDCs.

These results suggest that pDCs are incapable of priming alloreactive T cells in unirradiated mice as effectively as in irradiated mice. To test this hypothesis, we transferred CFSE-labeled BALB/c CD4⁺ T cells to unirradiated H2-Ab1^{-/-} mice

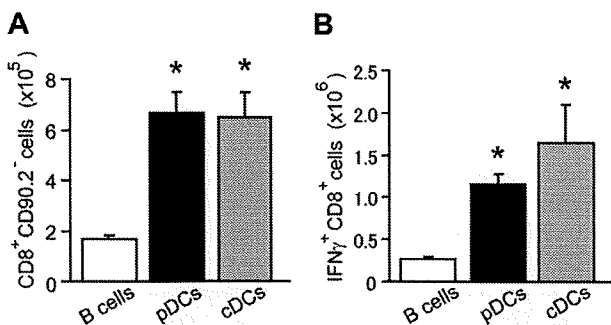


Figure 4. Alloantigen expression on pDCs alone is sufficient to stimulate alloreactive CD8⁺ T cells. CD8⁺ T cells from AKR (CD90.2⁻) mice were transferred to irradiated β2m^{-/-} C3H (CD90.2⁺) mice preinjected with WT B cells, pDCs, or cDCs. Expansion of CD90.2⁻ donor CD8⁺ T cells in mLN (A) and IFN-γ expression on donor CD8⁺ T cells in spleens (B) 6 days after transfer are shown as means plus or minus SEM. Data are representative of 2 similar experiments. *P < .05.

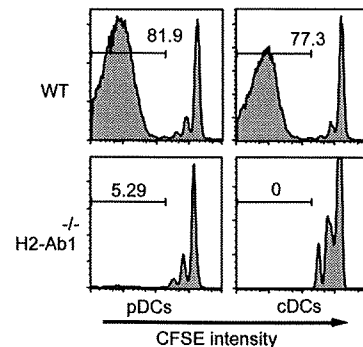
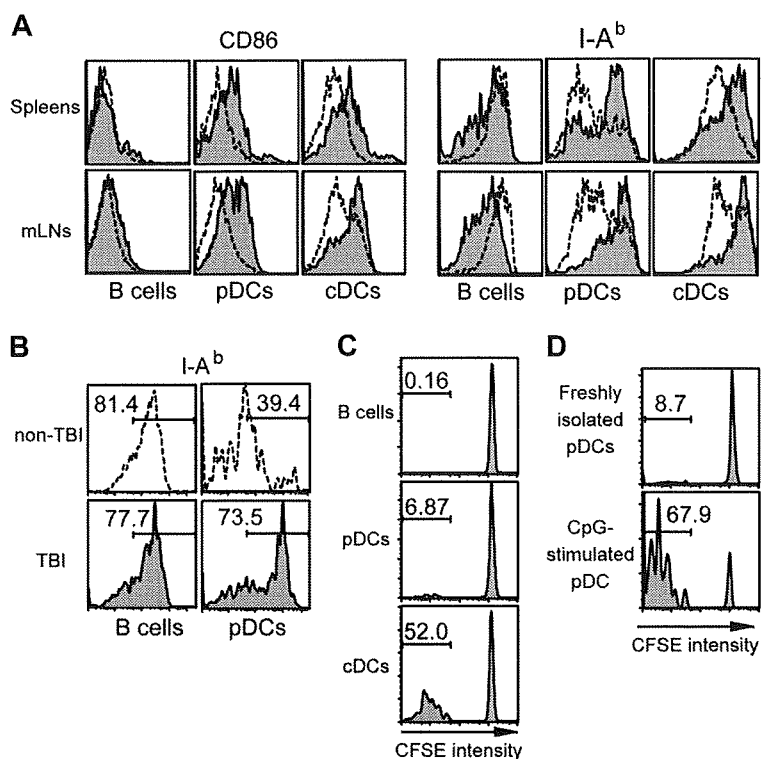


Figure 5. Cognate interaction between pDCs and T cells is required for T-cell activation. CFSE-labeled BALB/c CD4⁺ T cells were transferred to irradiated H2-Ab1^{-/-} mice preinjected with 2 × 10⁶ pDCs and cDCs isolated from WT or H2-Ab1^{-/-} B6 mice. Cell divisions of H-2K^d donor CD4⁺ T cells in the spleens 6 days after transfer are shown. Data are representative of 2 similar experiments.

Figure 6. Host irradiation is prerequisite for pDC maturation to prime T cells. (A) Expression of CD86 and I-A^b expression on B cells, pDCs, and cDCs in the spleen and mLNIs isolated from irradiated mice 10 hours after irradiation (filled histograms) and from unirradiated control mice (broken-lined open histograms). (B) Unirradiated (top) or irradiated (bottom) B6-Ly5a (CD45.1⁺) mice were injected with 80×10^6 BM cells and splenocytes isolated from FL-treated B6 (CD45.2⁺) mice. Expression of I-A^b on CD45.2⁺ B cells and pDCs in mLNIs 10 hours after injection is shown. (C) Aliquots of 20×10^6 CFSE-labeled BALB/c (H-2K^d) CD4⁺ T cells were transferred to unirradiated H2-Ab1^{-/-} mice preinjected with 6×10^6 WT pDCs or cDCs. Cell division of donor CD4⁺ T cells in the spleens on day +6 is shown. (D) Similarly, CFSE-labeled BALB/c CD4⁺ T cells were transferred to unirradiated H2-Ab1^{-/-} mice preinjected with freshly isolated pDCs or pDCs stimulated with CpG 1668 $1 \mu\text{M}$ for 24 hours in vitro. Cell division of donor CD4⁺ T cells in the spleens on day +6 is shown. Data are representative of 2 similar experiments.



preinjected with WT B cells, pDCs, or cDCs. Donor CD4⁺ T cells were significantly proliferated in mice preinjected with cDCs 6 days after transfer (Figure 6C), although this cell division appeared to be less potent compared with that in irradiated animals. In contrast, few cell divisions were observed in mice preinjected with B cells or pDCs. We next evaluated whether maturation of pDCs was critical for T-cell activation. Isolated pDCs were cultured with CpG 1668 for 24 hours and injected into unirradiated H2-Ab1^{-/-} mice, followed by the transfer of CFSE-labeled BALB/c CD4⁺ T cells. CpG-stimulated pDCs stimulate proliferation of donor T cells even in unirradiated mice (Figure 6D).

TLR signaling is not required for pDCs to prime alloreactive T cells

Stimulation with TLR ligands is crucial for the maturation and activation of pDCs.^{7,14} We, therefore, hypothesized that maturation of pDCs after TBI is mediated by TLR engagement. To test this hypothesis, we used TRIF/MyD88 DKO mice, where the TLR-dependent signaling pathway was critically abolished.²⁹ pDCs isolated from TRIF/MyD88 DKO mice were phenotypically identical to WT pDCs (data not shown) but did not respond to CpG or LPS stimulation (Figure 7A), as has been shown.²⁹ However, TRIF/MyD88 DKO cDCs and pDCs were capable of stimulating donor CD4⁺ T-cell division in irradiated H2-Ab1^{-/-} mice 6 days after transfer (Figure 7B) and induced significant pathologic GVHD (Figure 7C) as effectively as WT cDCs and pDCs. Thus, TLR signaling is not required for pDCs and cDCs to prime alloreactive T cells.

Discussion

GVHD is initiated by the interaction of alloreactive donor T cells and host DCs.^{25,28} Our GVHD model system using

MHC-deficient mice presents a stringent test on the allostimulatory functions of a subpopulation of APCs.²⁸ Using this model system with modification, we addressed whether pDCs are pathogenic in GVHD. We found that alloantigen expression on pDCs alone sufficiently stimulates donor T cells to trigger GVHD in the absence of other APC subsets. Induction of GVHD required cognate interaction of pDCs and T cells, since MHC class II expression on pDCs was absolutely required for stimulating donor CD4⁺ T cells. It has been suggested that pDCs are involved in the pathogenesis of SLE and psoriasis through IFN- α production^{23,24}; however, to our knowledge, our study is the first to directly demonstrate in vivo pathogenic role of pDCs as APCs in an antigen-specific T cell-mediated disease in the absence of other DC subsets.

Our results are in sharp contrast to previous reports suggesting that pDCs mediate tolerance in vivo. In cancer patients, pDCs are incapable of inducing antitumor immune responses but, instead, may induce regulatory T cells that inhibit immunity.^{16,17} In animal model of asthma, pDCs in the lung prevent asthmatic reactions to harmless inhaled antigens.¹⁵ In experimental cardiac transplantation, pDCs mediate tolerance and prolong survival of allografts.^{11,18,19} It has been suggested that pDCs functioning as APCs generate suppressive or regulatory T cells that mediate tolerance.^{12,13,16-18} In contrast, our study showed that alloantigen-presenting pDCs were incapable of suppressing activation of alloreactive T cells but, instead, induced immunity even in the absence of other APC subsets. Differences between previous studies and our study may be attributed to pretransplantation irradiation essential for performing HSCT, which was used in our study.

We have shown that TBI is critical for pDC maturation to prime T cells; TBI up-regulates expression of MHC and costimulatory molecules on pDCs. It has been shown that TBI induces phenotypic and functional maturation of cDCs.²⁷ Our results

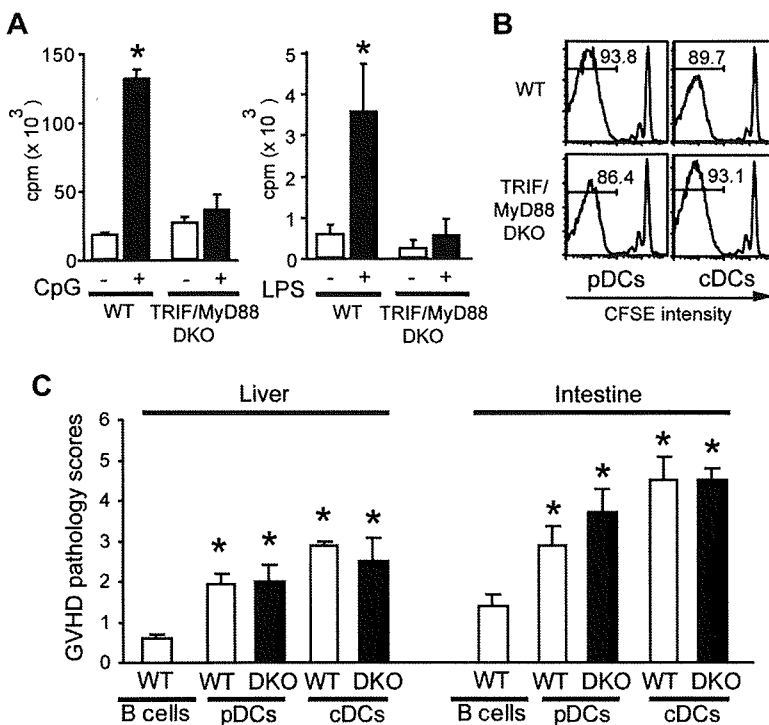


Figure 7. TLR signaling is not required for pDCs to prime alloreactive T cells. (A) A total of 2×10^5 BALB/c CD4⁺ T cells were cultured with 10^4 WT or TRIF/MyD88 DKO (DKO) pDCs with or without CpG 1668 $1 \mu\text{M}$ (left) or LPS $10 \mu\text{g/mL}$ (right) to determine cell proliferation. Data are shown as mean (\pm SD). (B,C) CFSE-labeled BALB/c CD4⁺ T cells (H-2K^d) were transferred to irradiated H2-Ab1^{-/-} mice preinjected with pDCs or cDCs isolated from WT or DKO B6 mice. Cell divisions of H-2K^d donor CD4⁺ cells in the spleens (B) and GVHD pathology scores in the liver and intestine (C) are shown. Data from 3 similar experiments are combined and shown as means plus or minus SEM ($n = 7$). * $P < .05$.

confirm and extend these findings. TBI also matures pDCs, and this process is dependent on a TBI-mediated inflammatory environment. Although pDCs need to be in a certain state of maturation to prime naive T cells, cDCs can stimulate donor T cells even in unirradiated mice. Thus, the capacity of pDCs to prime naive T cells is far less efficient than cDCs in an uninfamed environment. Host pDCs may not be responsible for the induction of transfusion-associated GVHD in humans.^{43,44}

It has been shown that TBI plays an important role in the pathogenesis of GVHD; GVHD is less severe in recipients that have had gentle rather than intensive preconditioning treatments.^{45,46} TBI activates and damages host tissue to secrete endogenous factors, such as heat-shock proteins and proinflammatory cytokines like tumor necrosis factor- α and IL-1. TBI-mediated gut injury allows the translocation of exogenous microbial products, such as LPS.^{27,45,47,48} DC maturation can be triggered by such endogenous and exogenous “danger signals” as well as by activated T cells, NK cells, and NKT cells.^{1,3,4,49} Since many of these stimuli bind to TLRs, we hypothesized that TLR signaling is required for pDC maturation. However, pDCs from TRIF/MyD88 DKO mice with defective TLR signaling²⁹ were activated by TBI and activate donor T cells to cause GVHD as potently as WT pDCs. Thus, pDCs can be matured by pathways other than TLR signaling, such as nucleotide-binding oligomerization domain-like receptors, retinoic acid-inducible gene I, melanoma differentiation-associated gene 5, DNA-dependent activator of interferon-regulatory factors, C-type lectin receptors, CD40 ligands, and cytokine and chemokine receptors.^{49,50}

Immature cDCs also play a role in maintaining tolerance, and thus, irrespective of the subset of DCs, DC maturation may be a control checkpoint in the initiation of immunity.^{3,51} Although this simple concept has been revised by several reports showing that mature cDCs and pDCs can induce regulatory T-cell responses,^{12,13} our results indicate that pDCs mature and acquire APC function to induce antigen-specific immunity in the inflamed tissue. Thus,

functional outcomes of pDC–T-cell interactions depend on the immunologic context of encounter.

It is important to note that pDCs mediate clinically and histologically standard GVHD that was not indistinguishable from cDC-mediated GVHD. Thus, our results further extend the current paradigm that host DCs play a critical role in initiating GVHD.^{25,28} Inactivation of host DCs can be a novel strategy to prevent GVHD.²⁵ Our results suggest that immunologic elimination of cDCs may not be sufficient for complete prevention of GVHD, thus providing important information for developing strategies aimed at inactivating host DCs to prevent GVHD.

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Authorship

Contribution: M.K. conducted research and wrote the paper; D.H., K.A., K.M., K.K., and H.N. conducted research; M.H., M.T., and K.A. designed the study; and T.T. designed the study and organized the data.

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Impaired Tax-specific T-cell responses with insufficient control of HTLV-1 in a subgroup of individuals at asymptomatic and smoldering stages

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Human T-cell leukemia virus type-1 (HTLV-1)-specific T-cell immunity, a potential antitumor surveillance system *in vivo*, is impaired in adult T-cell leukemia (ATL). In this study, we aimed to clarify whether the T-cell insufficiency in ATL is present before the disease onset or occurs as a consequence of the disease. We investigated T-cell responses against Tax protein in peripheral blood mononuclear cells (PBMCs) from individuals at earlier stages of HTLV-1-infection, including 21 asymptomatic HTLV-1 carriers (ACs) and four patients with smoldering-type ATL (sATL), whose peripheral lymphocyte count was in normal range. About 30% of samples tested showed clear Tax-specific interferon (IFN)- γ producing responses. Proviral loads in this group were significantly lower than those in the other less-specific response group. The latter group was further divided to two subgroups with or without emergence of Tax-specific responses following depletion of CC chemokine receptor 4 (CCR4)⁺ cells that contained HTLV-1-infected cells. In the PBMCs with Tax-specific responses, CD8⁺ cells efficiently suppressed HTLV-1 p19 production in culture. The remaining group without the emergence of Tax-specific response after CCR4⁺ cell-depletion included at least two sATL and one AC samples, which spontaneously produced HTLV-1 p19 in culture, where tetramer-binding, Tax-specific cytotoxic T-lymphocytes were either undetectable or unresponsive. Our results indicated that HTLV-1-specific T-cell responsiveness widely differed among HTLV-1 carriers, and that impairment of HTLV-1-specific T-cell responses was observed not only in advanced ATL patients but also in a subpopulation at earlier stages, which was associated with insufficient control of HTLV-1. (*Cancer Sci* 2009; 100: 481–489)

Human T-cell leukemia virus type 1 (HTLV-1) is the etiological agent of adult T-cell leukemia (ATL).^(1,2) Although the majority of HTLV-1-infected individuals remain asymptomatic throughout their lives, about 5% develop ATL during or after middle age and another small population develops HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) and a variety of chronic inflammatory diseases.^(3–7) Several epidemiological risk factors have been suggested to be associated with ATL development, including vertical transmission, gender (greater incidence in males than in females),^(4,8) and increased numbers of abnormal lymphocytes associated with elevated HTLV-1 proviral loads.^(9,10) However, elevation in HTLV-1 proviral loads is also a feature of HAM/TSP patients.^(7,11)

ATL is known to be an immunosuppressive condition.⁽³⁾ Recent reports have shown that ATL cells frequently express Foxp3 and the chemokine receptor CCR4, in addition to CD4 and CD25.^(12–16) These molecules are also expressed in regula-

tory T-cells (Tregs).^(17–20) Although isolated ATL cells do not always exhibit suppressive functions *in vitro*, the common phenotypes shared by ATL cells and Tregs suggest that ATL cells may share a common origin with Tregs, or possess immunoregulatory properties.⁽²¹⁾ General immunosuppression may be present not only in ATL patients, but also in asymptomatic HTLV-1 carriers (ACs) to some extent.^(22,23)

There is a clear difference between ATL and HAM/TSP patients in the host T-cell responses against HTLV-1. Outgrowth of CD8⁺ HTLV-1-specific cytotoxic T-lymphocytes (CTLs) in response to *in vitro* stimulation is frequently found in peripheral blood mononuclear cell (PBMC) cultures from HAM/TSP patients, but rarely observed in those from ATL patients.^(24–26) These CTLs have anti-HTLV-1 effects, as elimination of CD8⁺ cells among PBMCs from HAM/TSP patients induces HTLV-1 expression during subsequent cell culture.^(27,28) HTLV-1 Tax-specific CTL responses are strongly activated in some ATL patients who obtained complete remission after hematopoietic stem cell transplantation (HSCT), but are not observed in the same patients before transplantation.⁽²⁹⁾ These findings suggest that Tax-specific CTLs may play a role in immunosurveillance for HTLV-1 leukemogenesis.

Studies on a rat model have indicated that the otherwise-elevated proviral loads in orally HTLV-1-infected rats could be reduced by restoration of HTLV-1-specific T-cell responses.^(30,31) Furthermore, DNA vaccines or peptide vaccines targeting Tax, the major target antigen recognized by HTLV-1-specific T-cells, can induce antitumor immunity and eradicate HTLV-1-infected lymphomas.^(32,33) These observations imply that antitumor therapeutic vaccines targeting Tax might be promising.

It is important to clarify the immunological status of ACs, since insufficiency in host T-cell responses against HTLV-1 could be an immunological risk factor for ATL. HTLV-1-specific CTL responses are also detectable in ACs.^(34,35) However, because a wide survey for HTLV-1-specific T-cell immunity has never been carried out, the questions of the proportion of ACs with proper levels of immune responses and the possible existence of a population of ACs with insufficient anti-HTLV-1 responses before ATL onset, remain unresolved. One reason for the poor status of such immunological surveys among ACs is the absence of simple methods for measuring HTLV-1-specific T-cell responses, as they are restricted by individual human leukocyte antigens (HLAs).

We recently established a detection system for HTLV-1-specific T-cell responses using recombinant Tax proteins fused to

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glutathione-S-transferase (GST), in which Tax antigens are processed by antigen-presenting cells and capable of stimulating both CD4⁺ and CD8⁺ T-cells among self PBMCs.⁽³⁶⁾ In this study, by using this assay, we analyzed HTLV-1-specific T-cell responses in unselected ACs and smoldering-type ATL (sATL) patients. We examined sATL samples together because their peripheral lymphocyte numbers are in the normal range (< 4000/ μ L) and the prognoses vary among individuals.^(37,38) Here, we demonstrated wide diversity in T-cell response patterns against HTLV-1 in ACs and sATL patients. Among them, we found some individuals exhibiting impaired Tax-specific T-cell responses associated with poor control of HTLV-1 both in ACs and sATL patients.

Materials and Methods

Subjects. A total of 21 ACs, five HAM/TSP patients, four sATL patients, two chronic-type ATL (cATL) patients, and two acute ATL patients in long-term remission (>2 and >5 years) after allogeneic HSCT donated peripheral blood samples after providing written informed consent. PBMCs were isolated by Ficoll-Paque PLUS (GE Healthcare UK, Buckinghamshire, UK) density gradient centrifugation, and either used immediately or stored frozen in liquid nitrogen in Bambanker stock solution (NIPPON Genetics Co., Tokyo, Japan).

Separation of PBMC fractions. CD4⁺ or CD8⁺ cells were depleted from PBMCs by negative selection using 10-fold numbers of Dynabeads M-450 CD4 or CD8 (DynaL Biotec, Oslo, Norway), respectively, according to the manufacturer's instructions. CCR4⁺ cells were depleted from PBMCs using Dynabeads goat antimouse IgG (DynaL Biotec) following incubation with carboxyfluorescein-conjugated anti-CCR4 monoclonal antibody (mAb) for 45 min at 4°C. The resulting contamination by CD4⁺, CD8⁺, or CCR4⁺ cells was between 0.02% and 3.90% of the total lymphocytes, as analyzed by flow cytometry. The PBMC concentrations were adjusted to 1×10^6 cells/mL before depletion, and the resulting CD4⁺, CD8⁺, or CCR4⁺ cell-depleted fractions were resuspended in medium with the same initial volume, irrespective of the remaining cell numbers.

Recombinant proteins and peptides. GST-fusion proteins of HTLV-1 Tax-A, Tax-B, and Tax-C (corresponding to the N-terminal, central, and C-terminal regions of HTLV-1 Tax, respectively) were prepared as described previously.⁽³⁶⁾ Briefly, partially overlapping DNA fragments designated Tax-A, Tax-B, and Tax-C were inserted into pGEX-2T (GE Healthcare UK) to express the corresponding proteins fused to GST. DH5 α competent cells were transformed with these plasmids, and cultured in 2xYT medium supplemented with ampicillin and isopropyl- β -D-thiogalactopyranoside (IPTG) for protein expression. Individual GST-Tax proteins in inclusion bodies were extracted by sonication and purified using Glutathione Sepharose 4B affinity columns (GE Healthcare UK), followed by size exclusion gel chromatography. The purified proteins were stored at -80°C. The concentrations used were 12.5 μ g/mL for GST and 18.75 μ g/mL for a mixture of GST-Tax A, B, and C proteins (6.25 μ g/mL for each protein). In some experiments, a synthetic peptide corresponding to Tax 301-309 (SFHSLHLLF) and Tax 88-96 (KVLTPPITH), representing the major CTL epitopes restricted by HLA-A24 and A11, respectively, was used as an antigen at 10 μ M in PBMC cultures.^(29,39)

Assay for T-cell responses. Whole PBMCs (2×10^5 cells/well) or various cell-depleted PBMC fractions starting from the same number of whole PBMCs were incubated with various antigens in 96-well round-bottom culture plates in duplicate wells. The culture medium was RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL of penicillin, 100 μ g/mL of streptomycin, and 2 mg/mL of sodium bicarbonate. To avoid the potential influence of endotoxin contamination of

the recombinant proteins, 10 μ g/mL of polymyxin B was added to all assays. After 4 days of culture, the supernatants were harvested and stored at -20°C until analysis. The concentrations of interferon (IFN)- γ in the supernatants were measured using a Human IFN- γ ELISA Kit (BioSource, Camarillo, CA, USA) or OptEIA Human IFN- γ ELISA Set (BD Biosciences). The absorbances at 450 nm were measured using a microplate reader and analyzed with the Microplate Manager III software (Bio-Rad Laboratories). In some experiments, a Human Th1/Th2 Cytokine Kit for a Cytokine Beads Assay (CBA) (BD Biosciences) was used to measure various cytokines, including IFN- γ .

Flow cytometry. For cell surface phenotyping, phycoerythrin (PE)-Cy5-conjugated anti-CD4 and PE-Cy5-conjugated anti-CD8 mAbs, carboxyfluorescein-conjugated anti-CCR4 mAb, and appropriate isotype controls were used. Uncultured PBMCs were incubated with these mAbs individually or in combination for 30 min at 4°C, before being washed in phosphate-buffered saline (PBS) containing 1% FBS and fixed with 1% formaldehyde in PBS. For tetramer staining, PBMCs were stained with PE-Cy5-conjugated anti-CD8 mAb for 30 min at 4°C, and then with PE-conjugated HLA-A*1101/Tax88-96, HLA-A*1101/Tax272-280, or HLA-A*2402/Tax301-309 tetramers (National Institute of Allergy and Infectious Diseases Tetramer Facility, Emory University Vaccine Center, Atlanta, GA, USA) for 45 min at 4°C.^(29,39) The samples were analyzed using a FACSCalibur and the CellQuest software (BD Biosciences).

HTLV-1 antibody titer. The titers of HTLV-1-specific antibodies in the plasma samples were determined by the particle agglutination method by using Serodia HTLV-1 (FUJIREBIO, Tokyo, Japan) according to the manufacturer's instructions.

HTLV-1 proviral loads. HTLV-1 proviral loads in PBMCs were measured by quantitative real-time polymerase chain reaction (PCR) with HTLV-1 Tax-specific primers through the clinical diagnostic services of SRL Inc. (Tokyo, Japan) or the Group of Joint Study on Predisposing Factors of ATL Development (JSPFAD, Japan) as described previously.^(40,41) Proviral DNA copy numbers in various fractions of PBMC samples were measured by SYBR Green quantitative real-time PCR methods using Tax-specific primers (forward: 5'-cggataccagctactgtgtttggagactgt-3', reverse: 5'-gagccgataacgcgtccatcgatggggctcc-3') and control beta-globin primers (forward: 5'-acacaactgtgttcactage-3', reverse: 5'-caactcatccagttcacc-3').

Statistical analysis. The Mann-Whitney *U*-test was used to examine the statistical difference in HTLV-1 proviral loads between two groups by using the Graphpad Prism 4 (Graphpad Software). *P*-values <0.05 were considered significant.

Results

Detection of different patterns of Tax-specific T-cell responses in various diseases associated with HTLV-1 infection. In order to obtain typical patterns of T-cell responses detected by the Tax protein-based assay, we examined PBMCs from HTLV-1-infected patients with various clinical conditions (Fig. 1a). Two ATL patients (#37, #253) who had been in long-term complete remission after HSCT showed clear Tax-specific IFN- γ production, only against GST-Tax protein but not against control GST protein. PBMCs from HAM/TSP patients (#254, #259) produced high levels of IFN- γ in response to GST-Tax, but also in the presence of medium alone or the control GST. In contrast, PBMCs from two cATL patients (#227, #249) showed very weak responses to any stimulation. Although the PBMCs from uninfected individuals showed low levels of background responses that might involve macrophages or natural killer cells, the levels of IFN- γ production in cATL samples were even lower than the background responses.

HTLV-1-infected cells have been reported to express the chemokine receptor CCR4 frequently.⁽¹³⁾ As shown in Fig. 1(b),

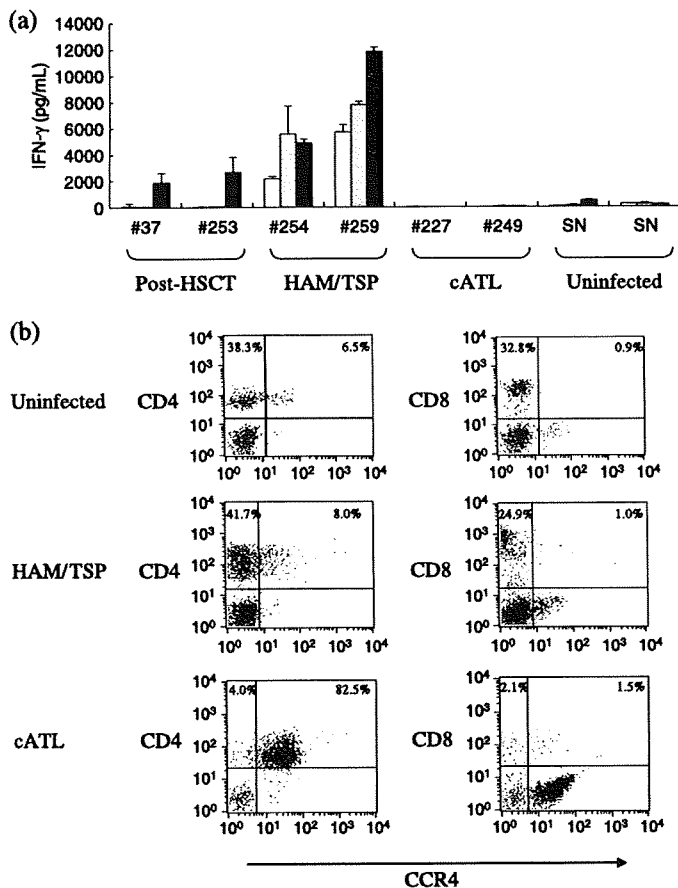


Fig. 1. Different patterns of Tax-specific T-cell responses in various diseases associated with human T-cell leukemia virus type-1 (HTLV-1) infection. (a) Peripheral blood mononuclear cells (PBMCs) (2.0×10^5 cells/well) from two post-hematopoietic stem cell transplantation (HSCT) adult T-cell leukemia (ATL) patients in long-term remission (#37, #253), two HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) patients (#254, #259), two chronic-type ATL (cATL) patients (#227, #249), and two seronegative uninfected subjects (SN) were cultured alone (open bars), with glutathione-S-transferase (GST) (gray bars) or with a mixture of GST-Tax proteins (black bars) in a total volume of 200 μ L of medium/well for 4 days. Interferon (IFN)- γ in the supernatants was measured by enzyme-linked immunosorbent assay. The results represent the mean \pm SD of duplicate wells. (b) Uncultured PBMCs from an uninfected subject, a HAM/TSP patient (#255), and a cATL patient (#227) were stained with carboxyfluorescein-conjugated CCR4 monoclonal antibody (mAb) together with phycoerythrin (PE)-Cy5-labeled CD4 (left) or CD8 (right) mAbs. Values indicate the percentages of positive cells in each quadrant analyzed by flow cytometry for a total of 10 000 gated lymphocytes.

PBMCs of an uninfected individual contained 44.8% CD4⁺ cells and 6.5% CD4⁺CCR4⁺ cells. In the cATL patient (#227), the proportion of CD4⁺CCR4⁺ cells was markedly elevated, consistent with previous reports.⁽¹³⁾ PBMCs from this cATL patient contained 86.5% CD4⁺ cells and 82.5% CD4⁺CCR4⁺ cells. In contrast, the proportion of CD4⁺CCR4⁺ cells in the PBMCs from the HAM/TSP patient (#255) was comparable with that in the uninfected individual.

Since the proportion of HTLV-1-infected cells in the peripheral lymphocytes was elevated in cATL, the poor T-cell responses in these samples may be partly explained by the relative scarcity of normal lymphocytes and antigen presenting cells. Such leukemic PBMCs are not suitable for evaluation with a protein-based assay, and we only used PBMCs with lymphocyte numbers within the normal range hereafter.

Diversity in Tax-specific T-cell responses in ACs and sATL patients.

Using the GST-Tax protein-based assay system, we next examined the T-cell responses of 21 ACs and four sATL patients. One of the sATL patients (#213) had polyclonal HTLV-1-infected cells but was categorized as sATL because of the number of abnormal lymphocytes was >5%. The hematologic and virologic profiles of the donors tested are summarized in Table 1. The numbers of peripheral lymphocytes of all ACs and sATL patients were within the normal range.

The IFN- γ production levels by whole PBMCs from the ACs and sATL patients in the absence or presence of GST or GST-Tax proteins are shown in Fig. 2(a). The levels and patterns of IFN- γ production varied widely among individuals. We divided the samples into two groups, according to Tax-specificity of the responses. This was assessed by calculating the ratio of IFN- γ produced in response to GST-Tax proteins divided by IFN- γ produced in response to GST alone for each sample (Tax/GST ratios). Tax-specific patterns were observed in samples from seven ACs (#228, #232, #238, #251, #258, #264, #277) and one sATL patient (#265) who had a localized skin lesion but was without apparent abnormal lymphocytes in the peripheral blood. The remaining samples showed less-specific patterns of IFN- γ production due to increased non-specific IFN- γ production or low IFN- γ production to any stimulation.

We compared the individual proviral loads between the two groups with Tax-specific and less-specific T-cell responses (Fig. 2b,c). The group with clear Tax-specific T-cell response possessed significantly lower proviral loads than the less-specific response group either in the total of ACs and sATL patients ($P = 0.0107$) or in ACs alone ($P = 0.0260$). The three sATL patients (#213, #252, #220) with highest proviral loads among those tested (>100 copies/1000 PBMCs), and several ACs with moderate levels of proviral loads (10–100 copies/1000 PBMCs) were in the less-specific response group. However there were also some ACs who exhibited low T-cell responses and low proviral loads (<10 copies/1000 PBMCs).

Involvement of CCR4⁺ cells in high background IFN- γ production by PBMCs from HAM/TSP patients. We next assessed what cells in the PBMCs were responsible for the high background IFN- γ production observed in HAM/TSP patients by depleting CD4⁺, CD8⁺, or CCR4⁺ cells from PBMCs prior to the assay. In order to see the effects of cell depletion, the cell concentrations were equalized before cell depletion, and the resulting cell fractions were resuspended in the same volume, irrespective of the final cell numbers. The results are shown in Fig. 3(a). Whole PBMCs from a HAM/TSP patient (#255) showed high levels of non-specific IFN- γ production with or without GST-Tax proteins. When the CD4⁺ cells were depleted, decreased but significant levels of IFN- γ were only produced in response to GST-Tax proteins. CD8⁺ cell depletion did not markedly alter the non-specific responses. CCR4⁺ cell depletion reduced the non-specific responses, but retained Tax-specific IFN- γ production at a level comparable to that of whole PBMCs. These results indicated that CCR4⁺ PBMC population from this patient mainly contributed to the non-specific responses in the assay, and that the effector of the Tax-specific responses was included in the CCR4⁺ population that contained CD4⁺ and CD8⁺ cells.

We also measured the HTLV-1 p19 levels in the supernatants of these PBMC cultures after 7 days, and found that HTLV-1 p19 became detectable only in the CD8⁺ cell-depleted fraction but not in the whole or CD4⁺ cell-depleted or CCR4⁺ cell-depleted fractions (Fig. 3b). Since the starting PBMC number before cell-depletion in each fraction was equivalent, the increase in p19 production in the CD8⁺ cell-depleted fraction indicated that CD8⁺ cells served as suppressors of viral expression in culture.

Reduction in the non-specific IFN- γ production by CCR4⁺ cell-depletion and enhancement in p19 production by CD8⁺

Table 1. Blood samples from asymptomatic HTLV-1 carriers (ACs) and smoldering ATL (sATL) patients tested

ID	Age	Sex	Clinical status	WBC/ μ L	Lymphocyte (%)	Abnormal lymphocyte (%)	Provirus DNA copies/1000 PBMCs	Plasma HTLV-1 antibody titer [†]
#211	20s	F	AC	4500	34	0	33	>8192
#213	50s	F	sATL [‡]	7500	36	8	200	4096
#215	20s	M	AC	8000	55	0	13	2048
#216	70s	F	AC	4200	31	3	39	>8192
#217	70s	F	AC	6800	51	0	14	>8192
#218	50s	F	AC	7500	55	0	<1	1024
#219	60s	F	AC	7200	43	0	<1	1024
#220	50s	M	sATL	4800	28	13	277	>8192
#223	60s	M	AC	4200	26	0	<1	1024
#226	50s	M	AC	5700	38	0	28	2048
#228	60s	M	AC	5900	59	0	<1	256
#232	30s	F	AC	5800	46	0	<1	>8192
#236	30s	F	AC	6500	39	0	22	>8192
#238	60s	F	AC	5700	51	0	2	1024
#243	50s	F	AC	4100	58	0	3	2048
#244	50s	F	AC	4900	27	3	63	1024
#245	40s	F	AC	5000	46	1	58	1024
#246	50s	M	AC	4600	37	0	2	>8192
#251	60s	M	AC	4800	50	0	2	2048
#252	50s	F	sATL	4100	38	11	207	>8192
#258	60s	M	AC	6400	30	3	8	256
#263	60s	M	AC	3900	49	2	1	2048
#264	50s	F	AC	3000	37	0	<1	256
#265	60s	F	sATL [§]	4700	38	1	2	2048
#277	50s	F	AC	4100	29	0	<1	256

[†]Measured by a particle agglutination method.

[‡]Diagnosed as sATL because of increased abnormal lymphocyte number, although the infected cells were polyclonal.

[§]Diagnosed as sATL because of the presence of skin lesions.

HTLV-1, human T-cell leukemia virus type-1; ACs, asymptomatic HTLV-1 carriers; ATL, adult T-cell leukemia; sATL, smoldering-type ATL; PBMC, peripheral blood mononuclear cell; WBC, white blood cell.

cell-depletion were also observed in the samples from two other HAM/TSP patients (#288 and #290) (Fig. 3c,d).

The prevalence of HTLV-1-infected cells in the CCR4⁺ cell fraction was further confirmed by a real-time PCR method in uncultured PBMCs from subjects #255 (HAM/TSP) and #211 (AC), although there were still detectable levels of proviruses left in the CCR4⁺ cell-depleted fractions (Fig. 3e).

Poor effects of CCR4⁺ cell depletion on Tax-specific T-cell responses in PBMC cultures from some ACs and sATL patients. We then assessed whether ACs and sATL samples with apparently less-specific responses actually possessed Tax-specific responses, similarly to the samples from HAM/TSP patients. We examined the effects of CCR4⁺ cell-depletion on Tax-specific T-cell responses and the effects of CD8⁺ cell-depletion on HTLV-1 p19 production in the PBMC samples from several available ACs and sATL patients with less-specific responses and elevated proviral loads (Fig. 4).

PBMCs from AC #238 were examined as a control that exhibited clear Tax-specific T-cell responses. As shown in Fig. 4, the Tax-specific pattern in this sample was not altered by CCR4⁺ cell depletion. HTLV-1 p19 production in the PBMC culture of #238 was very low, consistent with the low proviral load in this subject.

In the sample from AC #211, which had a moderately elevated level of proviral load (10–100 copies/1000 PBMCs), whole PBMCs showed a less-specific response pattern with high background IFN- γ production. This was improved to a Tax-specific pattern by depletion of CCR4⁺ cells. HTLV-1 p19 production in CD8⁺ cell-depleted fraction from this subject was 10 times higher than that in whole PBMCs, indicating that CD8⁺ cells efficiently suppressed HTLV-1 p19 production in culture.

The whole PBMCs from AC #244, possessing a proviral load similar to that of subject #211, also showed a less-specific

response pattern. CCR4⁺ cell depletion reduced spontaneous IFN- γ production but did not improve the difference between the responses to control GST and GST-Tax by much. Whole PBMCs from subject #244 spontaneously produced a substantial amount of HTLV-1 p19, which was slightly enhanced by depletion of CD8⁺ cells.

PBMCs from sATL patient #213, who had a high proviral load (>100 copies/1000 PBMCs), spontaneously produced significant levels of IFN- γ irrespective of the stimulation. Depletion of CCR4⁺ cells slightly reduced the general levels of IFN- γ production, but did not improve the non-specific pattern. Unfractionated PBMCs from this subject produced high levels of HTLV-1 p19, and CD8⁺ cell depletion did not alter the levels of HTLV-1 p19 at all.

PBMCs from sATL patient #252, who also had a high proviral load, showed very low levels of IFN- γ production in response to GST or GST-Tax proteins. CCR4⁺ cell depletion from these PBMCs did not improve the scale or specificity of the IFN- γ production by these PBMCs. Whole PBMCs from subject #252 spontaneously produced a significant amount of HTLV-1 p19, which was slightly increased by CD8⁺ cell depletion.

Thus, CCR4⁺ cell depletion revealed Tax-specific T-cell responses in some, but not all, samples with less-specific responses. Samples that did not show a Tax-specific pattern after CCR4⁺ cell depletion were associated with insufficient control of HTLV-1 production in culture.

Unresponsiveness of Tax-specific CTLs in PBMCs from an AC. Finally, we investigated whether the five subjects shown in Fig. 4 had Tax-specific CTLs, using a flow cytometric analysis with tetramers. As shown in Fig. 5(a), the PBMCs from AC #238 contained HLA-A*1101/Tax88-96 tetramer-binding CTLs,

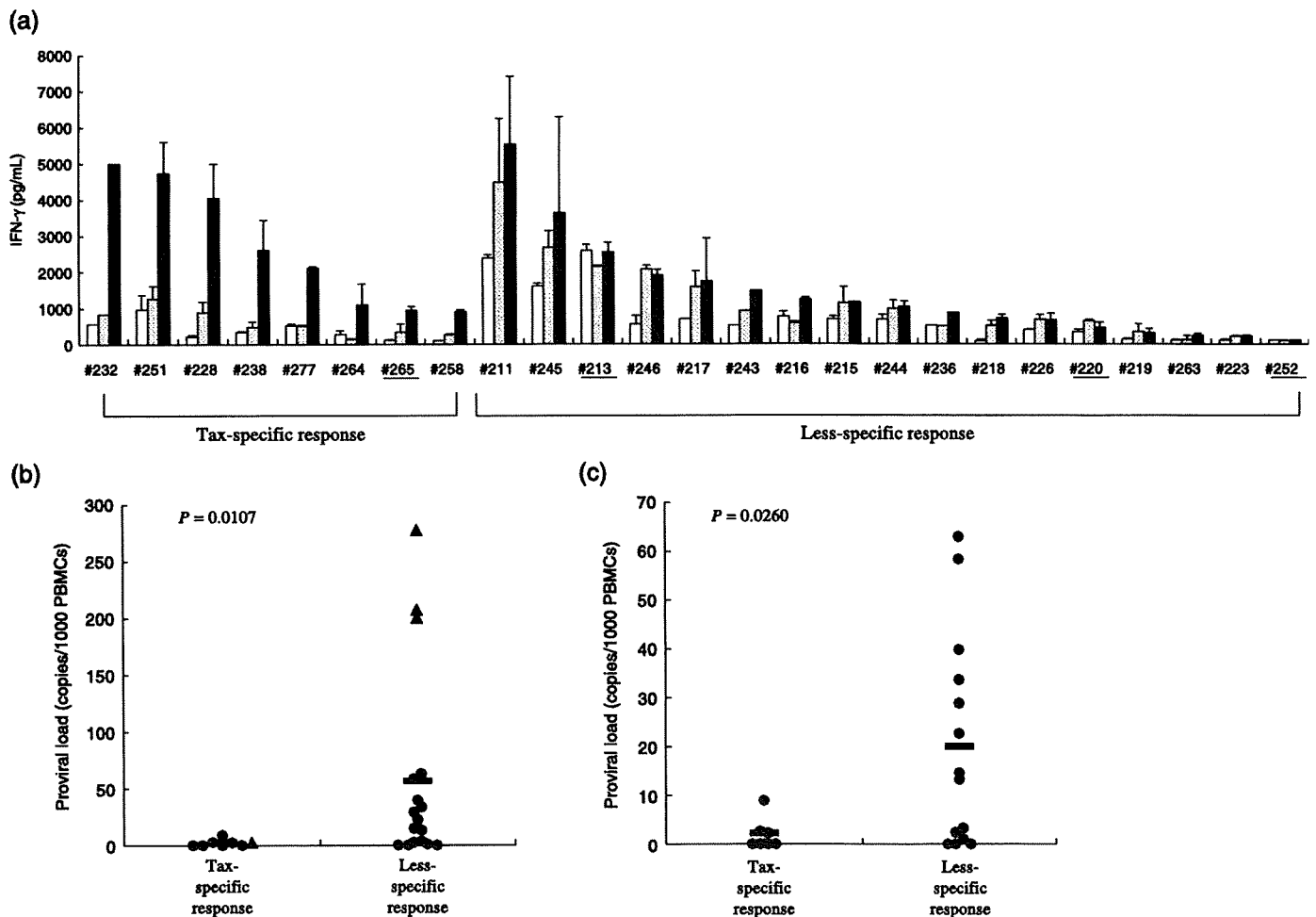


Fig. 2. Diversity in Tax-specific T-cell responses among asymptomatic human T-cell leukemia virus type-1 (HTLV-1) carriers (ACs) and smoldering-type adult T-cell leukemia (sATL) patients. (a) Peripheral blood mononuclear cells (PBMCs) isolated from 21 ACs and 4 sATL patients (underline) were incubated alone (open bars), with glutathione-S-transferase (GST) (gray bars) or with a mixture of GST-Tax proteins (black bars) for 4 days, and interferon (IFN)- γ amounts in the supernatants were measured by enzyme-linked immunosorbent assay and a Cytokine Beads Assay in part. The results were divided into Tax-specific or less-specific response groups depending on the ratios of anti-GST-Tax/anti-GST IFN- γ production were more than 2.5 or not, and then aligned in the order of absolute values of IFN- γ production against GST-Tax proteins in each group. (b, c) HTLV-1 proviral loads of all the ACs and sATL patients tested (b) or ACs alone (c) were indicated. The mean values of proviral loads (bars) in Tax-specific and less-specific response groups were 2.0 and 56.9 copies/1000 PBMCs, respectively ($P = 0.0107$) in (b), and 1.9 and 20.1 copies/1000 PBMCs, respectively ($P = 0.0260$) in (c). AC, closed circle; sATL: closed triangle.

and ACs #211 and #244 were positive for HLA-A*2402/Tax301-309 tetramer-binding CTLs. In the PBMCs from subjects #213 and #252 (sATL), tetramer-binding cells were not detectable, although these subjects were positive for HLA-A24 and A11, and HLA-A11, respectively (data not shown).

We examined the activity of Tax-specific CTLs by stimulating CCR4⁺ cell-depleted PBMCs from three ACs, which contained tetramer-binding CTLs, with oligopeptides corresponding to the tetramers in culture. As shown in Fig. 5(b), CCR4⁺ cell-depleted PBMCs from subjects #238 and #211 produced IFN- γ in response to Tax88-96 and Tax301-309 peptides, respectively, whereas those from subject #244 did not respond to Tax 301-309 peptides. These results are consistent with the results of the assay using GST-Tax proteins and suggest that Tax-specific CTLs in subject #244 might be in an anergic state.

Discussion

In the present study, we investigated HTLV-1-specific T-cell responses in unselected HTLV-1-infected individuals without any bias of HLAs by using a GST-Tax protein-based assay

system. This assay detected clear Tax-specific responses in ATL patients in complete remission, less-specific responses in HAM/TSP patients, and very weak responses in cATL patients. Interestingly, all of these patterns were observed in PBMCs from ACs and sATL patients, indicating wide diversity in Tax-specific T-cell responsiveness at these stages. Most importantly, some individuals at these stages exhibited impaired Tax-specific T-cell responses and elevated proviral loads, indicating that such conditions are present before an overt leukemia stage.

High background responses have been an obstacle to evaluate T-cell responses in HTLV-1-infected individuals. This may be similar to the phenomenon known as 'spontaneous proliferation' of PBMCs from HAM/TSP patients and ACs.⁽⁴²⁻⁴⁴⁾ In our study, depletion of CCR4⁺ cells containing HTLV-1-infected cells from the PBMCs reduced this spontaneous response and revealed a Tax-specific T-cell response pattern in the samples from several HAM/TSP patients and ACs with strong non-specific responses (Figs 3 and 4). The sources of spontaneous IFN- γ production may be either HTLV-1-specific T-cells reacting with coexisting infected cells in culture, or HTLV-1-infected cells themselves, or both.^(45,46) As CCR4⁺ cell depletion from the PBMCs reduced the

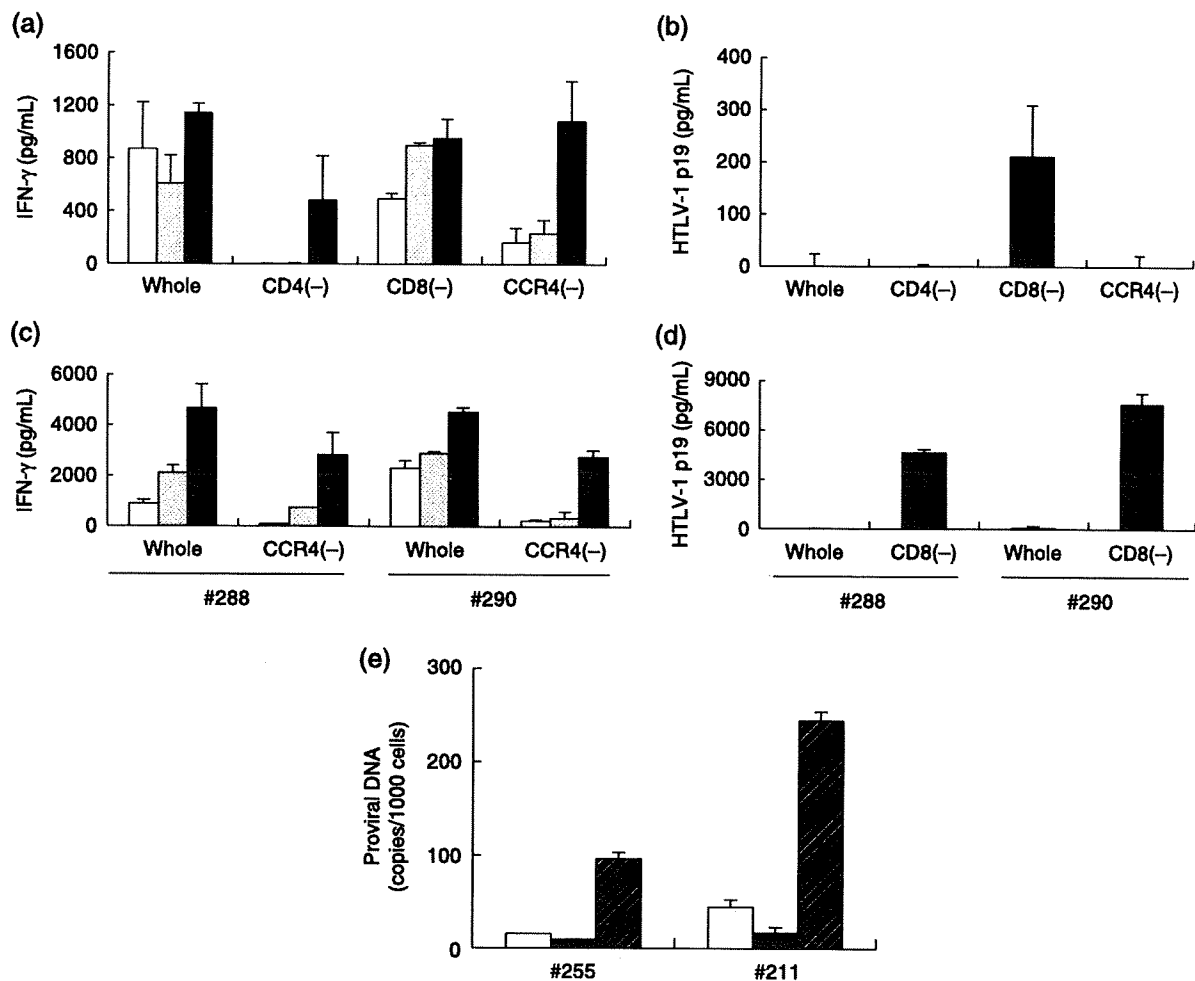


Fig. 3. Involvement of CCR4⁺ cells in high background interferon (IFN)- γ production by peripheral blood mononuclear cells (PBMCs) from human T-cell leukemia virus type-1 (HTLV-1)-associated myelopathy/tropical spastic paraparesis (HAM/TSP) patients. PBMCs from a HAM/TSP patient (#255) were equally divided into four fractions. One fraction was used directly (whole) and the other three fractions were used after depletion of CD4⁺ [CD4(-)], CD8⁺ [CD8(-)], or CCR4⁺ [CCR4(-)] cells. The cell numbers in each fraction were 2.0×10^5 , 0.8×10^5 , 1.1×10^5 , and 1.3×10^5 cells/well, respectively. (a) The fractions were cultured alone (open bars), with glutathione-S-transferase (GST) (gray bars), or with a mixture of GST-Tax proteins (black bars) for 4 days. IFN- γ in the supernatants was measured by enzyme-linked immunosorbent assay (ELISA). (b) The levels of HTLV-1 p19 antigen in the culture supernatants of the PBMC fractions in the absence of any stimulation were measured by ELISA at 7 days after the initiation of culture. The results represent the mean \pm SD of duplicate wells. (c, d) Whole and fractionated PBMCs from two other HAM/TSP patients (#288 and #290) were similarly examined for IFN- γ production for 4 days against medium (open bars), GST (gray bars), or GST-Tax proteins (black bars) (c), and HTLV-1 p19 production for 7 days of culture (d). The cell numbers of whole, CCR4(-), and CD8(-) PBMCs in each well were 2.0×10^5 , 1.7×10^5 , and 1.2×10^5 for patient #288, 2.1×10^5 , 0.9×10^5 , and 0.9×10^5 for patient #290, respectively. (e) HTLV-1 provirus numbers (copies/1000 cells) in whole (open bars), CCR4⁺ cell-depleted (closed bars), and CCR4⁺ (hatched bars) PBMC fractions before culture from subjects #255 (HAM/TSP) and #211 (AC) were measured by real-time polymerase chain reaction methods. The results represent the mean \pm SD of duplicate samples.

number of HTLV-1-infected cells without losing CD8⁺ cells, T-cell response against exogenously added Tax proteins became clearly detectable. CD8⁺ cells in these PBMCs effectively suppressed HTLV-1 p19 production in culture, presumably by killing infected cells.

Although the number of samples available for detailed analysis was limited, PBMCs from at least two sATL patients (#213 and #252) with high proviral loads (>100 copies/1000 cells) exhibited impaired T-cell responses even after CCR4⁺ cell depletion. CD8⁺ cells of these subjects had poor effects on HTLV-1 p19 production (Fig. 4). This was not attributable to the scarcity of non-ATL cells, as these PBMCs contained 16.5% and 12.0% CD8⁺ cells, respectively. HTLV-1-infected cells were the most likely source of IFN- γ production in whole and CCR4⁻ PBMC cultures from subject #213, as HTLV-1 p19 was detected even in the CCR4⁺

cell-depleted fraction during further culture (data not shown), presumably because of expansion of the remaining infected cells in the initially CCR4⁻ cell culture, in the absence of any anti-Tax response. HTLV-1-infected cells from subject #252 expressed viral antigens but not IFN- γ .

ACs with less-specific T-cell responses and moderate levels of proviral loads (10–100 copies/1000 PBMCs) seemed to be a mixed population in regards to Tax-specific response. CCR4⁺ cell-depleted PBMCs showed Tax-specific T-cell response in subject #211, but did not markedly do so in subject #244. Similarly, CD8⁺ cell-mediated control on HTLV-1 p19 production in #211 was more efficient than in #244. The results of peptide stimulation also indicated impaired status of Tax-specific response in #244 (Figs 4 and 5). However, the level of p19 production in the CD8⁺ cell-depleted cell fraction was even higher

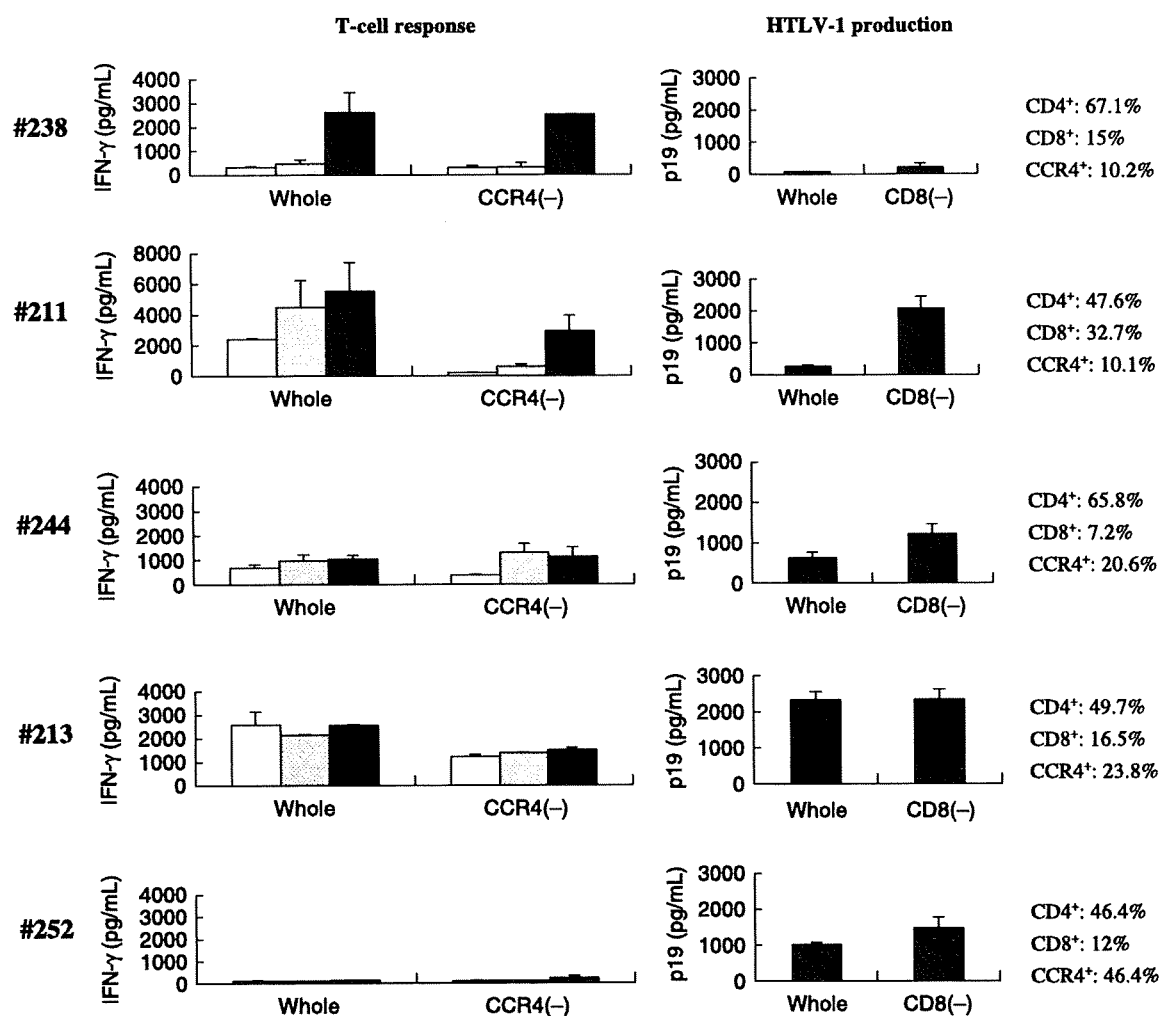


Fig. 4. Tax-specific T-cell response associated with CD8⁺ cell-mediated control of human T-cell leukemia virus type-1 (HTLV-1) production in peripheral blood mononuclear cell (PBMC) cultures from asymptomatic HTLV-1 carriers (ACs) and smoldering-type adult T-cell leukemia (sATL) patients. Whole and CCR4⁺ cell-depleted [CCR4(-)] PBMC fractions prepared from subjects #238 (AC), #211 (AC), #244 (AC), #213 (sATL), and #252 (sATL) were cultured alone (open bars), with glutathione-S-transferase (GST) (gray bars), or with a mixture of GST-Tax proteins (black bars) for 4 days. Interferon (IFN)- γ produced in the supernatants was measured by enzyme-linked immunosorbent assay (left panels). The levels of HTLV-1 p19 production in whole and CD8⁺ cell-depleted [CD8(-)] PBMC cultures from the same donors were measured on day 7 (right panels). The ratios of CD4⁺, CD8⁺, and CCR4⁺ cells (%) in uncultured PBMCs from each subject were indicated at right, as determined by flow cytometry.

in #211. Nevertheless, #211 and #244 had comparable levels of proviral loads (33 and 63 copies/1000 PBMCs, respectively). These results suggested that proviral load might represent equilibrium between growth capabilities of infected cells and the T-cell response against them.

It is of note that some ACs (#219, #223, #263) exhibited very low anti-Tax T-cell responses that were not improved by CCR4⁺ cell depletion, and also had low proviral load. HTLV-1-infected cells in these individuals might have low proliferative abilities. Since these subjects maintained considerable levels of anti-HTLV-1 antibodies in the plasma (1:1024–2048, Table 1), poor T-cell responsiveness cannot be explained merely by the scarcity of viral antigens. The mechanisms of general and/or HTLV-1-specific immune suppression in HTLV-1 infection remain to be clarified.

In conclusion, ACs and sATL patients consist of diverse subpopulations with different patterns of T-cell responses against HTLV-1, containing a subpopulation exhibiting impaired HTLV-1-specific T-cell responses and insufficient control of HTLV-1-infected cells. This strongly suggests that impairment

of HTLV-1-specific T-cell response is not a consequence of advanced ATL but present at early stages or even before the disease onset. Although there must be multiple steps towards ATL development, impaired HTLV-1-specific T-cell response could be one of the underlying conditions that allows expansion of HTLV-1 infected cells *in vivo*. Reactivation of HTLV-1-specific T-cell response by immunotherapeutic strategies such as vaccines in the subpopulation with insufficient T-cell response might contribute to the recovery of host control on HTLV-1-infected cells *in vivo*.

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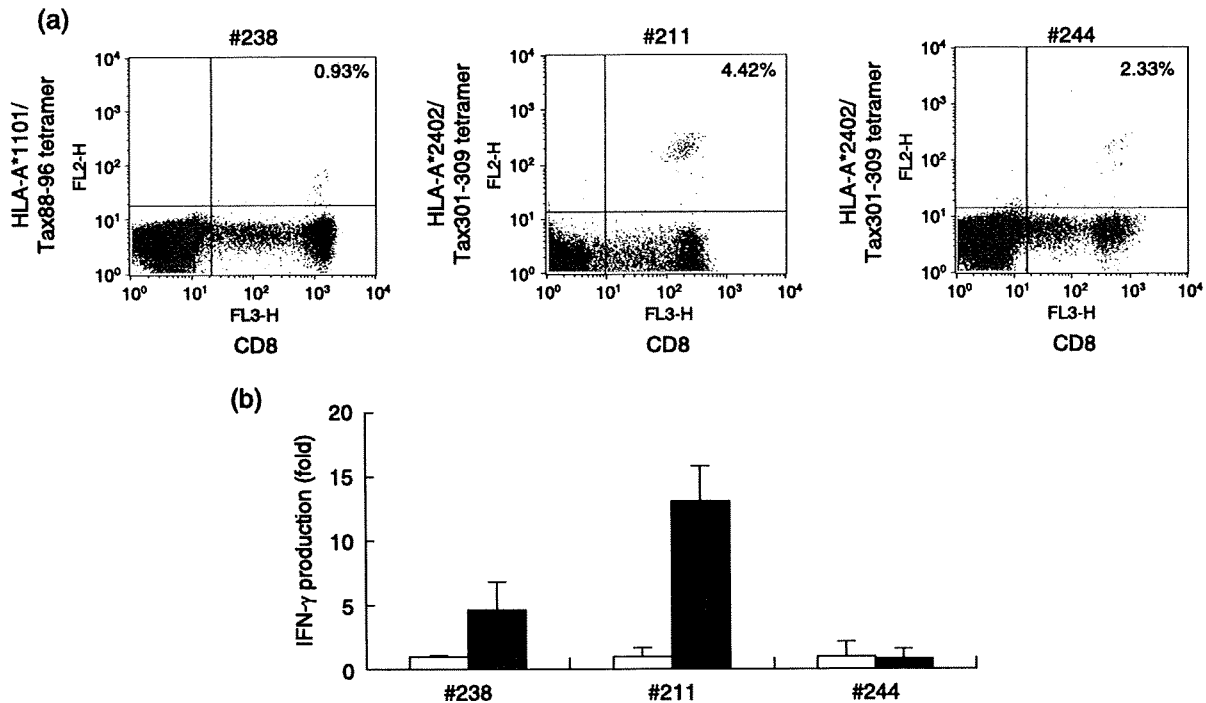


Fig. 5. Discrepancy between the presence of Tax-specific cytotoxic T-lymphocytes (CTLs) and interferon (IFN)- γ production in peripheral blood mononuclear cells (PBMCs) from asymptomatic human T-cell leukemia virus type-1 (HTLV-1) carriers (ACs). (a) Uncultured PBMCs from indicated subjects were stained with phycoerythrin (PE)-Cy5-conjugated CD8 monoclonal antibody (mAb) together with PE-conjugated HLA-A*1101/Tax88-96 tetramers for subject #238, and HLA-A*2402/Tax301-309 tetramers for subjects #211 and #244. The percentages of tetramer⁺ cells among CD8⁺ cells are indicated, as analyzed by flow cytometry. (b) CCR4⁺ cell-depleted PBMCs from the same subjects were incubated with control dimethylsulfoxide (DMSO) (open bars) or 10 μ M of Tax peptides (closed bars) for 4 days, and the amounts of IFN- γ in the supernatants were measured by enzyme-linked immunosorbent assay. Tax peptides used were Tax 88-96 for subject #238 and Tax 301-309 for subjects #211 and #244. The values represent folds of IFN- γ production against control DMSO, and indicated as the mean \pm SD of duplicate samples.

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