CASE REPORT

A 65-YEAR-OLD JAPANESE woman complained of persistent fatigue and weight loss of 8 kg in 2 weeks. The laboratory findings showed that her white blood cell count was elevated to 16 800/μL, of which abnormal lymphocytes accounted for 18%, and seropositivity for human T-cell leukemia virus type-1 (HTLV-1). Monoclonal integration of HTLV-1 was revealed by Southern blotting of DNA from peripheral blood. She was diagnosed as ATL, chronic type, in April 2004. Since then, she had experienced repeating infectious episodes and systemic lymph node swelling. On April 2005, she began to receive systemic chemotherapy composed of sobuzoxane (400 mg/day), etoposide (25 mg/day) and prednisolone (10 mg/day) p.o. twice a week because of disease progression to acute type which was accompanied by new ATL involvement in her right breast region and right axilla lymphadenopathy. As her disease was refractory to this regimen, she received four cycles of THP-COP regimen (cyclophosphamide, pirarubicin, vincristine and prednisolone) from August 2005 through October 2005 (Fig. 1a). She achieved a partial response and was followed up without subsequent chemotherapy including steroids for 1.4 years, but her disease progressed with markedly increased ATL cells

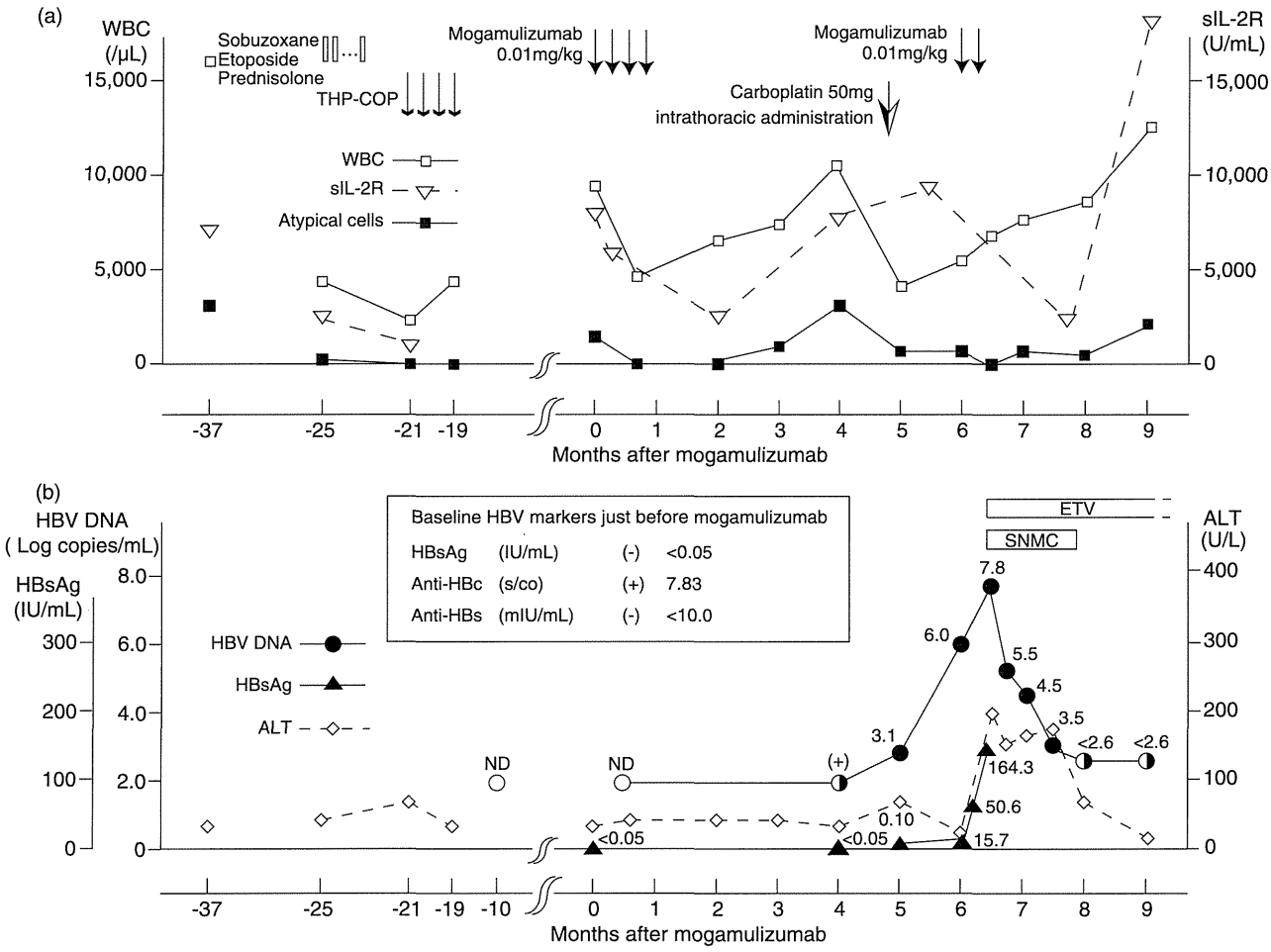

Figure 1 Clinical course and kinetics of HBV markers in a patient with adult T-cell leukemia–lymphoma before and after the anti-CC chemokine receptor 4 monoclonal antibody mogamulizumab treatment. ALT, alanine aminotransferase; anti-HBc, antibody against hepatitis core antigen; anti-HBs, antibody against hepatitis surface antigen; ETV, entecavir; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; ND, not detectable; sIL-2R, soluble interleukin-2 receptor; SNMC, Stronger Neo-Minophagen C; THP-COP, cyclophosphamide, pirarubicin, vincristine and prednisolone; WBC, white blood cells.

and an elevated lactate dehydrogenase value in peripheral blood in March 2007. She was enrolled into a phase 1 study for dose-finding of the anti-CCR4 antibody, mogamulizumab,[9] and received this antibody at 0.01 mg/kg by i.v. infusion once a week for 4 weeks (Fig. 1a, thin arrows). No combination of other anticancer chemotherapy was performed and no steroids were given, except for allergic prophylaxis. She was HBsAg negative at baseline on enrollment in the phase 1 study. Retrospective analysis using preserved samples revealed that she was anti-hepatitis B core positive, anti-hepatitis B surface negative, and HBV DNA was undetectable at baseline, attributed to previously resolved HBV infection (Fig. 1b). After mogamulizumab, ATL cells disappeared immediately from the peripheral blood, the nodal disease partially improved and no severe adverse event was observed. However, at 9 weeks after the end of mogamulizumab, the ATL cells reappeared in the peripheral blood. Furthermore, she received intrathoracic administration of carboplatin for involvement of ATL (right pleural effusion) in August 2007. During the next month, her cervical lymph nodes enlarged rapidly and we decided to re-treat with mogamulizumab because of the previous efficacy and safety of this antibody. After two doses of mogamulizumab, she was hospitalized in emergency due to ALT flare on October 2007 (Fig. 1b, 6.5 months after mogamulizumab). The laboratory findings showed that HBsAg had become positive and her HBV DNA levels increased to 7.8 log copies/mL, suggesting that the liver damage was caused by HBV reactivation. Entecavir (0.5 mg/day) and Stronger Neo-Minophagen C (40 mg/day) were given immediately and hepatitis B improved gradually (with ALT peaking at 205 U/L) for approximately 2 months. Entecavir was effective in controlling hepatitis B, and was continued for 1.5 years without any severe adverse events.

DISCUSSION

THE PRESENTED CASE is the first report of HBV reactivation in a HBsAg negative patient receiving mogamulizumab. We analyzed preserved samples retrospectively and showed that her liver damage was attributable to HBV reactivation. Also, those analyses showed the following important findings regarding the kinetics of HBV DNA during reactivation: First, HBV DNA was undetectable at baseline, before administration of mogamulizumab. Elevated HBV DNA levels were detectable, in which polymerase chain reaction (PCR) signals were only detected 10 weeks prior to the development of hepatitis and 13 weeks after the end of this antibody treatment. HBV DNA levels, measured by PCR-based assay, increased rapidly from 3.1 to 6.0 log copies/mL for 1 month and, finally, up to 7.8 log copies/mL. Second, the elevated HBV DNA levels preceded the detection of HBsAg (Architect Assay; Abbott Laboratories, North Chicago, IL, USA) by 1 month. Third, the patient was infected with HBV genotype C with a point mutation in the precore regions (G1896A) which might have been associated with the rapidly increasing kinetics of HBV DNA levels in this case.

How was the anti-CCR4 antibody mogamulizumab involved in the HBV reactivation? CCR4 is a chemokine receptor expressed on T-helper type 2 and regulatory T cells, and is thought to carry an important role in maintaining the balance of the human immune system.[7-9] It is difficult to demonstrate how mogamulizumab caused HBV reactivation in this case; the reduction of CCR4-expressing cells following this antibody treatment might have been associated with imbalance of antiviral immunity, resulting in the development of hepatitis due to HBV reactivation. Other than mogamulizumab, the intrathoracic administration of carboplatin and the ATL disease progression are considered to be factors potentially influencing HBV reactivation. However, retrospective analysis showed that HBV DNA levels were detectable in the peripheral blood before administration of carboplatin, suggesting that carboplatin is unlikely to have been mainly involved in the HBV reactivation. ATL is often diagnosed with a compromised immune system, and the disease progression might have been associated with reactivation of the virus. Interestingly, the timing of the rapid increase in ATL cells in the peripheral blood coincided with that of HBV replication in this case. However, disease progression of ATL alone is very unlikely to have caused the HBV reactivation because reactivation did not occur during the previous ATL progression.

To prevent hepatitis due to HBV reactivation, what lesson can we learn from this case? HBV reactivation following immunosuppressive therapy may lead to acute liver failure or fulminant hepatitis, and the patients have poor prognosis regardless of intensive antiviral treatment.[11,12] For preventing HBV reactivation in patients with previously resolved HBV infection, monitoring of HBV DNA-guided preemptive antiviral therapy is recommended in some guidelines,[13,14] however, the evidence of optimal interval of HBV DNA monitoring is limited. Most recently, monthly monitoring of HBV DNA was shown to effectively prevent HBV reactivation in patients with previously resolved HBV

infection who received rituximab plus steroids containing chemotherapy.[15] The kinetics of HBV reactivation in this case strongly suggested that monthly monitoring of HBV DNA could prevent hepatitis even in such a highly replicative clone with a precore mutation.

In summary, we first reported HBV reactivation following treatment with the anti-CCR4 antibody mogamulizumab and revealed the detailed kinetics of HBV replication during reactivation. Further well-designed studies are warranted to address the mechanisms of HBV reactivation and to establish standard management for reactivation in patients with previously resolved HBV infection, following anticancer chemotherapy and immunosuppressive therapy.

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Regular Article

LYMPHOID NEOPLASIA

Development of a novel redirected T-cell–based adoptive immunotherapy targeting human telomerase reverse transcriptase for adult T-cell leukemia

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Key Points

- The efficacy and safety of a novel redirected T-cell–based adoptive immunotherapy targeting hTERT for patients with adult T-cell leukemia.
- hTERT-specific T-cell receptor gene-transduced $CD8^+$ T cells lyse ATL cells, but not normal cells, both in vitro and in vivo.

Although adult T-cell leukemia (ATL) has a poor prognosis, successful allogeneic hematopoietic stem cell transplantation (allo-HSCT) in some cases suggests that a cellular immune-mediated strategy can be effective. So far, however, no effective target for anti-ATL immunotherapy has been defined. Here we demonstrated for the first time that human telomerase reverse transcriptase (hTERT) is a promising therapeutic target for ATL, and we developed a novel redirected T-cell–based immunotherapy targeting hTERT. *hTERT* messenger RNA was produced abundantly in ATL tumor cells but not in steady-state normal cells. Rearranged human leukocyte antigen-A*24:02 (HLA-A*24:02) –restricted and $hTERT_{461-469}$ nonameric peptide-specific T-cell receptor (TCR) α/β genes were cloned from our previously established cytotoxic T lymphocyte clone (K3-1) and inserted into a novel retroviral TCR expression vector encoding small interfering RNAs for endogenous *TCR* genes in redirected T cells (hTERT-*siTCR* vector). Consequently, allogeneic or autologous gene-modified $CD8^+$ T cells prepared using the hTERT-*siTCR* vector successfully killed ATL tumor cells, but not normal cells including steady-state hematopoietic progenitors, in an HLA-A*24:02-restricted manner both in vitro and in vivo. Our experimental observations support the development of a novel hTERT-targeting redirected T-cell–based adoptive immunotherapy for ATL patients, especially those for whom suitable allo-HSCT donors are lacking. (*Blood.* 2013;121(24):4894-4901)

Introduction

Adult T-cell leukemia (ATL) is an aggressive peripheral T-cell neoplasm caused by human T-cell lymphotropic virus I (HTLV-I).[1] It is estimated that there are more than 1 million HTLV-I carriers in Japan, about 5% of whom develop ATL at around 60 years of age or older.[2] Because ATL tumor cells soon acquire chemotherapy resistance and compromise host immunity against infectious pathogens, ATL has a poor prognosis.[3] Although most ATL patients are ineligible for allogeneic hematopoietic stem cell transplantation (allo-HSCT) because of advanced age, age-related comorbidity, or lack of suitable donors,[4] the number of ATL patients who are treated successfully with allo-HSCT and achieve prolonged survival has been increasing.[5] The graft-versus-ATL effect observed in ATL patients treated successfully with allo-HSCT[5] strongly suggests that a cellular immune-mediated approach for ATL can be clinically effective. With regard to cellular immunotherapy for ATL (unlike Epstein-Barr virus [EBV]-associated malignancy[6]), targeting of antigens associated with HTLV-I (the causative virus of ATL) such as Tax[7] and HBZ[8] still remains controversial, and the recently proposed NY-ESO-1[9] (a cancer-testis antigen) still awaits clinical validation. Thus, at this time, no effective therapeutic target antigen for anti-ATL immunotherapy has been clinically defined.

Human telomerase reverse transcriptase (hTERT), which is a component of human telomerase and a catalytic subunit for telomere elongation, is activated in almost all cancer cells, including hematologic malignancies, but not in normal cells.[10] In HTLV-I–infected cells and ATL tumor cells, Tax or interleukin-2 (IL-2) signaling strongly activates the *hTERT* promoter through the nuclear factor-κB or PI3K pathway,[11-13] suggesting that expression of hTERT protein would be upregulated in ATL tumor cells. Clinical trials of anticancer immunotherapy targeting hTERT have already been conducted, and both the safety and induction of immune responses to hTERT have been reproducibly confirmed.[10,14-17] In our previous studies, we defined a [human leukocyte antigen] HLA-A*24:02-restricted $hTERT_{461-469}$ nonameric peptide (VYGFVRACL) that was capable of inducing antileukemia cytotoxic T lymphocytes (CTLs),[18] and we subsequently established a CTL clone, K3-1, specific for this

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epitope.[19] We previously conducted a phase I/II clinical trial of hTERT peptide vaccine for treatment of HLA-A*24:02$^+$ patients with lung cancer and metastatic renal cell cancer.[20] These achievements strongly encouraged us to further explore cellular immune-mediated treatment of ATL targeting hTERT. Because of concern over the potential regulatory T-cell function of ATL tumor cells,[21] in this study we focused on developing a redirected T-cell–based immunotherapy targeting hTERT rather than using an $hTERT_{461\text{-}469}$ peptide vaccine. Recently developed forms of anticancer immunotherapy using gene-modified T cells that redirect defined tumor-associated antigens have been shown to have clinical promise.[22-25] To this end, therefore, we first cloned the rearranged HLA-A*24:02-restricted and $hTERT_{461\text{-}469}$-specific T-cell receptor α/β (*TCR-α/β*) genes from K3-1 and inserted them into a novel *TCR* gene expression vector carrying silencers for endogenous TCRs (*siTCR* vector)[26] in redirected T cells (hTERT-*siTCR* vector). Notably, we used a souped-up second-generation 2A peptide-based *siTCR* vector that achieved an increased level of expression of the introduced TCR.[27]

In this study, we used the newly established hTERT-*siTCR* vector to examine the feasibility of a novel redirected T-cell–based adoptive immunotherapy targeting hTERT for treatment of ATL.

Patients and methods

Cell lines, freshly isolated leukemia cells, and normal cells

Approval for this study was obtained from the institutional review board of Ehime University Hospital. Written informed consent was obtained from all patients, healthy volunteers, and parents of cord blood donors in accordance with the Declaration of Helsinki.

B-lymphoblastoid cell lines (B-LCLs) were established by transformation of peripheral blood B lymphocytes with EBV. ATN-1,[28] TL-Om1,[29] HUT102[29], and TL-MAT[30] were human T-cell lines established from ATL patients, and TL-Su,[31] MT-1,[32] MT-2[32], and MT-4[33] were human T-cell lines transformed by HTLV-I infection. LCLs, T2-A24,[19] K562 (American Type Culture Collection [ATCC]), and human T-cell lines (except TL-Om1), maintenance of which requires 10 U/mL recombinant human IL-2 (rhIL-2) (R&D Systems), were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. The HLA-A*24:02 gene-transduced K562 (K562-A24) was maintained in culture medium supplemented with 1.0 μg/mL puromycin (Sigma-Aldrich). Peripheral blood mononuclear cells (PBMCs) from ATL patients and healthy donors and cord blood mononuclear cells (CBMCs) from healthy donors were isolated by density gradient centrifugation and stored in liquid nitrogen until use. All samples from ATL patients contained more than 90% ATL cells. CD4$^+$ T cells, CD14$^+$ cells from PBMCs, and CD34$^+$ cells from CBMCs were isolated by using CD4$^+$ cell-, CD14$^+$ cell-, or CD34$^+$ cell-isolating immunomagnetic beads (MACS beads; Miltenyi Biotec), respectively. IL-2–dependent CD4$^+$ cell lines induced by HTLV-I infection were generated as reported previously.[8]

Cloning of full-length *TCR* α and β chain genes and construction of hTERT-*siTCR* retroviral vector

HLA-A*24:02-restricted and $hTERT_{461\text{-}469}$ nonameric peptide (VYGFVRACL)-specific *TCR-α/β* genes were cloned from our previously established CTL clone, K3-1,[19] by using the 5′ rapid amplification of complementary DNA ends method (Clontech). The rearranged *TCR-α/β* genes of K3-1 expressed the germ line gene segments *TRAV29DV5/TRAJ34/TRAC* and *TRBV20-1/TRBJ2-1/TRBC2*, respectively. The retroviral vector expressing K3-1–derived *TCR* genes was constructed as reported previously.[26,27,34] Briefly, the constant regions of the hTERT-specific *TCR-α/β* genes were codon optimized and then integrated into a novel Splice-b2Aa-siTCR–based retroviral vector encoding small interfering RNAs that complementarily bind to the constant regions of the endogenous *TCR-α/β* genes (hTERT-*siTCR* vector).[27]

Establishment of hTERT-*siTCR*–transduced CD8$^+$ T-cell lines

Isolated CD8$^+$ T cells from PBMCs of healthy volunteers or ATL patients using CD8$^+$ cell-isolating MACS beads and stimulation with 1 μg/mL anti-CD3 monoclonal antibody (mAb; OKT-3; BioLegend) were cultured in GT-T503 (Takara Bio) supplemented with 5% human serum, 0.2% human albumin, 50 U/mL rhIL-2, 5 ng/mL rhIL-7 (R&D Systems), 10 ng/mL rhIL-15 (PeproTech), and 10 ng/mL rhIL-21 (Shenandoah Biotechnology). Then, CD8$^+$ T cells were transfected with the hTERT-*siTCR* retroviral vector using RetroNectin (Takara Bio) -coated plates as described previously.[34] In some experiments, because TRBV20-1 is specifically labeled with anti-Vβ2 mAb (IMGT Web resources: http://www.imgt.org/), Vβ2-positive cells among hTERT-*siTCR*–transduced CD8$^+$ T cells (hTERT-*siTCR*/CD8) were further isolated by using fluorescein isothiocyanate (FITC) –conjugated Vβ2 mAb (Beckman Coulter) and anti-FITC–conjugated MACS beads. To measure the expression levels of the introduced hTERT-specific TCR in gene-modified CD8$^+$ T cells, the cells were labeled with anti-CD8 (BD Biosciences) and anti-Vβ2 mAbs and phycoerythrin-conjugated HLA-A*24:02/$hTERT_{461\text{-}469}$ tetramer or HLA-A*24:02/HIV-1 $Env_{584\text{-}592}$ (RYLRDQQLL) tetramer, as a negative control.[19] Labeled cells were analyzed by using a Gallios flow cytometer (Beckman Coulter) and FlowJo Version 7.2.2 software (TreeStar). To expand the hTERT-*siTCR*/CD8 cells, they were stimulated weekly with mitomycin-C (Kyowa Hakko) –treated and $hTERT_{461\text{-}469}$ peptide–pulsed HLA-A*24:02$^+$ LCLs.

Cytotoxicity assays

Standard ^{51}Cr-release assays were performed as described previously.[35] Briefly, 5×10^3 unpulsed or peptide-pulsed target cells were labeled with ^{51}Cr ($Na_2{}^{51}CrO_4$; MP Bio Japan) and incubated at various ratios with effector cells in 200 μL of culture medium in 96-well round-bottomed plates. To assess HLA class I restriction, target cells were incubated with 10 μg/μL anti-HLA class I framework mAb (clone w6/32; ATCC) or a control anti-HLA -DR mAb (clone L243; ATCC) for 1 hour, then incubated with effector cells for 5 hours. The percentage of specific lysis was calculated as (experimental release cpm – spontaneous release cpm)/(maximal release cpm – spontaneous release cpm) × 100 (%). In some experiments, time-lapse imaging was used. Ten thousand ATL cells lentivirally gene-modified to express monomeric Azami-Green (Amalgaam) were cocultivated with 5×10^4 effector cells expressing hTERT-specific TCR (at an effector:target ratio of 5:1) for 12 hours in culture medium supplemented with 10 μg/mL propidium iodide (Sigma) to label dead cells red by using a glass dish for microscopic observation of live cells (iBIDI-dish1 Hi-Q4; Nikon). Images were acquired by using a systemic bio-imaging tool (BioStation IM; Nikon). To examine the cytotoxicity of these effector cells against early-differentiated and highly proliferating subsets of hematopoietic progenitor cells, CB-CD34$^+$ cells cultured by using a hematopoietic cell expansion medium (StemSpan CC100 and StemSpan SFEM; Stem Cell) for 7 days were subjected to flow-based cytotoxicity assay. 7-Aminoactinomycin D (7-AAD) –positive dead cells in each subset were examined by flow cytometry.

Quantitative analysis of hTERT mRNA expression

Quantitative real-time PCR (qRT-PCR) for *hTERT* messenger RNA (mRNA) was performed as described previously.[36] Briefly, after complementary DNA was synthesized, qRT-PCR for *hTERT* mRNA (NM_198253) was performed by using the QuantiTect SYBR green PCR Kit (QIAGEN) and primers as follows: forward, 5′-TTCTTGTTGGTGACACCTCACCTC-3′; reverse, 5′-CAGCCATACTCAGGGACACCTC-3′ (Takara Bio). Human hypoxanthine phosphoribosyltransferase 1 (*hHPRT1*) mRNA (NM_000194) was prepared and used as an internal control. Samples were analyzed by using an ABI Prism 7500 Sequence Detection System (Applied Biosystems). The expression level of *hTERT* mRNA was corrected by reference to that of *hHPRT1* mRNA, and the amount of *hTERT* mRNA relative to that in PBMCs was calculated by the comparative threshold cycle method. K562, which strongly expresses *hTERT* mRNA, was used as an internal control.

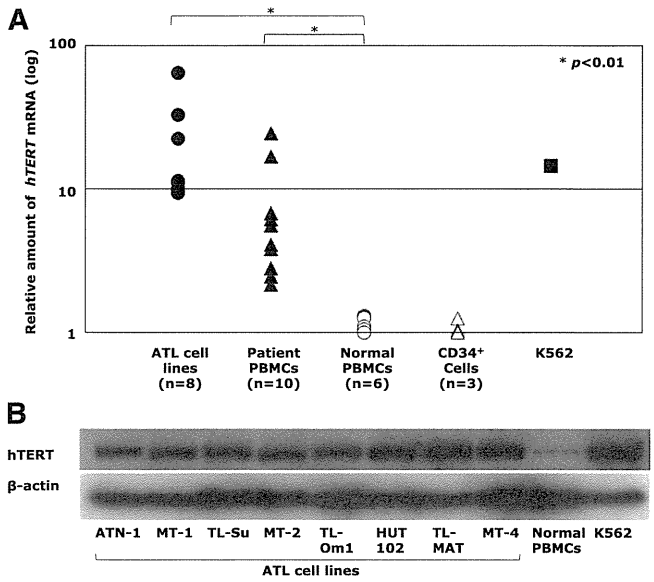

Figure 1. Abundant expression of hTERT in ATL tumor cells. (A) Expression of *hTERT* mRNA in ATL/HTLV-I infected cell lines (■), freshly isolated ATL tumor cells from patients (▲), normal PBMCs (○), and CB-CD34$^+$ cells (△) were examined by qRT-PCR. The level of *hTERT* mRNA expression in the K562 leukemia cell line (●) was used as an internal control. The expression level of *hTERT* mRNA in each sample was calculated relative to that of PBMCs. *hTERT* mRNA expression relative to normal PBMCs was 21.3 ± 17.9 for the ATL/HTLV-I–infected cell line, 7.48 ± 6.89 for freshly isolated ATL tumor cells, and 1.10 ± 0.12 for CB-CD34$^+$ (mean ± standard deviation [SD]). The ATL/HTLV-I–infected cell line and freshly isolated ATL tumor cells expressed *hTERT* mRNA abundantly and significantly (*$P < .01$). (B) Expression of hTERT protein in ATL cell lines and normal PBMCs was confirmed by western blotting.

Western blotting of hTERT protein

For analysis of protein expression, western blotting was performed as described previously.[35] Briefly, cell lysates were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (e-PAGEL, ATTO) and blotted onto polyvinylidene difluoride membranes (Bio-Rad Laboratories). The blots were incubated first with anti-hTERT rabbit mAb (Millipore), then with horseradish peroxidase–conjugated anti-rabbit immunoglobulin G Ab (GE Healthcare). The probed proteins were visualized by using an enhanced chemiluminescence system (GE Healthcare). Subsequently, the blotted membranes were stripped and reprobed with anti–β-actin mouse mAb (Sigma-Aldrich) to confirm equivalent protein loading between samples.

Detection of hTERT$_{461\text{-}469}$–specific CTL precursors in the periphery of ATL patients

PBMCs from HLA-A*24:02$^+$, HLA-A*24:02$^-$ ATL patients, or HLA-A*24:02$^+$ healthy individuals were seeded in 24-well plates at 1.5 × 10^6 per well in the presence of the hTERT$_{461\text{-}469}$ peptide at a concentration of 1 μM in GT-T503 medium supplemented with 5% human serum and 10 U/mL IL-2. After culturing for 14 days, cultured PBMCs were stained with FITC-conjugated anti-CD8 mAb and HLA-A*24:02/hTERT$_{461\text{-}469}$ tetramer or control tetramer at a concentration of 20 μg/mL at 4°C for 20 minutes. Subsequently, the stained cells were analyzed by flow cytometry.

IFN-γ secretion assay

hTERT-*siTCR*/CD8 or K3-1 (2 × 10^4) cells were incubated with 2 × 10^4 hTERT$_{461\text{-}469}$ peptide-pulsed (1 μM) or unpulsed K562-A24 or K562 cells for 24 hours. Interferon gamma (IFN-γ) in the culture supernatant was measured by using an enzyme-linked immunosorbent assay kit (Pierce). Enzyme-linked immunospot assays were used to detect the epitope-responsive IFN-γ production mediated by hTERT$_{461\text{-}469}$-specific CTL precursors in the periphery of ATL patients as described previously.[34]

Anti-ATL tumor effect of hTERT-*siTCR*–transduced CD8$^+$ T cells in xenografted mouse models

To assess the in vivo anti-ATL tumor effect mediated by hTERT-*siTCR*/CD8, a bioluminescence assay using a xenografted mouse model was used. First, we lentivirally generated a luciferase gene–transduced HLA-A*24:02$^+$ ATL cell line, ATN-1 (ATN-1/luc). For measurement, anesthetized xenografted mice were given an intraperitoneal injection of 2.5 mg/body VivoGlo luciferin (Caliper Life Science), and images were acquired for 5 to 10 minutes by using an AEQUORIA luminescence imaging system (Hamamatsu Photonics). The acquired photon counts were analyzed by using AQUACOSMOS software (Hamamatsu Photonics).

Six-week-old NOD/scid/γc^{null} (NOG) female mice[37] were purchased from the Central Institute for Experimental Animals and maintained in the institutional animal facility at Ehime University. All in vivo experiments were approved by the Ehime University animal care committee. For the Winn assay, 5 × 10^5 ATN-1/luc cells and 2.5 × 10^6 hTERT-*siTCR*/CD8 or non–gene-modified CD8$^+$ T cells (NGM/CD8) were subcutaneously inoculated into the abdominal wall of NOG mice that had been pretreated with 1 Gy irradiation. Thereafter, 2.5 × 10^6 effector cells of each type were administered weekly to the corresponding mice, respectively, via the tail vein for a total of 3 times. For the adoptive transfer experiments, similarly pretreated mice were intravenously inoculated with 5 × 10^5 ATN-1/luc cells. After 4 days, mice started to receive intravenously infused 5 × 10^6 hTERT-*siTCR*/CD8 or NGM/CD8, respectively, for a total of 5 times. These mice were serially monitored for tumor growth determined by photon counts acquired every 7 days until they were euthanized owing to disease progression.

Statistical analysis

The Mann-Whitney U test was used to assess differences between two groups; a P value of $< .05$ was considered significant.

Results

ATL tumor cells abundantly express *hTERT* mRNA and hTERT protein

The expression level of *hTERT* mRNA in the ATL/HTLV-I–infected cell line (n = 8), freshly isolated tumor cells from ATL patients (n = 10), normal PBMCs from healthy individuals (n = 6), and CD34$^+$ cells from normal CBMCs (CB-CD34$^+$) (n = 3) were measured by using the qRT-PCR method. *hTERT* mRNA expression relative to normal PBMCs was 21.3 ± 17.9 for the ATL/HTLV-I–infected cell line, 7.48 ± 6.89 for freshly isolated ATL tumor cells, and 1.10 ± 0.12 for CB-CD34$^+$ cells (mean ± standard deviation). In Figure 1A, the ATL/HTLV-I–infected cell line and freshly isolated ATL tumor cells, but not CB-CD34$^+$, abundantly produced *hTERT* mRNA in comparison with normal PBMCs, the difference being statistically significant. The P value was .002 for the ATL/HTLV-I–infected cell line, .001 for freshly isolated ATL tumor cells, and .243 for CB-CD34$^+$ cells. Similarly, western blotting demonstrated abundant expression of hTERT protein in the ATL tumor cells (Figure 1B).

Circulatory hTERT$_{461\text{-}469}$-specific CTL precursors were exclusively detectable in the periphery of HLA-A*24:02$^+$ ATL patients

Next, by using the tetramer assay, we examined circulatory hTERT$_{461\text{-}469}$-specific CTL precursors in PBMCs from HLA-A*24:02$^+$ ATL patients (n = 7), HLA-A*24:02$^-$ ATL patients (n = 3) before chemotherapy, and HLA-A*24:02$^+$ healthy individuals as controls (n = 6). Since freshly isolated PB lymphocytes were almost

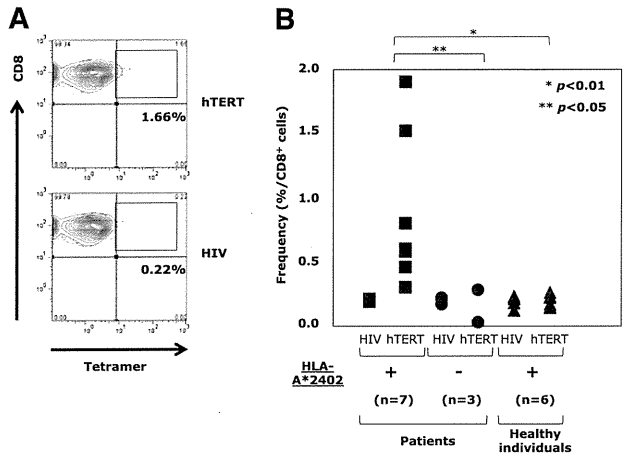

Figure 2. Detection of circulatory hTERT$_{461-469}$-specific CTL precursors in the periphery of ATL patients. (A) hTERT$_{461-469}$-specific CTL precursors in PBMCs repetitively stimulated with hTERT$_{461-469}$ peptide from HLA-A*24:02$^+$ ATL patients were detected by using HLA-A*24:02/hTERT$_{461-469}$ tetramer. A representative case is shown. HLA-A*24:02/HIV tetramer was used as a negative control. (B) In comparison with HLA-A*24:02$^-$ ATL patients (●) (n = 3) and HLA-A*24:02$^+$ healthy individuals (▲) (n = 6), the frequency of hTERT$_{461-469}$-specific CTL precursors in HLA-A*24:02$^+$ ATL patients (■) (n = 7) was significantly high (*P < .01; **P < .05). The frequency was 0.88% ± 0.55% for HLA-A*24:02$^+$ ATL patients, 0.11% ± 0.1% for HLA-A*24:02$^-$ ATL patients, and 0.2% ± 0.04% for HLA-A*24:02$^+$ healthy individuals (mean ± SD).

negative for tetramer staining, PBMCs stimulated with hTERT$_{461-469}$ peptide were analyzed. A representative example of an HLA-A*24:02$^+$ ATL patient is shown in Figure 2A. The frequencies of hTERT$_{461-469}$-specific CTL precursors in HLA-A*24:02$^+$ and HLA-A*24:02$^-$ ATL patients and HLA-A*24:02$^+$ healthy individuals are summarized in Figure 2B. hTERT$_{461-469}$-specific CTL precursors were detected at 0.88% ± 0.55% in HLA-A*24:02$^+$ ATL patients, being significantly more frequent than in HLA-A*24:02$^-$ ATL patients (0.11% ± 0.1%; P < .05) or HLA-A*24:02$^+$ healthy individuals (0.2% ± 0.04%; P < .01). These observations confirmed the presence of primed memory CD8$^+$ T cells with hTERT$_{461-469}$ epitope/HLA-A*24:02 complex (ie, that the hTERT$_{461-469}$ epitope must be naturally immunogenic) in HLA-A*24:02$^+$ ATL patients.

hTERT-*siTCR*–transduced CD8$^+$ T cells exert anti-ATL reactivity in vitro

The hTERT-*siTCR* gene was retrovirally introduced into normal CD8$^+$ T cells. Transduction efficiency determined by expression of Vβ2 on the gene-modified T cells was 85% to 95% (data not shown), and almost 50% of the transfectants were positive for HLA-A*24:02/hTERT$_{461-469}$ tetramer (Figure 3A). The cognate epitope specificity and HLA-A*24:02 restriction were examined by using standard ^{51}Cr-release assays (Figure 3B). Because expression of *hTERT* mRNA in LCLs was upregulated (supplemental Figure 2C), hTERT peptide-unpulsed HLA-A*24:02$^+$ LCLs were killed to some extent, reflecting the presence of endogenously processed hTERT (Figure 3B). Such epitope-specific cytotoxicity mediated by hTERT-*siTCR*/CD8 was obviously attenuated by anti-HLA class I mAb, but not by anti-HLA-DR mAb (Figure 3C). The antigen sensitivity to cognate hTERT$_{461-469}$ peptide mediated by hTERT-*siTCR*/CD8 (shown in Figure 3D) was similar to that of the parental CTL clone, K3-1 (Figure 3E-F).

hTERT-*siTCR*/CD8 dose-dependently killed the HLA-A*24:02$^+$ ATL/HTLV-I–infected cell lines ATN-1, TL-Su, and MT-2, but not the HLA-A*24:02$^-$ TL-Om1, HUT102, and MT-4 (Figure 4A). Additionally, the tumoricidal effect mediated by hTERT-*siTCR*/CD8 was abrogated by anti-HLA class I mAb, but not by anti-HLA-DR mAb (Figure 4B). Furthermore, time-lapse imaging directly demonstrated this tumoricidal activity of hTERT-*siTCR*/CD8 against HLA-A*24:02$^+$ ATN-1, but not that against HLA-A*24:02$^-$ HUT102 or K562 (negative control) (supplemental Fig 1-(1)). We then examined the tumoricidal activity against freshly isolated ATL tumor cells and found that these transfectants also dose-dependently killed HLA-A*24:02$^+$, but not -A*24:02$^-$ freshly isolated ATL tumor cells (Figure 5A).

Conversely, as shown in Figure 5B, neither HLA-A*24:02$^+$ normal CD4$^+$ T cells (the normal counterpart of ATL tumor cells)

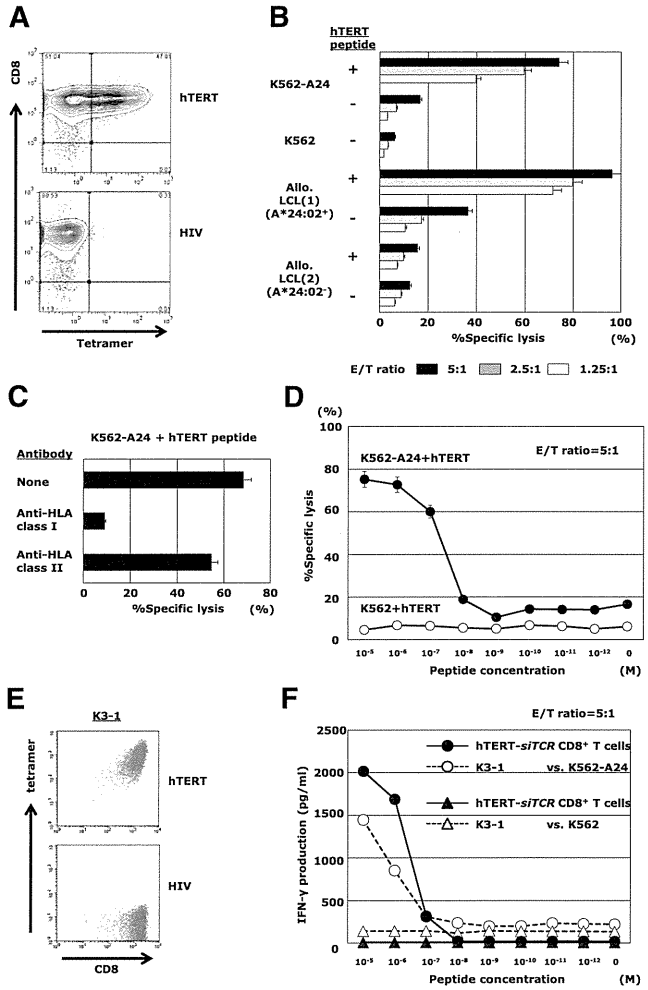

Figure 3. hTERT-*siTCR*–transduced CD8$^+$ T cells display epitope-specific responsiveness. (A) Representative flow cytometry plots showing staining of hTERT-*siTCR*–transduced CD8$^+$ T cells with HLA-A*24:02/hTERT$_{461-469}$ tetramer. HLA-A*24:02/HIV tetramer was used as a negative control. (B) ^{51}Cr-release assays were conducted by using hTERT-*siTCR*–transduced CD8$^+$ T cells with unpulsed or hTERT$_{461-469}$ peptide-loaded (1 μM) K562-A24, K562, HLA-A*24:02$^+$, or HLA-A*24:02$^-$ allogeneic B-LCLs at the indicated effector:target (E/T) ratios. (C) Effect of HLA class I and class II blockade on the cytotoxic activity of hTERT-*siTCR*–transduced CD8$^+$ T cells against the cognate peptide-pulsed (1 μM) K562-A24 was determined by ^{51}Cr-release assays at an E/T ratio of 5:1. (D) hTERT-*siTCR*–transduced CD8$^+$ T cells were tested in ^{51}Cr release assays against K562 (negative control) and K562-A24 cells pulsed with the indicated concentrations of hTERT$_{461-469}$ peptide at an E/T ratio of 5:1. Error bars represent SDs. (E) Representative flow cytometry plots showing staining of K3-1 with the HLA-A*24:02/hTERT$_{461-469}$ tetramer (upper) and the irrelevant HLA-A*24:02/HIV-1 Env$_{584-592}$ tetramer (negative control; bottom). (F) IFN-γ production by hTERT-*siTCR*–transduced CD8$^+$ T cells was measured by using a format similar to that described for panel D. The parental K3-1 CTL clone was tested in parallel.

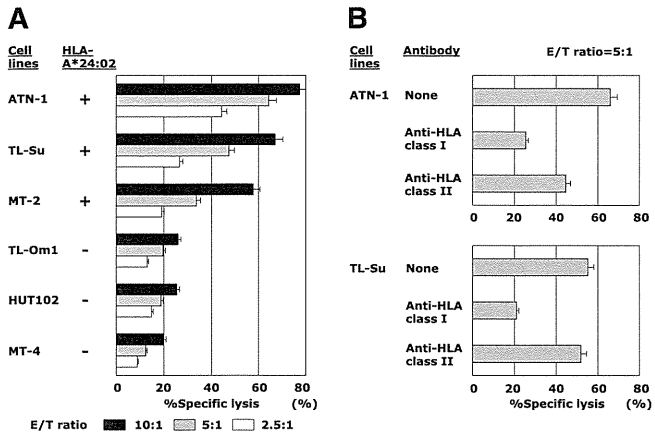

Figure 4. Cytotoxic activity of hTERT-*siTCR*–transduced CD8$^+$ T cells against ATL/HTLV-I–infected cell lines. (A) Cytotoxic activity of hTERT-*siTCR*–transduced CD8$^+$ T cells against HLA-A*24:02$^+$ or HLA-A*24:02$^-$ ATL/HTLV-I–infected cell lines was tested in ^{51}Cr-release assays at the indicated E/T ratios. All tested ATL/HTLV-I–infected cell lines overexpressed *hTERT* mRNA and protein, as shown in Figure 1. (B) Effect of HLA class I and class II blockade on the cytotoxic activity of hTERT-*siTCR*–transduced CD8$^+$ T cells against ATN-1 and TL-Su was tested in ^{51}Cr-release assays at an E/T ratio of 5:1.

nor HLA-A*24:02$^+$ normal CB-CD34$^+$ cells as normal hematopoietic progenitor cells were killed. In the same experiment, newly established IL-2–dependent HTLV-I–infected CD4$^+$ T cells (Patient #1 and Patient #2), but not the corresponding original normal/HTLV-I$^-$ CD4$^+$ T cells (Patient #1 and Patient #2), became to some extent sensitive to the same transfectants as the level of *hTERT* mRNA expression increased (Figure 5B). This observation confirmed that not only ATL tumor cells, but also HTLV-I–infected cells from which ATL tumor cells were derived could be killed by these hTERT-specific effector cells.

Next, because the majority of ATL patients were of an advanced age and were therefore ineligible for allo-HSCT, we examined the tumoricidal activity against autologous ATL tumor cells mediated by gene-modified PB-CD8$^+$ T cells from the patient (Figure 6). Although PB-CD8$^+$ T cells from heavily pretreated ATL patients were sometimes difficult to subject to *TCR* gene modification and ex vivo expansion, hTERT-*siTCR*/CD8 cells generated from HLA-A*24:02$^+$ patients (n = 3) were able to substantially lyse autologous ATL tumor cells in proportion to the corresponding level of *hTERT* mRNA expression. Autologous CD14$^+$ PB monocytes were used as a negative control because they lacked expression of *hTERT* mRNA. These results demonstrated that hTERT-*siTCR*/CD8 cells were able to exert tumoricidal activity against ATL tumor cells through recognition of the hTERT$_{461\text{-}469}$ epitope/HLA-A*24:02 complex, which is naturally presented on the surface of ATL tumor cells.

hTERT-*siTCR*–transduced CD8$^+$ T cells display in vivo anti-ATL reactivity

In vivo anti-ATL reactivity mediated by hTERT-*siTCR*/CD8 cells was assessed by using a xenografted mouse model and bioluminescence assay. Serial bioluminescence assay images were simultaneously acquired.

In the Winn assay (Figure 7A), tumor cell growth in NOG mice treated with hTERT-*siTCR*/CD8 (n = 2) was completely inhibited for longer than 6 months. In contrast, when compared with non-treated NOG mice (n = 2) in which the inoculated ATL tumor mass rapidly enlarged, activated NGM/CD8 (n = 2) did suppress ATL tumor growth to some degree, but eventually huge tumor masses developed within 2 months. In a therapeutic adoptive transfer model (Figure 7B), the tumor cell growth in mice treated with hTERT-*siTCR*/CD8 (n = 2) was obviously suppressed within the 8-week observation period, in contrast to that in mice treated with NGM/CD8 (n = 2) and that in control mice (n = 2).

Discussion

Although ATL still has a poor prognosis, the clinical presence of the graft-versus-ATL in patients treated successfully by allo-HSCT has encouraged the search for a novel cellular immune-mediated treatment of ATL. Unlike EBV-related malignancy,[6] the feasibility of HTLV-I–associated Tax[7] and HBZ[8] proteins as therapeutic targets of anti-ATL immunotherapy still remains controversial. Therefore, in this study, we explored the feasibility of a novel therapeutic target other than one associated with HTLV-I. Consequently, we demonstrated for the first time that hTERT was a promising therapeutic target for anti-ATL adoptive immunotherapy. Freshly isolated ATL tumor cells produced *hTERT* mRNA abundantly, and HLA-A*24:02-restricted and hTERT$_{461\text{-}469}$-specific CTL precursors were detected in the periphery of HLA-A*24:02$^+$ ATL patients. These findings suggested that naturally processed and presented hTERT$_{461\text{-}469}$/HLA-A*24:02 complex on the surface of ATL tumor cells was sufficiently immunogenic to be recognized by the target-specific CTLs in HLA-A*24:02$^+$ ATL patients. Additionally, *hTERT* mRNA expression in newly generated HTLV-I–infected CD4$^+$ T cells was upregulated, and these cells became sensitive to gene-modified hTERT-specific CTLs (Figure 5B). The involvement of Tax[12] and HBZ[38] in upregulation of the *hTERT* gene in

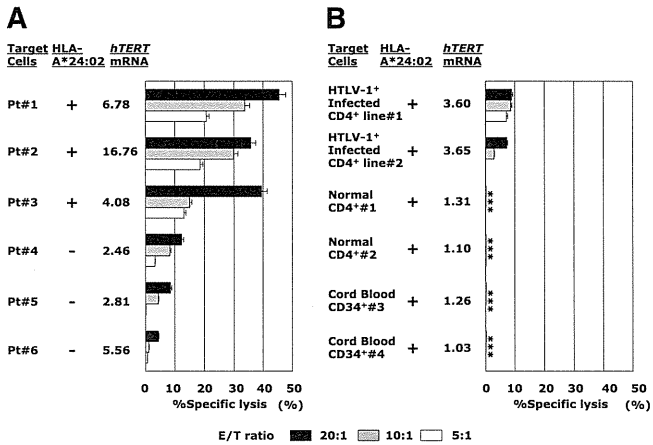

Figure 5. hTERT-*siTCR*–transduced CD8$^+$ T cells kill freshly isolated ATL cells and newly HTLV-I–infected CD4$^+$ T cells, but not normal cells, in vitro. (A) Freshly isolated HLA-A*24:02$^+$ (n = 3) or HLA-A*24:02$^-$ (n = 3) ATL tumor cells overexpressing *hTERT* mRNA were used as targets in ^{51}Cr-release assays with hTERT-*siTCR*–transduced CD8$^+$ T cells at the indicated E/T ratios. (B) The same hTERT-*siTCR*–transduced CD8$^+$ T cells used in panel A at the same E/T ratios were tested in ^{51}Cr-release assays against newly generated HLA-A*24:02$^+$ HTLV-I–infected CD4$^+$ T cells (n = 2) representing HTLV-I carrier CD4$^+$ T cells, original HLA-A*24:02$^+$ normal CD4$^+$ T cells (n = 2) representing the normal counterpart ATL tumor cells (corresponding number indicating cells from the identical donor), and HLA-A*24:02$^+$ normal CB-CD34$^+$ cells (n = 2) encompassing steady-state normal hematopoietic progenitor cells. Listed levels of expression of *hTERT* mRNA are those relative to the mean levels of expression across 6 PBMC samples from healthy donors determined by qRT-PCR and calculated by using the comparative threshold cycle method. Error bars represent SDs (* indicates less than detectable).

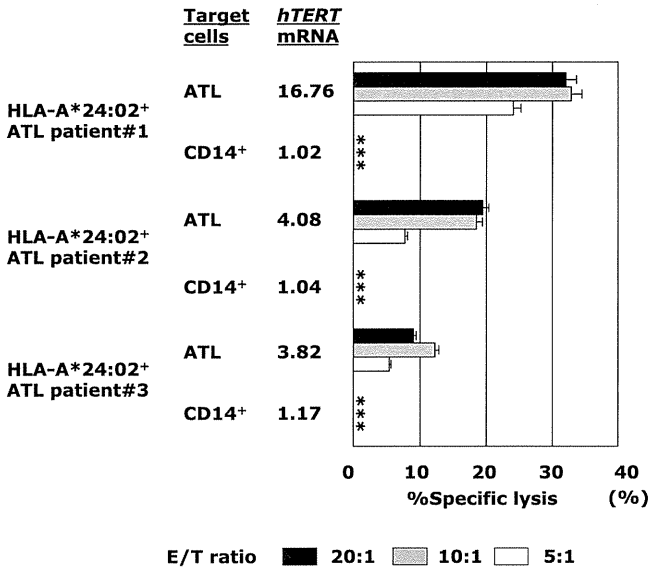

Figure 6. hTERT-*siTCR*–transduced CD8$^+$ T cells kill freshly isolated autologous ATL tumor cells on the basis of hTERT expression levels. Cytotoxic activity of hTERT-*siTCR*–transduced CD8$^+$ T cells obtained from HLA-A*24:02$^+$ ATL patients (n = 3) against autologous freshly isolated ATL tumor cells and autologous peripheral CD14$^+$ cells (negative control) was tested in ^{51}Cr-release assays at the indicated E/T ratios. *hTERT* mRNA in each patient's ATL tumor cells is listed using a format similar to that used in Figure 5. Error bars represent SDs (* indicates less than detectable).

HTLV-I–infected immortalized CD4$^+$ T cells and ATL tumor cells has been reported previously. Initially, it might seem more realistic to develop an hTERT$_{461\text{-}469}$ peptide vaccine for treatment of HLA-A*24:02$^+$ ATL patients. However, because we were concerned that CTL induction of hTERT peptide vaccine might have a tendency to be impeded by the regulatory T-cell function of ATL tumor cells,[21] we focused on developing a redirected T-cell–based adoptive immunotherapy targeting hTERT to allow administration of a number of hTERT-specific CTLs directly.

To this end, we cloned the full-length rearranged *TCR-α/β* genes from K3-1, the HLA-A*24:02-restricted and hTERT$_{461\text{-}469}$-specific CTL clone.[19] With codon optimization of the constant regions, we inserted them into our new souped-up second-generation 2A peptide-based *siTCR* vector to accomplish an increased expression level of the introduced TCR, carrying small interfering RNAs for the endogenous *TCR-α/β* genes in the redirected T cells (hTERT-*siTCR* vector).[26,27,34] The *siTCR* vector system makes it possible to simultaneously accomplish profound suppression of endogenous *TCR* genes and markedly increase the cell-surface expression of the introduced TCR, resulting in upregulated antitumor reactivity,[34] thus leading to inhibition of mispaired TCR formation between the endogenous and introduced TCR-α and -β chains, and lowering the potential risk of lethal graft-versus-host disease.[39] We found that both allogeneic and autologous gene-modified CD8$^+$ T cells using the hTERT-*siTCR* vector successfully killed ATL tumor cells both in vitro and in vivo (Figures 4-7), but not normal cells, including steady-state hematopoietic progenitor cells (Figure 5B). The introduced cytocidal activity against ATL tumor cells mediated by these gene-modified CTLs was actually accomplished through recognition of the HLA-A*24:02/hTERT$_{461\text{-}469}$ complex on the surface of ATL tumor cells (Figures 3 and 4).

Clinical studies of anticancer immunotherapy targeting hTERT have not demonstrated any significant adverse events so far.[14-17,20] However, for clinical application, because a number of activated gene-modified hTERT-specific CTLs would be administered at once, it would again be necessary to be mindful of on-target adverse events against normal tissues that constitutively express the *hTERT* gene.[10,40] Notably, any impairment of hematopoiesis would be the major concern. In this study, both allogeneic and autologous gene-modified effector CD8$^+$ T cells expressing hTERT-specific TCR from adult peripheral lymphocytes, and CB lymphocytes did not kill CB-CD34$^+$ cells representing steady-state hematopoietic progenitors (Figure 5B). By using cytokine-driven myeloid differentiation with CB-CD34$^+$ cells, gene-modified CTLs targeting hTERT showed a slight cytocidal effect against differentiated and highly proliferating subsets of CD34$^+$CD33$^+$ and CD34$^-$CD33$^+$ cells but spared CD34$^+$CD33^{dim} cells (supplemental Fig 2A). Additionally, contrary to resting CD4$^+$ cells and CD19$^+$ cells, highly mitotic polyhydroxic acid–stimulated CD4$^+$ cells and CD19$^+$ EBV LCLs became sensitive to effector CTLs because of increased expression of *hTERT* mRNA, the latter being more salient (Figure 5B and supplemental Fig 2B). Taken together, our findings suggest that gene-modified hTERT-specific CTLs will

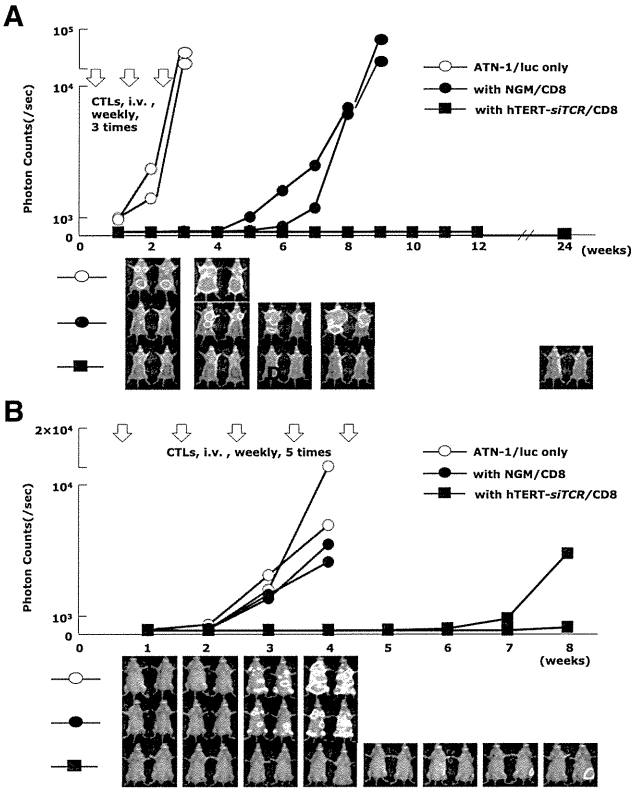

Figure 7. Anti-ATL reactivity of hTERT-*siTCR*–transduced CD8$^+$ T cells in vivo. (A) Winn assay. NOG mice were coinjected with a luciferase-transduced HLA-A*24:02$^+$ ATL cell line (ATN-1/luc) (5×10^5) and either 2.5×10^6 hTERT-*siTCR*–transduced (hTERT-*siTCR*/CD8) or NGM/CD8$^+$ T cells (n = 2 per group). Subsequently, 3 weekly infusions of the respective CD8$^+$ T-cell populations (2.5×10^6 cells per infusion) were administered intravenously (i.v.). Tumor growth was monitored every 7 days by using bioluminescence assay. Nontreated ATN-1/luc cells were similarly inoculated into NOG mice (n = 2) as a control. Although NGM/CD8 activated using OKT-3 and rhIL-2 suppressed tumor growth to some extent, hTERT-*siTCR*/CD8 durably suppressed tumor growth for longer than 6 months. (B) Therapeutic adaptive transfer model. NOG mice were intravenously inoculated with 5×10^5 ATN-1/luc cells. Four days later, intravenous administration of either 5×10^6 hTERT-*siTCR*/CD8 or NGM/CD8 (n = 2 per group) was started once a week for a total of 5 infusions. NOG mice given only ATN-1/luc cells (n = 2) were used as a control. In comparison with NGM/CD8, therapeutically infused hTERT-*siTCR*/CD8 also obviously suppressed the tumor cell growth within the 8-week observation period. Serial images of the bioluminescence assay demonstrate tumor growth in each group.

spare steady-state hematopoietic progenitor cells. However, to ensure safety, it would be better to avoid the active recovery phase of bone marrow after chemotherapy, notably under granulocyte colony-stimulating factor support, and also the acute infectious period in which immune-cell components are stimulated.

Another likely problem in clinical practice is that heavily pretreated peripheral lymphocytes from ATL patients might fail to proliferate. Proliferative activity of therapeutically infused gene-modified T cells in vivo is an important prerequisite for a successful outcome.[41] In this connection, although the control of treatment-related graft-versus-host disease still remains unsolved, use of CB lymphocytes has been investigated.[42] In this study, gene-modified CB-CD8$^+$ T cells from 2 donors successfully killed ATL tumor cells but spared autologous steady-state CB-CD34$^+$ cells (supplemental Figure 1-(2)). Compelling lack of suitable allo-HSCT donors for patients of advance age with ATL will encourage the application of CB transplantation using reduced-intensity preconditioning in the near future. Genetic redirection of CB lymphocytes using tumor antigen–specific *TCR* gene transfer will also play a considerable role.

Conversely, because hTERT is overexpressed in various kinds of cancer,[10] this approach may have widespread potential clinical application. Furthermore, the clinical availability of a new defucosylated anti-CCR4 mAb for treatment of ATL[43] can be reasonably anticipated to diminish regulatory T cells, the key player in the immunosuppressive microenvironment in patients with cancer,[44] because CCR4 is also expressed on regulatory T cells.[45] Therefore, hTERT-targeting immunotherapy after preconditioning with this anti-CCR4 mAb may become a realistically promising treatment option not only for ATL, but also for other malignancies.

In summary, using a newly established hTERT-*siTCR* vector, we have demonstrated the feasibility of anti-ATL redirected T-cell–based adoptive immunotherapy targeting hTERT, notably for patients who are ineligible for allo-HSCT. Further studies will be needed to investigate the clinical safety and utility of this novel therapy.

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Authorship

Contribution: Y.M. performed the research and wrote the paper; H.F. designed and performed the research, wrote and edited the paper and provided financial support; H.A., F.O., and T.O. performed the research and discussed the experimental results; T.A. interpreted the experimental results and provided financial support; T.I., S.O., J.M., K.K., and H.S. provided materials and discussed the experimental results; and M.Y. discussed and interpreted the experimental results and provided financial support.

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Anti-CCR4 mAb selectively depletes effector-type FoxP3$^+$CD4$^+$ regulatory T cells, evoking antitumor immune responses in humans

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CD4$^+$ Treg cells expressing the transcription factor FOXP3 (forkhead box P3) are abundant in tumor tissues and appear to hinder the induction of effective antitumor immunity. A substantial number of T cells, including Treg cells, in tumor tissues and peripheral blood express C-C chemokine receptor 4 (CCR4). Here we show that CCR4 was specifically expressed by a subset of terminally differentiated and most suppressive CD45RA$^-$FOXP3^{hi}CD4$^+$ Treg cells [designated effector Treg (eTreg) cells], but not by CD45RA$^+$FOXP3^{lo}CD4$^+$ naive Treg cells, in peripheral blood of healthy individuals and cancer patients. In melanoma tissues, CCR4$^+$ eTreg cells were predominant among tumor-infiltrating FOXP3$^+$ T cells and much higher in frequency compared with those in peripheral blood. With peripheral blood lymphocytes from healthy individuals and melanoma patients, ex vivo depletion of CCR4$^+$ T cells and subsequent in vitro stimulation of the depleted cell population with the cancer/testis antigen NY-ESO-1 efficiently induced NY-ESO-1–specific CD4$^+$ T cells. Nondepletion failed in the induction. The magnitude of the responses was comparable with total removal of FOXP3$^+$ Treg cells by CD25$^+$ T-cell depletion. CCR4$^+$ T-cell depletion also augmented in vitro induction of NY-ESO-1–specific CD8$^+$ T cells in melanoma patients. Furthermore, in vivo administration of anti-CCR4 mAb markedly reduced the eTreg-cell fraction and augmented NY-ESO-1–specific CD8$^+$ T-cell responses in an adult T-cell leukemia-lymphoma patient whose leukemic cells expressed NY-ESO-1. Collectively, these findings indicate that anti-CCR4 mAb treatment is instrumental for evoking and augmenting antitumor immunity in cancer patients by selectively depleting eTreg cells.

cancer immunotherapy | immunomodulation

Naturally occurring CD25$^+$CD4$^+$ regulatory T (Treg) cells expressing the transcription factor forkhead box P3 (FOXP3) are indispensable for the maintenance of immunological self-tolerance and homeostasis (1, 2). Given that most tumor-associated antigens are antigenically normal self-constituents (3–5), it is likely that natural FOXP3$^+$ Treg cells engaged in self-tolerance concurrently hinder immune surveillance against cancer in healthy individuals and also hamper the development of effective antitumor immunity in tumor-bearing patients. Indeed FOXP3$^+$CD25$^+$CD4$^+$ Treg cells are abundant in tumor tissues (6–10), and their depletion augments spontaneous and vaccine-induced antitumor immune responses in animal models (10, 11). In humans, increased numbers of FOXP3$^+$CD25$^+$CD4$^+$ Treg cells and, in particular, decreased ratios of CD8$^+$ T cells to FOXP3$^+$CD25$^+$CD4$^+$ Treg cells among tumor-infiltrating lymphocytes (TIL) are well correlated with poor prognosis in various types of cancers (6, 7, 10). Some clinical studies have shown the potential of depleting CD25-expressing lymphocytes to augment antitumor immune responses (12, 13); yet other similar studies failed to support the effects (10, 14, 15). Because activated effector T cells also express CD25, and their production of IL-2 is required for the expansion of CD8$^+$ cytotoxic lymphocytes, CD25-based cell depletion may reduce activated effector T cells as well, cancelling the effect of Treg-cell depletion to augment antitumor immunity (10). In addition, it has been demonstrated in animal models that depletion of Treg cells as a whole can trigger autoimmunity (1, 16, 17). Therefore, a current key issue is to determine how Treg cells can be controlled to evoke and enhance antitumor immunity without affecting effector T cells or eliciting deleterious autoimmunity.

Human FOXP3$^+$CD4$^+$ T cells are heterogenous in phenotype and function (2). These cells can be dissected into three subpopulations by the expression levels of FOXP3 and the cell-surface molecules CD45RA and CD25: (*i*) FOXP3^{hi}CD45RA$^-$CD25^{hi} cells, designated effector Treg (eTreg) cells, which are terminally differentiating and highly suppressive; (*ii*) FOXP3^{lo}CD45RA$^+$CD25^{lo} cells, designated naive Treg cells, which differentiate into eTreg cells upon antigenic stimulation; and (*iii*) FOXP3^{lo}CD45RA$^-$CD25^{lo} non-Treg cells, which do not possess suppressive activity but secrete proinflammatory cytokines (18). In principle, these distinct properties of FOXP3$^+$ T-cell subpopulations can be exploited to augment antitumor immunity without inducing autoimmunity, for example, by depleting a particular Treg-cell subpopulation rather than whole Foxp3$^+$-cell population. One of

Significance

Regulatory T (Treg) cells expressing the transcription factor FOXP3 play a critical role in suppressing antitumor immune responses. Here we found that, compared with peripheral blood T cells, tumor-infiltrating T cells contained a higher frequency of effector Tregs, which are defined as FOXP3^{hi} and CD45RA$^-$, terminally differentiated, and most suppressive. Effector Treg cells, but not FOXP3^{lo} and CD45RA$^+$ naïve Treg cells, predominantly expressed C-C chemokine receptor 4 (CCR4) in both cancer tissues and peripheral blood. In vivo or in vitro anti-CCR4 mAb treatment selectively depleted effector Treg cells and efficiently induced tumor-antigen-specific CD4$^+$ and CD8$^+$ T cells. Thus, cell-depleting anti-CCR4 mAb therapy is instrumental for evoking and enhancing tumor immunity in humans via selectively removing effector-type FOXP3$^+$ Treg cells.

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the candidate molecules for such differential control of Treg-cell subpopulations is chemokine receptors, which allow Treg cells to migrate to a specific inflammation site via sensing specific chemokine milieu (19).

It has been shown that tumor-infiltrating macrophages and tumor cells produce the chemokine (C-C motif) ligand 22 (CCL22), which chemoattracts Treg cells as well as effector T cells expressing C-C chemokine receptor type 4 (CCR4) (6, 10, 20). In this report, we have addressed whether CCR4-targeting treatment is able to selectively reduce a particular Treg-cell subpopulation, rather than whole Treg population, and thereby elicit or augment in vitro and in vivo antitumor immune responses in humans.

Results

Depletion of CCR4$^+$ T Cells Predominantly Depletes eTreg Cells. In peripheral blood mononuclear cells (PBMCs) of healthy individuals, CCR4$^+$ T cells were present in both FOXP3$^+$ and FOXP3$^-$ T-cell fractions, and FOXP3^{hi} cells in particular were CCR4$^+$ (Fig. 1*A*). When FOXP3$^+$ T cells were classified into three populations by the levels of FOXP3 and CD45RA expression (18), FOXP3^{hi}CD45RA$^-$ eTreg cells (Fr. II) predominantly expressed CCR4 at the protein and mRNA level (Fig. 1*A*, and Figs. S1 and S2*A*). In contrast, FOXP3^{lo}CD45RA$^+$ naive Treg cells (Fr. I) scarcely expressed the molecule, whereas FOXP3^{lo}CD45RA$^-$ non-Treg cells (Fr. III) exhibited a moderate expression. Among FOXP3$^-$ cells, some CD45RA$^-$CD4$^+$ memory or activated T cells expressed CCR4, whereas CD45RA$^+$ CD4$^+$ naive T cells did not. CD25 expression was well correlated with CCR4 expression with the highest CD25 expression by eTreg cells (Fr. II). Analyses of multiple samples of PBMCs from healthy individuals showed similar patterns of CCR4 expression by FOXP3 subsets (Fig. 1*B*). CD8$^+$ T cells, natural killer (NK) cells, CD14$^+$ monocytes/macrophages, dendritic cells, and B cells hardly expressed CCR4 at the protein and mRNA level (Fig. S2). In vitro depletion of CCR4$^+$ cells from PBMCs by magnet-bead sorting with anti-CCR4 mAb predominantly decreased CD4$^+$FOXP3^{hi} CD45RA$^-$ eTreg cells (Fr. II) and, to a lesser extent, CD4$^+$ FOXP3^{lo}CD45RA$^-$ non-Treg cells (Fr. III), but spared CD4$^+$ FOXP3^{lo}CD45RA$^+$ naive Treg cells (Fr. I) and FOXP3$^-$ cells (Fr. IV and V) (Fig. 1*C*). In contrast with anti-CCR4 mAb treatment, similar in vitro cell depletion with anti-CD25 mAb significantly reduced all of the FOXP3$^+$ subpopulations (Fr I, II, and III) and, to a lesser extent, FOXP3$^-$CD45RA$^-$CD4$^+$ activated or memory T cells (Fr. IV), with a relative increase in FOXP3$^-$ CD45RA$^+$CD4$^+$ naive T cells (Fr. V) (Fig. 1*D*). PBMCs of melanoma patients showed similar patterns of CCR4 expression by FOXP3$^+$ subpopulations and similar changes in the composition of FOXP3$^+$ T-cell subsets after in vitro CCR4$^+$ T-cell depletion (Fig. S3).

Taking these data together, we find that CCR4 is predominantly expressed by eTreg cells and depletion of CCR4$^+$ cells results in selective reduction of eTreg cells, while preserving naive Treg cells and the majority of FOXP3$^-$CD4$^+$ T cells.

Tumor-Infiltrating Treg Cells Exhibit the eTreg-Cell Phenotype and Can Be Depleted in Vitro by Anti-CCR4 mAb. Although there is accumulating data that FOXP3$^+$ T cells predominantly infiltrate into tumor tissues (6, 7, 10, 21), their detailed phenotypes remain to be determined. Our analysis of TILs in nine melanoma samples revealed infiltration of a high percentage of CCR4$^+$ T cells, the majority of which were CD4$^+$FOXP3^{hi}CD45RA$^-$ eTreg cells (Fr. II), with only a small number of CD4$^+$FOXP3^{lo}CD45RA$^+$ naive Treg cells (Fr. I) (Fig. 2*A*). In vitro depletion of CCR4$^+$ T cells indeed dramatically reduced these tumor-infiltrating eTreg cells (Fig. 2*B*), indicating that anti-CCR4 mAb treatment is able to selectively deplete eTreg cells abundantly infiltrating into tumors.

In Vitro Induction of NY-ESO-1–Specific CD4$^+$ T Cells After CCR4$^+$ T-Cell Depletion from PBMCs of Healthy Donors and Melanoma Patients. With the efficient depletion of the eTreg-cell population by in vitro anti-CCR4 mAb treatment, we next examined

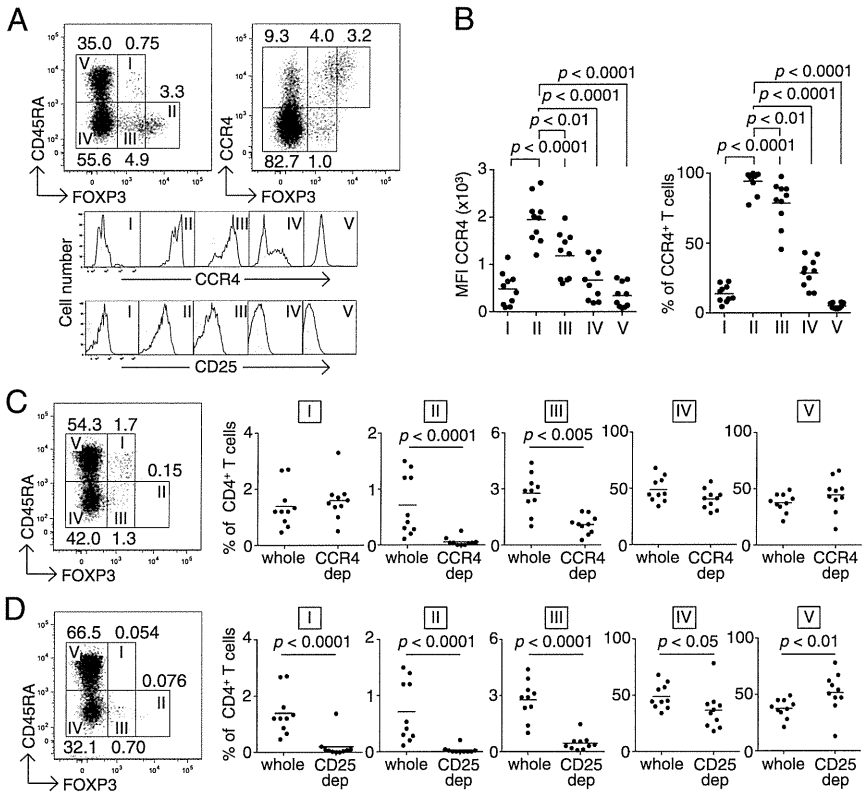

Fig. 1. Reduction of eTreg cells by in vitro depletion of CCR4-expressing T cells. (*A*) CCR4 and CD25 expression by subpopulations of FOXP3$^+$ Treg cells in PBMCs from healthy donors. CCR4 and CD25 expression levels were evaluated for each fraction. Representative data from 10 healthy donors are shown. (*B*) Median fluorescence intensity (MFI, *Left*) and frequency (*Right*) of CCR4 expression by each fraction of T cells in PBMCs of healthy donors (n =10). (*C*) Changes in the proportion of T-cell subpopulations after CCR4$^+$ T-cell depletion (CCR4 dep) (n = 10). (*D*) Changes in the proportion of T-cell subpopulations after CD25$^+$ T-cell depletion (CD25 dep) (n = 10). The numbers in *A*, *C*, and *D* indicate the percentage of gated CD4$^+$ T cells. Representative staining profiles in *A*, *C*, and *D* are from the same donor, and the same PBMC samples were analyzed in *B*–*D*.

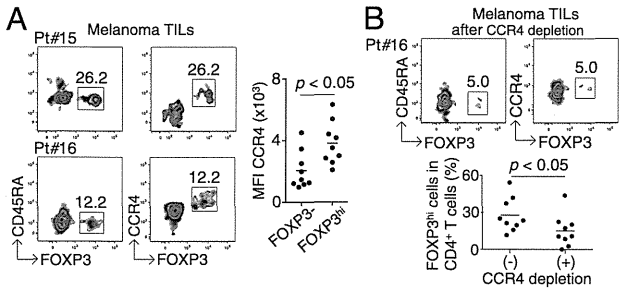

Fig. 2. Predominant infiltration of CCR4$^+$ eTreg cells into melanoma tissues. (*A*) CCR4 expression by melanoma-infiltrating T cells. CD4$^+$ T cells from melanoma sites were fractionated into subpopulations based on the expression of CCR4, CD45RA, and FOXP3; CCR4 expression by each fraction was analyzed. Data from two representative patients are shown. (*Right*) Summary of MFI of CCR4 expression by FOXP3$^-$ or FOXP3$^+$ cells (n = 9). (*B*) CCR4$^+$ CD4$^+$ T cells from melanoma tissues (Pt #16) were depleted of CCR4$^+$ T cells and then analyzed for the proportion of FOXP3^{hi} eTreg cells. (*Lower*) Percentages of FOXP3^{hi} cells among CD4$^+$ T cells after CCR4$^+$ cell depletion or nondepletion (n = 9). The numbers in *A* and *B* indicate the percentage of gated CD4$^+$ T cells.

whether CCR4$^+$ T-cell depletion from PBMCs of healthy donors was able to induce tumor antigen-specific CD4$^+$ T cells. We assessed specific T-cell responses to NY-ESO-1, a cancer/testis antigen, which is normally expressed by human germ-line cells and also by various types of cancer cells (4, 22). CCR4$^-$CD4$^+$ T cells or CD25$^-$CD4$^+$ T cells were cultured with CD4$^-$CD8$^-$ PBMCs as antigen-presenting cells (APCs), which were pulsed overnight with series of overlapping peptides covering the entire sequence of the NY-ESO-1 protein and X-irradiated (35 Gy) before use, as previously described (23, 24). Fifteen to 20 d later, NY-ESO-1–specific CD4$^+$ T cells secreting IFN-γ were enumerated by enzyme-linked immunospot (ELISpot) assay. Significant numbers of IFN-γ–secreting NY-ESO-1–specific CD4$^+$ T cells were induced in 7 of 16 healthy donors (43.8%), but only in the cultures with CCR4$^+$ or CD25$^+$ T-cell–depleted T cells (Fig. 3*A*, and summarized in Table S1). Furthermore, the frequencies of IFN-γ–secreting NY-ESO-1–specific CD4$^+$ T cells were higher after CCR4$^+$ T-cell depletion compared with CD25$^+$ T-cell depletion in five of seven healthy donors (71.4%) (Table S1). This result could be attributed in part to possible depletion of NY-ESO-1–specific CD25$^+$ activated T cells by anti-CD25 mAb treatment. The NY-ESO-1–specific CD4$^+$ T cells produced IFN-γ and TNF-α (Fig. 3*B*). Those cells induced in vitro after CCR4$^+$ T-cell depletion recognized NY-ESO-1 peptides at the concentration as low as 0.1 μM (Fig. 3*C*), and also NY-ESO-1 peptides produced by natural processing of the NY-ESO-1 protein by APCs, as previously shown with CD25$^+$ T-cell depletion (22, 24) (Fig. 3*D*).

We also attempted to determine whether Treg-cell depletion would evoke anti–NY-ESO-1 responses in apparently nonresponsive melanoma patients. With PBMCs from patients bearing NY-ESO-1–expressing melanomas, but without detectable NY-ESO-1–specific Ab in the sera, in vitro depletion of CCR4$^+$ or CD25$^+$ T cells and subsequent in vitro peptide stimulation induced IFN-γ– and TNF-α–secreting NY-ESO-1–specific CD4$^+$ T cells in three of eight patients (37.5%) (Fig. S4 *A* and *B* and Table S2). These NY-ESO-1–specific CD4$^+$ T cells appeared to express high-avidity T-cell receptors that recognized NY-ESO-1 peptides at a concentration as low as 0.1 μM, as seen with healthy donor T cells (Fig. S4*C*).

Thus, in healthy individuals as well as melanoma patients who had not raised spontaneous NY-ESO-1 immune responses, removal of eTreg cells by CCR4$^+$ T-cell depletion is able to efficiently induce high-avidity NY-ESO-1–specific CD4$^+$ T cells secreting effector cytokines.

CCR4$^+$ T-Cell Depletion Augments in Vitro Induction of NY-ESO-1–Specific CD8$^+$ T Cells from PBMCs of Melanoma Patients. PBMCs from melanoma patients were subjected to in vitro depletion with anti-CCR4 mAb or anti-CD25 mAb, and cultured with NY-ESO-1 peptide capable of binding to HLA class I of each patient. Seven to 10 d later, NY-ESO-1–specific CD8$^+$ T cells were detected by NY-ESO-1/HLA tetramers and analyzed for intracellular cytokine production. NY-ESO-1–specific CD8$^+$ T cells were induced in four of six patients (66.7%), and the responses were markedly augmented after depletion of CCR4$^+$ or CD25$^+$ cells (Fig. 4*A*). In addition, these NY-ESO-1–specific CD8$^+$ T cells recognized an HLA-matched malignant melanoma cell line and secreted IFN-γ and TNF-α (Fig. 4*B*). For example, Pt. #9 (HLA-A*02/29, B*44/27, C*03/04) harbored not only HLA-C*03–restricted NY-ESO-1–specific CD8$^+$ T-cells detected by HLA Cw*0304/NY-ESO-1 tetramers, but also those NY-ESO-1–specific CD8$^+$ T cells that recognized the SK-MEL 37 melanoma line (A*0201$^+$, NY-ESO-1$^+$) in an HLA-A2–restricted manner.

We also examined whether NY-ESO-1–specific CD8$^+$ T cells could be induced by directly adding mAb into cell cultures. Addition of anti-CD25 mAb or anti-CCR4 mAb reduced the frequency of CD4$^+$FOXP3^{hi}CD45RA$^-$ eTreg cells (Fr. II) (Fig. S5).

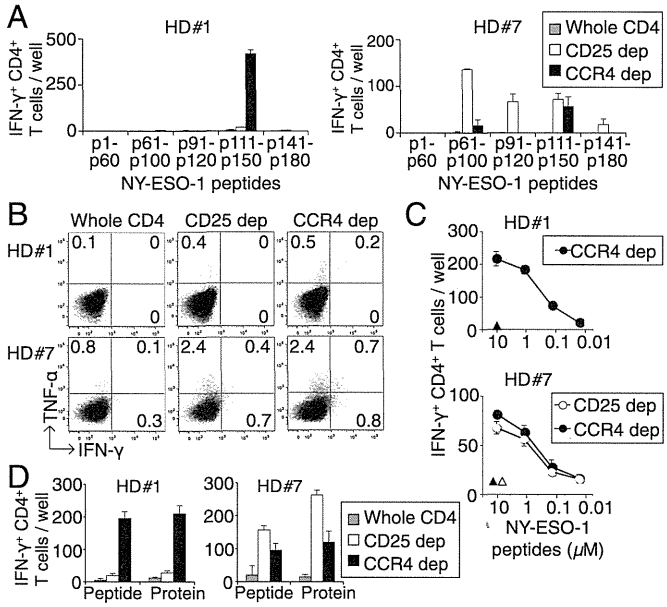

Fig. 3. Induction of cancer/testes antigen-specific CD4$^+$ T cells by depletion of CCR4- or CD25-expressing T cells in healthy donors. (*A*) CD4$^+$ T-cell responses to NY-ESO-1 peptides after depletion of CCR4$^+$ or CD25$^+$ T cells. CD4$^+$ T cells prepared from PBMCs of healthy donors were presensitized with APCs pulsed with NY-ESO-1 peptide covering the entire sequence of NY-ESO-1. Results of 2 (HD#1 and HD#7) among 16 healthy donors are shown. The numbers of IFN-γ–secreting CD4$^+$ T cells were assessed by ELISpot assay. (*B*) Intracellular cytokine secretion of CD4$^+$ T cells shown in *A*. The numbers in figures indicate the percentage of gated CD4$^+$ T cells. (*C*) Peptide dose-dependent recognition of NY-ESO-1–specific IFN-γ–secreting CD4$^+$ T cells. NY-ESO-1–specific CD4$^+$ T cells derived from CCR4$^+$ or CD25$^+$ T-cell–depleted cells (CCR4 dep and CD25 dep, respectively) were cultured with autologous activated T-cell APCs pulsed with graded amounts of NY-ESO-1 peptides and assessed for the number of IFN-γ–secreting cells as in *A*. Triangles indicate responses to control peptide at 10 μM. (*D*) Recognition of naturally processed NY-ESO-1 protein antigen by NY-ESO-1–specific CD4$^+$ T cells derived from whole CD4$^+$, CCR4$^+$ cell-depleted, or CD25$^+$ cell-depleted cells. NY-ESO-1–specific CD4$^+$ T cells from two healthy donors were cultured with autologous dendritic cells pulsed with NY-ESO-1 or control protein, or with NY-ESO-1 or control peptide. The experiments were independently performed twice with similar results.

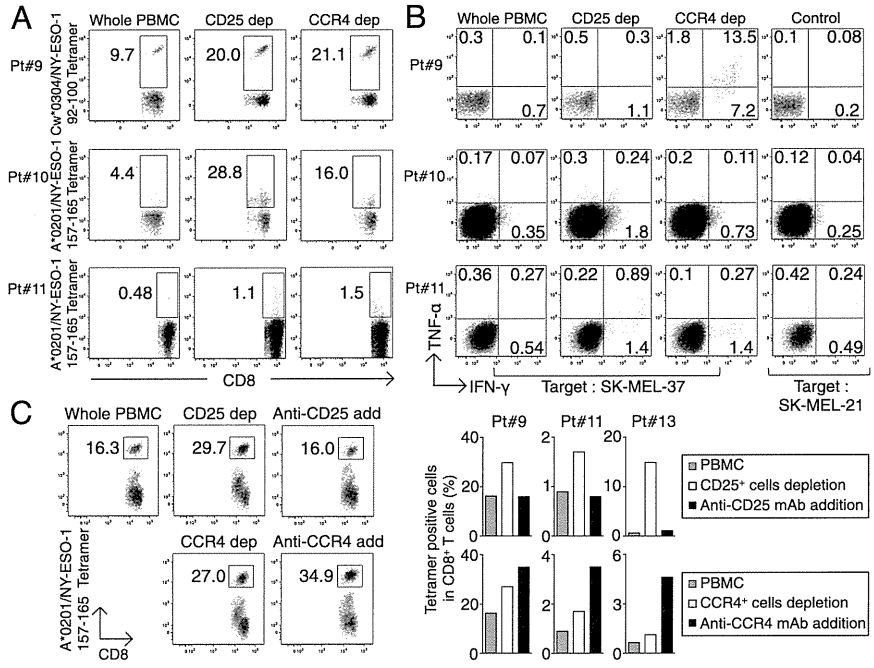

Fig. 4. Augmentation of NY-ESO-1–specific $CD8^+$ T-cell induction in melanoma patients by in vitro $CCR4^+$ T-cell depletion. (*A*) Induction of NY-ESO-1–specific $CD8^+$ T cells. Unfractionated PBMCs, or PBMCs depleted of $CD25^+$ or $CCR4^+$ cells, were prepared from melanoma patients (n = 6), and presensitized in peptides capable of binding to patients' HLA. NY-ESO-1–specific $CD8^+$ T cells were analyzed with NY-ESO-1/HLA tetramers (Pt. #9: A*02/29, B*44/27, C*03/04, Pt. #10: A*02/11, B*35/44, C*04/05, and Pt. #11: A*02/-, B*13/18, C*06/07). (*B*) Cytokine secretion of NY-ESO-1–specific $CD8^+$ T cells upon recognition of the HLA-A*0201$^+$ melanoma cell line SK-MEL 37 (NY-ESO-1$^+$), or SK-MEL-21 (NY-ESO-1$^-$) analyzed by intracellular cytokine staining. Data from three representative patients are shown. (*C*) Induction of antigen-specific $CD8^+$ T cells by addition (add) of anti-CD25 or anti-CCR4 mAb (KM2160) to cell cultures, or by $CCR4^+$ or $CD25^+$ cell depletion or nondepletion, as shown in *A* (Pt. #13 A02/03, B07/41, C07/17). A representative result (*Left*) and summary of three melanoma patients (*Right*) are shown. The numbers in the panels indicate the percentage of gated $CD8^+$ T cells. These experiments were performed independently at least twice with similar results.

Interestingly, although NY-ESO-1–specific $CD8^+$ T-cell induction was augmented in the cell culture containing anti-CCR4 mAb, the addition of anti-CD25 mAb reduced the frequency of NY-ESO-1–specific $CD8^+$ T cells (Fig. 4*C*), indicating that it might have killed some $CD25^+CD8^+$ activated effector T cells in addition to $CD25^+CD4^+$ Treg cells.

These results indicate that depletion of $CCR4^+$ T cells before in vitro induction or even simple incubation with anti-CCR4 mAb during the induction effectively augments NY-ESO-1–specific $CD8^+$ T-cell responses by selectively reducing eTreg cells.

Anti-CCR4 mAb Administration into Adult T-Cell Leukemia-Lymphoma Patients Reduces $CD4^+FOXP3^{hi}CD45RA^-$ eTreg Cells and Augments NY-ESO-1–Specific $CD8^+$ T-Cell Responses. In adult T-cell leukemia-lymphoma (ATL), which is caused by human T-lymphotropic virus 1 infection, ATL cells are $CD4^+$ and the majority—if not all—of them express FOXP3, CD25, CTLA-4, and CCR4, thus resembling naturally occurring $FOXP3^+$ Treg cells (25–28). Although it is currently difficult to discriminate whether anti-CCR4 mAb reduces ATL cells or normal $FOXP3^+$ Treg cells (29), we examined whether in vivo administration of anti-CCR4 mAb (Mogamulizumab), which has a cell-depleting effect by antibody-dependent cellular cytotoxicity, was able to reduce $FOXP3^+$ cells or a subpopulation thereof. Analysis of PBMCs from ATL patients collected before and after anti-CCR4 mAb therapy revealed that $CD4^+FOXP3^{hi}CD45RA^-$ cells including both ATL cells and eTreg cells were markedly reduced after the therapy (Fig. 5*A*). In addition, in a patient whose ATL cells expressed NY-ESO-1, NY-ESO-1–specific $CD8^+$ T cells producing IFN-γ and TNF-α were induced after several rounds of anti-CCR4 mAb administration (Fig. 5*B*). NY-ESO-1–specific $CD8^+$ T cells producing these cytokines were much higher in frequency than NY-ESO-1–specific $CD8^+$ T cells detected by NY-ESO-1/HLA-B*3501 tetramers, suggesting that this patient additionally possessed $CD8^+$ T cells recognizing other epitopes of NY-ESO-1. These results collectively indicate that anti-CCR4 mAb therapy for ATL is able to selectively deplete eTreg cells as well as ATL cells in vivo, and induce/augment tumor antigen-specific T-cell responses, although it is possible that anti-CCR4 mAb-induced reduction of $FOXP3^+$ ATL cells, which reportedly exhibit a Treg-cell–like in vitro suppressive activity (27, 28), might also contribute to the augmentation of immune responses.

Discussion

Accumulating evidence indicates that effective cancer immunotherapy needs to control $FOXP3^+$ Treg cells naturally present in the immune system and abundantly infiltrating into tumor tissues (10, 11, 30). Here, we have shown that $CD4^+FOXP3^{hi}CD45RA^-$ eTreg cells, which are terminally differentiated and most suppressive, highly express CCR4, that they are predominant among $FOXP3^+$ T cells infiltrating into tumor tissues (e.g., melanoma), and that specific depletion of eTreg cells in vivo or in vitro by anti-CCR4 mAb evoked tumor antigen-specific immune responses mediated by $CD4^+$ and $CD8^+$ T cells in healthy individuals and cancer patients.

Besides high expression of CCR4 in eTreg cells, CCR4 is expressed, although to a lesser extent, in non-Treg $CD4^+$ T-cell fractions [i.e., the $FOXP3^{lo}CD45RA^-$ cells (Fr. III) and $FOXP3^-CD45RA^-$ cells (Fr. IV)]. The former are capable of secreting cytokines, such as IL-4 and IL-17, as previously reported with PBMCs of healthy individuals (18). It has also been shown that Th2 cells and a fraction of central memory $CD8^+$ T cells express CCR4 (31–33). It is thus likely that tumor-infiltrating activated macrophages, and presumably some tumor cells produce CCL22, which predominantly chemoattracts and recruits from peripheral blood both $CCR4^+$ eTreg and $CCR4^+$ effector T cells that recognize tumor-associated antigens (such as cancer/testis antigen) and presumably self-antigens released from tumor cells (6, 10, 21, 34). However, the frequency of IL-4– or IL-17–secreting $CD4^+$ T cells were much lower than eTreg cells among $CCR4^+CD4^+$ T cells in PBMCs and TILs in melanoma tissues of nontreated patients; and CCR4 expression by $CD8^+$ TILs were limited. Moreover, addition of anti-CCR4 mAb into in vitro peptide stimulation more effectively induced antigen-specific $CD8^+$ T cells than $CCR4^+$ T-cell depletion, indicating that anti-CCR4 mAb had reduced eTreg cells but spared $CD8^+$ effector T cells. The result contrasted with the addition of anti-CD25 mAb, which appeared to deplete $CD25^+CD8^+$ T cells and cancel the enhancing effect of Treg-cell depletion. These results taken together indicate that anti-CCR4 mAb treatment to augment antitumor immunity mainly target $CCR4^+$ eTreg cells

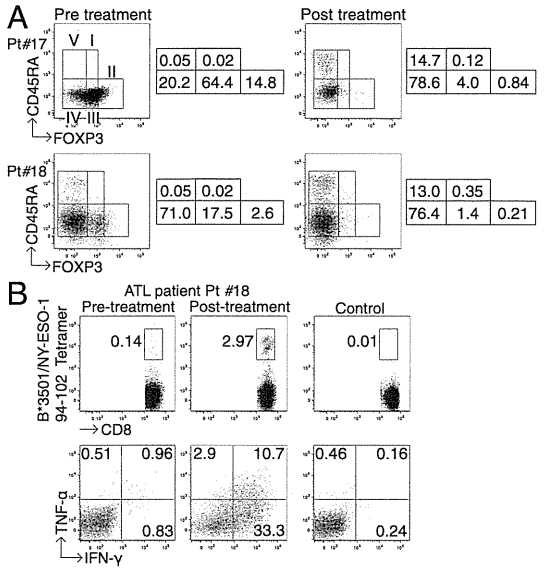

Fig. 5. Reduction of $CD4^+FOXP3^{hi}CD45RA^-$ T cells and augmentation of NY-ESO-1–specific $CD8^+$ T-cell responses in ATL patients after anti-CCR4 mAb (Mogamulizumab) therapy. (*A*) $FOXP3^+$ Treg-cell subpopulations in PBMCs from two ATL patients (Pt. #17: acute type, HLA-A*2402/-, B*3901/5401, C*0102/0702 and Pt. #18: lymphoma type, HLA-A*0201/3101, B*3501/4002, C*0303/0401) before and after anti-CCR4 mAb therapy. These experiments were performed at least twice with similar results. The numbers indicate the percentage of gated $CD4^+$ T cells. (*B*) Analysis of NY-ESO-1–specific $CD8^+$ T-cell induction before and after anti-CCR4 mAb therapy. PBMCs from Pt. #18 were presensitized in the presence of APCs pulsed with NY-ESO-1_{91-110} peptide corresponding to the patient's HLA. NY-ESO-1–specific $CD8^+$ T cells were detected with NY-ESO-1/HLA tetramers, and cytokine secretion of these NY-ESO-1–specific $CD8^+$ T cells upon recognition of autologous activated T-cell APCs pulsed with NY-ESO-1_{91-110} or control peptide was analyzed by intracellular cytokine staining. The numbers in figures indicate the percentage of gated $CD8^+$ T cells. The result was derived from a single assay because of limited availability of the patient's samples.

in tumor tissues and the regional lymph nodes, as well as peripheral blood, which would otherwise be a reservoir of fresh tumor-infiltrating Treg cells. Further study is warranted to determine whether depletion of $CCR4^+CD4^+$ and $CD8^+$ effector T cells in vivo affects antitumor immunity to a clinically significant extent.

Both NY-ESO-1–specific $CD4^+$ and $CD8^+$ T cells induced by in vitro anti-CCR4 mAb treatment possessed high-avidity T-cell receptors, and responded to dendritic cells processing tumor antigens and histocompatible tumor cell lines, respectively. This finding raises the issue of whether Treg depletion by anti-CCR4 mAb activates and expands already present antigen-primed effector T cells or newly induces effector T cells from a naive T-cell pool. We previously showed that in vitro NY-ESO-1-peptide stimulation following $CD25^+CD4^+$ T-cell depletion could activate NY-ESO-1–specific naive $CD4^+$ T-cell precursors in healthy individuals and in melanoma patients who possessed NY-ESO-1–expressing tumors but failed to develop anti-NY-ESO-1 Ab (23). In contrast, most NY-ESO-1–specific $CD4^+$ T cells in melanoma patients who had spontaneously developed anti–NY-ESO-1 Ab were derived from a memory population and could be activated even in the presence of $CD25^+CD4^+$ Treg cells (23). In addition, following vaccination of ovarian cancer patients with a HLA-DP–restricted NY-ESO-1 peptide, development of NY-ESO-1–specific high-avidity effector T cells from naive T cells was hampered by the presence of $CD25^+CD4^+$ Treg cells, although the vaccination could expand low-avidity NY-ESO-1–specific $CD4^+$ T cells that were apparently present in an effector/memory fraction before the vaccination (24). These results collectively indicate that elimination of eTreg cells by $CCR4^+$ T-cell depletion abrogates Treg cell-mediated suppression on NY-ESO-1–specific high-avidity naive T-cell precursors, allowing their activation and differentiation into high-avidity effector T cells capable of mediating strong antitumor immune responses. This successful induction of tumor antigen-specific $CD4^+$ and $CD8^+$ T cells indicates that the combination of anti-CCR4 mAb administration and vaccination with tumor antigens, such as NY-ESO-1, could be an ideal strategy for immunotherapy of a variety of cancers including ATL, which express NY-ESO-1 (35).

On the other hand, it was noted that not all healthy individuals or melanoma patients developed NY-ESO-1–specific T cells in vitro after Treg depletion for several possible reasons. For example, individuals who do not have a proper HLA haplotype may fail to select NY-ESO-1–reactive T cells thymically (22), hence possessing few NY-ESO-1–specific T-cell precursors. Other types of suppressor cells (such as myeloid-derived suppressor cells, immunosuppressive macrophages, and $Foxp3^-$ Treg cells) might contribute to inhibiting the induction of the responses (30). Alternatively, T cells specific for NY-ESO-1, a cancer/testis antigen, may also be subjected to other mechanisms of immunological self-tolerance—for example, anergy—hence being hyporesponsive to the antigen (36). These possibilities are under investigation to make anti-CCR4 mAb therapy more effective.

Would in vivo anti-CCR4 mAb treatment to deplete Treg cells elicit harmful autoimmunity? It has been shown in animal models that a longer period and a more profound degree of Treg-cell depletion is required to elicit clinically and histologically evident autoimmunity than evoking effective antitumor immunity (37, 38). In humans, naive Treg cells are generally well preserved in peripheral blood in cancer patients, even if they are low in frequency in tumor tissues. Furthermore, $CCR4^+$ T-cell depletion selectively eliminates eTreg cells but spares naive Treg cells. Assuming that effective tumor immunity can be evoked without significant autoimmunity via controlling the degree and duration of Treg-cell depletion, it is likely that, although anti-CCR4 mAb administrations reduce eTreg cells in the immune system during the treatment, the residual $CCR4^-$ eTreg cells (as shown in Fig. 2), including those which have newly differentiated from naive Treg cells, are sufficient to prevent deleterious autoimmunity. Supporting this notion, only a minor population of ATL patients treated with anti–CCR4 mAb experienced severe immune-related adverse events, except skin rashes (29). Anti-CCR4 mAb therapy can therefore be a unique cancer immunotherapy aiming at depleting eTreg cells without clinically serious adverse effects that would be incurred by total Treg-cell depletion or functional blockade (39).

The critical roles of CCR4 in Treg-cell recruitment to tumors have been reported with various types of human cancers, such as malignant lymphomas, gastric, ovarian, and breast cancers (10). $CCR4^+$ eTreg cells abundantly and predominantly infiltrated into gastric and esophageal cancers as observed with melanoma. Although it remains to be determined whether every cancer tissue has predominant infiltration of $CCR4^+$ eTreg cells, it is envisaged that possible combination of anti-CCR4 mAb treatment, tumor antigen immunization, and antibody-mediated immune checkpoint blockade will further increase clinical efficacy of cancer immunotherapy.

Materials and Methods

Donor Samples. PBMCs were obtained from healthy donors, malignant melanoma patients with NY-ESO-1 expression, and ATL patients. To collect tumor-infiltrating T cells, melanoma tissues were minced and treated with gentleMACS Dissociator (Miltenyi Biotec). All healthy donors were subjects with no history of autoimmune disease. All donors provided written informed consent before sampling according to the Declaration of Helsinki. The present study was approved by the institutional ethics committees of Osaka University, Osaka, Japan and Landesarztekammer Hessen, Frankfurt, Germany.

Antibodies and Peptides. The information of antibodies and synthetic peptides is provided in *SI Materials and Methods*.

Preparation of CD25$^-$ or CCR4$^-$ Cells. PBMCs or CD4$^+$ T cells were treated with biotin-anti-CD25 mAb (BC96) or biotin-anti-CCR4 (1G1) mAb (0.01 mg/mL), otherwise specified, for 15 min at 4 °C. Subsequently, anti-Biotin MicroBeads (Miltenyi Biotec) were added as described in the manufacturer's protocol, then washed using PBS containing 2% (vol/vol) FCS. CD25$^-$ or CCR4$^-$ cells were separated on autoMACS Pro Separator (Miltenyi Biotec).

In Vitro Sensitization of NY-ESO-1–Specific CD4$^+$ T Cells. NY-ESO-1–specific CD4$^+$ T cells were presensitized as previously described (23, 24) and in *SI Materials and Methods*.

In Vitro Sensitization of NY-ESO-1–Specific CD8$^+$ T Cells. For in vitro sensitization of NY-ESO-1–specific CD8$^+$ T cells, 1.5–2 × 10^6 cells were cultured with NY-ESO-1 peptides (NY-ESO-$1_{157-165}$ for HLA-A*0201 restricted, NY-ESO-1_{92-100} for HLA-Cw*0304 restricted, NY-ESO-1_{91-110} for HLA-B*3501 restricted, 10 μM) (22, 23) in a 48-well dish or round-bottom 96-well plate. After 8 h, one-half of the medium was replaced by fresh medium containing IL-2 (20 U/mL) and IL-7 (40 ng/mL) and repeated twice per week. In some assays, purified anti-CD25 (M-A251) mAb or anti-CCR4 (KM2160) mAb (1 μg/mL) was included in some wells during the entire period of culture.

ELISpot Assay. The number of IFN-γ–secreting NY-ESO-1–specific CD4$^+$ T cells was assessed by ELISpot assay as previously described (23, 24) and in *SI Materials and Methods*.

Intracellular Cytokine Secretion Assay. The presensitized CD4$^+$ and CD8$^+$ T cells were restimulated with peptide-pulsed autologous activated T-cell APCs, SK-MEL-21 cells (NY-ESO-1$^-$, HLA-A*0201$^+$), or SK-MEL-37 cells (NY-ESO-1$^+$, HLA-A*0201$^+$) for 1 h, after which GolgiStop reagent (BD Biosciences) was added. Subsequently, cells were cultured for another 6–8 h at 37 °C. Cells were stained for cell surface markers and then for intracellular cytokines using BD Cytofix/Cytoperm Buffer and BD Perm/Wash Buffer (BD Biosciences). Results were analyzed by flow cytometry (BD LSRFortessa; BD Biosciences) and FlowJo v9.6.2 software (TreeStar).

Tetramer Assay. Tetramer staining was performed as previously described (35, 40) and in *SI Materials and Methods*.

Preparation of Dendritic Cells. Dendritic cells were prepared as previously described (24) and in *SI Materials and Methods*.

Statistical Analysis. The significance of the difference in each data between two groups was assessed by a Mann–Whitney test using Prism version 6 software (GraphPad). *P* values less than 0.05 were considered significant.

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Potential Contribution of a Novel Tax Epitope–Specific CD4$^+$ T Cells to Graft-versus-Tax Effect in Adult T Cell Leukemia Patients after Allogeneic Hematopoietic Stem Cell Transplantation

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Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is an effective treatment for adult T cell leukemia/lymphoma (ATL) caused by human T cell leukemia virus type 1 (HTLV-1). We previously reported that Tax-specific CD8$^+$ cytotoxic T lymphocyte (CTL) contributed to graft-versus-ATL effects in ATL patients after allo-HSCT. However, the role of HTLV-1–specific CD4$^+$ T cells in the effects remains unclear. In this study, we showed that Tax-specific CD4$^+$ as well as CD8$^+$ T cell responses were induced in some ATL patients following allo-HSCT. To further analyze HTLV-1–specific CD4$^+$ T cell responses, we identified a novel HLA-DRB1*0101–restricted epitope, Tax155–167, recognized by HTLV-1–specific CD4$^+$ Th1-like cells, a major population of HTLV-1–specific CD4$^+$ T cell line, which was established from an ATL patient at 180 d after allo-HSCT from an unrelated seronegative donor by in vitro stimulation with HTLV-1–infected cells from the same patient. Costimulation of PBMCs with both the identified epitope (Tax155–167) and known CTL epitope peptides markedly enhanced the expansion of Tax-specific CD8$^+$ T cells in PBMCs compared with stimulation with CTL epitope peptide alone in all three HLA-DRB1*0101$^+$ patients post–allo-HSCT tested. In addition, direct detection using newly generated HLA-DRB1*0101/Tax155–167 tetramers revealed that Tax155–167-specific CD4$^+$ T cells were present in all HTLV-1–infected individuals tested, regardless of HSCT. These results suggest that Tax155–167 may be the dominant epitope recognized by HTLV-1–specific CD4$^+$ T cells in HLA-DRB1*0101$^+$–infected individuals and that Tax-specific CD4$^+$ T cells may augment the graft-versus-Tax effects via efficient induction of Tax-specific CD8$^+$ T cell responses. *The Journal of Immunology*, 2013, 190: 4382–4392.

Human T cell leukemia virus type 1 (HTLV-1) is the causative agent of a highly aggressive CD4$^+$ T cell malignancy, adult T cell leukemia/lymphoma (ATL) (1, 2). This virus has infected 10–20 million people worldwide, especially in southern Japan, the Caribbean basin, South America, Melanesia, and equatorial Africa (3). Approximately 5% of HTLV-1–seropositive individuals develop ATL, and another 2–3% develop a slow progressive neurologic disorder known as HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP) or various chronic inflammatory diseases (4). The majority of HTLV-1–infected individuals remain asymptomatic throughout their lives.

ATL is characterized by extremely poor prognosis, mainly because of intrinsic drug resistance to cytotoxic agents. It has been reported that allogeneic hematopoietic stem cell transplantation (allo-HSCT), but not autologous HSCT, improved the outcome of ATL (5, 6). In previous clinical studies carried out by the ATL allo-HSCT Study Group, the overall survival rate within 3 y after allo-HSCT with reduced intensity conditioning (RIC) was 36% (7). HTLV-1 proviral load became and remained undetectable in some ATL patients with complete remission after allo-HSCT, suggesting that it is an effective treatment for ATL (7–9). In these studies, we reported that donor-derived HTLV-1 Tax-specific CD8$^+$ CTLs were induced in some ATL patients who achieved complete remission after allo-HSCT (10). These CTLs were able to lyse recipient–derived HTLV-1–infected T cells in vitro, suggesting potential contributions to graft-versus-leukemia effects. CD8$^+$ T cells, especially CTLs, generally play an important role in controlling viral replication in various infections, such as those

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Abbreviations used in this article: AC, asymptomatic carrier; allo-HSCT, allogeneic stem cell transplantation; ATL, adult T cell leukemia/lymphoma; HAM/TSP, HTLV-1–associated myelopathy/tropical spastic paraparesis; HTLV-1, human T cell leukemia virus type 1; ILT, IL-2–dependent T cell line; LCL, lymphoblastoid B cell line; rhIL-2, recombinant human IL-2; RIC, reduced intensity conditioning; Treg, regulatory T.

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involving HIV, hepatitis B virus, and hepatitis C virus. In HTLV-1 infection, HTLV-1–specific $CD8^+$ T cells predominantly recognize the Tax Ag and are believed to contribute to controlling infected cells (11, 12). A high frequency of functional Tax-specific $CD8^+$ T cells can be detected in HAM/TSP patients and some asymptomatic carriers (ACs), whereas most ATL patients and a small population of ACs show severely reduced Tax-specific $CD8^+$ T cell responses (13, 14). The mechanism underlying the suppression of HTLV-1–specific $CD8^+$ T cell responses in these patients has not yet been fully elucidated.

For induction and maintenance of virus-specific CTLs, virus-specific $CD4^+$ Th cell responses are required in many virus infections (15–19). However, there are only a few reports of HTLV-1–specific Th cell responses (20–23), presumably because of their susceptibility to HTLV-1 infection in vivo and in vitro (24). Preferential HTLV-1 infection in HTLV-1–specific $CD4^+$ T cells could be one of the reasons for immune suppression in ATL patients. In addition, it has been reported that a higher frequency of $CD4^+FOXP3^+$ regulatory T (Treg) cells is observed in infected individuals compared with uninfected healthy donors. The frequency of Tax^- Treg cells, which are a major population of Treg cells in infected individuals, is negatively correlated with HTLV-1–specific CTL responses (25). HTLV-1 basic leucine zipper factor might also be involved in immune suppression, because HTLV-1 basic leucine zipper was constitutively expressed in infected cells (26) and inhibited the activity of IFN-γ promoters by suppressing NFAT and AP-1 signaling pathways, resulting in the impaired secretion of Th1 cytokines from $CD4^+$ Th cells in a transgenic mouse model (27) These reports suggest that both the dysfunction of HTLV-1–specific $CD4^+$ Th cells and the increased number of uninfected Treg cells might be implicated in the immunosuppression observed in ATL patients. Conversely, in HAM/TSP patients, $CD4^+$ T cells are predominantly found in early active inflammatory spinal cord lesions (28, 29) with spontaneous production of proinflammatory, neurotoxic cytokines, such as IFN-γ and TNF-α (30), suggesting their contributions to the pathogenesis of HAM/TSP. However, the precise roles of HTLV-1–specific $CD4^+$ T cells in HTLV-1 infection remain unclear.

In some ATL patients who achieved complete remission after allo-HSCT, it has been suggested that donor-derived HTLV-1 Tax-specific CTLs may contribute to elimination of ATL cells (graft-versus-Tax effects) (10). We believe that $CD4^+$ T cells also play a critical role in the graft-versus-ATL effects because $CD4^+$ T cells are required for induction and maintenance of optimal CTL responses (15–19). It therefore is important to clarify the role of HTLV-1–specific $CD4^+$ T cells in the effects for understanding HTLV-1–specific T cell immunity in ATL patients after allo-HSCT and for developing new vaccine strategies to prevent recurrence of ATL.

Several studies have reported some HTLV-1–specific $CD4^+$ T cell epitopes restricted by different HLA haplotypes (20–23). The helper functions of these epitopes in HTLV-1–specific CTL responses in HTLV-1–infected individuals have not been well understood. However, Jacobson et al. (20) showed that $CD4^+$ T cells specific for Env gp46 196–209, an epitope restricted by HLA-DQ5 or -DRw16, exhibited a cytotoxic function by directly recognizing HTLV-1–infected cells. This observation raises the possibility that some HTLV-1–specific $CD4^+$ T cells may contribute to the graft-versus-ATL effects through their cytotoxic function in ATL patients after allo-HSCT.

In the current study, we demonstrated that both $CD4^+$ and $CD8^+$ Tax-specific T cell responses were induced in patients after allo-HSCT with RIC for ATL. To further analyze HTLV-1–specific $CD4^+$ T cell responses in ATL patients after allo-HSCT, we determined a novel HLA-DRB1*0101–resricted epitope, Tax155–167, recognized by HTLV-1–specific $CD4^+$ Th1-like cells, a major population of HTLV-1–specific $CD4^+$ T (T4) cell line, which was established from a patient in complete remission following allo-HSCT with RIC. Costimulation with oligopeptides corresponding to the Th1 epitope, Tax155–167, together with a known CTL epitope led to robust expansion of Tax-specific $CD8^+$ T cells in PBMCs from three HLA-DRB1*0101^+ patients after allo-HSCT tested. Furthermore, Tax155–167-specific $CD4^+$ T cells were found to be maintained in all HTLV-1–infected HLA-DRB1*0101^+ individuals tested, regardless of HSCT, by direct detection with newly generated HLA-DRB1*0101/Tax155–167 tetramers. Our results suggest that Tax155–167 may be a dominant epitope recognized by HTLV-1–specific $CD4^+$ T cells in HTLV-1–infected individuals carrying HLA-DRB1*0101 and that Tax-specific $CD4^+$ T cells may strengthen the graft-versus-ATL effects through efficient induction of Tax-specific CTL responses.

Materials and Methods

Subjects

A total of 18 ATL patients who underwent allo-HSCT with RIC regimen, and one HTLV-1–seronegative (#365) and two seropositive donors (one AC #310 and one HAM/TSP patient #294) carrying HLA-DRB1*0101 donated peripheral blood samples after providing written informed consent. Approximately one-half of these patients received allogeneic peripheral blood stem cell transplantation from HLA-A-, B-, and -DR–identical sibling donors. The other half received allogeneic bone marrow cells from HLA-A–, B–, and DR–identical seronegative unrelated donors (Table I). These patients were the participants of clinical studies organized by the ATL allo-HSCT Study Group, supported by the Ministry of Health, Welfare, and Labor of Japan. This study was also reviewed and approved by the Institutional Ethical Committee Review Board of the Tokyo Medical and Dental University.

Generation of cell lines derived from patients and donors

PBMCs were isolated using Ficoll-Paque PLUS (GE Healthcare, Buckinghamshire, U.K.) density gradient centrifugation and stored in liquid nitrogen in Bambanker stock solution (NIPPON Genetics, Tokyo, Japan) until required. These were used in part to obtain HTLV-1–infected IL-2–dependent T cell lines (ILT) and EBV-transformed lymphoblastoid B cell lines (LCL). ILT-#350 was spontaneously immortalized during long-term culture of PBMCs from patient #350 before allo-HSCT and maintained in RPMI 1640 medium (Life Technologies, Grand Island, NY) containing 20% FCS (Sigma Aldrich, St. Louis, MO) and 30 U/ml recombinant human IL-2 (rhIL-2; Shionogi, Osaka, Japan). LCL-#307, -#341, and -#350 were established by maintaining PBMCs from ATL patients #307, #341, and #350, respectively, after allo-HSCT. These PBMCs were maintained in RPMI 1640 medium containing 20% FCS, following infection with the EBV-containing culture supernatant of the B95-8 cell line, LCL-Kan, derived from a healthy individual was also used.

Synthetic peptides

A total of 18 overlapping peptides, 12- to 25-mer in length, spanning the central region of Tax (residues 103–246) were purchased and used for epitope mapping (Scrum Tokyo, Japan) (Table II). HLA-A*2402–restricted CTL epitopes (Tax301–309, SFHSLHLLF) (10) were used for in vitro stimulation of Tax-specific CTLs (Hokudo, Sapporo, Japan).

GST–Tax fusion protein–based immunoassay

HTLV-1 Tax-specific T cell responses were evaluated using GST–fusion proteins of the N-terminal (residues 1–127), central (residues 113–237), and C-terminal (residues 224–353) regions of HTLV-1 Tax (GST–Tax-A, -B, and -C, respectively) as described previously (13, 31). PBMCs (1×10^6 cells/ml) were incubated with or without a mixture of GST–Tax-A, -B, and -C proteins (GST–TaxABC) in 200 μl RPMI 1640 medium supplemented with 10% FCS. After 4 d, the supernatant was collected, and the concentration of IFN-γ in the supernatant was determined using an OptiEIA Human IFN-γ ELISA Kit (BD Biosciences, San Jose, CA). The minimum detectable dose for this assay was determined to be 23.5 pg/ml IFN-γ. $CD8^+$ cells were depleted from PBMCs by negative selection using Dynabeads M-450 CD8 (Invitrogen, Carlsbad, CA), according to the

manufacturer's instructions. For cytokine profiling of a HTLV-1–specific $CD4^+$ T cell line, cells were stimulated with formaldehyde-fixed ILT-#350 for 48 h. Culture supernatant was collected, and various cytokines were measured using a Human Th1/Th2/Th17 Cytokine Kit for a Cytokine Beads Array (BD Biosciences).

Induction of HTLV-1–specific $CD4^+$ T cell line (T4 cells)

PBMCs (1×10^6 cells/ml) from patient #350, in complete remission at 180 d after allo-HSCT, were cultured for 2 wk with 100 nM Tax301–309 peptide in 96-well round-bottom tissue culture plate (BD Biosciences) in a final volume of 200 μl RPMI 1640 medium with 20% FCS and 10 U/ml rhIL-2. $CD4^+$ cells were then isolated by negative selection using a Human CD4 T lymphocyte Enrichment Set-DM (BD Biosciences) and maintained in RPMI 1640 medium with 20% FCS and 100 U/ml rhIL-2. Cells (1×10^6 cells/ml) were stimulated with formaldehyde-fixed ILT-#350 (2.5×10^5 cells/ml) every 2–3 wk. After multiple rounds of stimulation, the resulting $CD4^+$ T cell line was assessed for HTLV-1 specificity by comparing IFN-γ production against ILT-#350 to that against an HTLV-1–negative cell line, LCL-#350.

RT-PCR

Total RNA from cells was isolated using Isogen (Nippon Gene, Tokyo, Japan) and Turbo DNA-free (Life Technologies). First-strand cDNA was prepared from 0.5 μg RNA using ReverTra Ace and $Oligo(dT)_{20}$ primers provided in a ReverTra Ace-α-kit (Toyobo, Osaka, Japan). PCRs were performed in 50 μl reaction mixture containing ReverTra Dash (Toyobo), 0.5 μM of each HTLV-1 pX-specific primer (pX1, 5′-CCA CTT CCC AGG GTT TAG ACA GAT CTT C-3′ and pX4, 5′-TTC CTT ATC CCT CGA CTC CCC TCC TTC CCC-3′), and 2 μl cDNA. GAPDH-specific primers (GAPDH5′, 5′-ACC ACA GTC CAT GCC ATC AC-3′; GAPDH3′, 5′-TCC ACC ACC CTG TTG CTG TA-3′) were used as an internal control. The thermal cycling conditions comprised an initial activation step at 94°C for 1 min, followed by 30 cycles of denaturation (98°C, 10 s), annealing (60°C, 2 s), and extension (74°C, 30 s). The PCR amplicons were visualized by ethidium bromide staining following 2% (w/v) agarose gel electrophoresis.

Flow cytometry

For cell surface staining, the following fluorochrome-conjugated mouse anti-human mAbs were used: CD3-FITC (UCHT1; BioLegend, San Diego, CA), CD4-FITC (RPA-T4; BioLegend), CD8-FITC (RPA-T8; BioLegend), and CD8-PE-Cy5 (HIT8a; BD Biosciences, San Jose, CA). For tetramer staining, PE-conjugated HLA-A*0201/Tax11–19, HLA-A*1101/Tax88–96, HLA-A*1101/Tax272–280, and HLA-A*2402/Tax301–309 tetramers were purchased from Medical & Biological Laboratories (Nagoya, Japan). PE-conjugated HLA-DRB1*0101/Tax155–167 tetramer were newly generated through the custom service of Medical & Biological Laboratories. Whole-blood or cultured cells were stained with PE-conjugated Tax/HLA tetramer in conjunction with CD3-FITC and CD8-PE-Cy5 or CD4-PE-Cy5. For whole-blood samples, RBCs were lysed and fixed in BD FACS lysing solution (BD Biosciences) before washing. Samples were analyzed on a FACSCalibur (BD Biosciences), and data analyses were performed using FlowJo software (Tree Star, Ashland, OR).

Epitope mapping

T4 cells (3×10^5 cells/ml) were stimulated with LCL-#350, pulsed with various concentrations of synthetic peptides for 1 h at 37°C, at a responder/stimulator (R/S) ratio of 3. The culture supernatant was collected at 6 h poststimulation, and peptide-specific IFN-γ production from T4 cells was determined by ELISA.

HLA class II restriction assay

T4 cells (5×10^5 cells/ml) were cocultured for 6 h with ILT-#350 (1×10^5 cells/ml) in the presence or absence of anti-human HLA-DR (10 μg/ml; L243; BioLegend), anti-human HLA-DQ (10 μg/ml; SPVL3; Beckman Coulter, Fullerton, CA), or anti–HLA-ABC (10 μg/ml; W6/32; BioLegend). The IFN-γ in the supernatant was measured by ELISA.

To identify a HLA class II molecule responsible for Ag presentation to T4 cells, Tax155–167 peptide-specific IFN-γ responses were evaluated using various HLA-typed LCLs (LCL-#350, LCL-#341, LCL-#307, and LCL-Kan). These LCLs (1×10^5 cells/ml) were pulsed with 100 ng/ml Tax155–167 peptide for 1 h, fixed with 2% formaldehyde, and then cultured with T4 cells (3×10^5 cells/ml) for 6 h. The culture supernatant was collected, and IFN-γ in the supernatant was measured by ELISA.

Tetramer-based proliferation assay

PBMCs (1.0×10^6 cells/ml) were cultured for 13 or 14 d with or without 100 nM antigenic peptides in the presence of 10 U/ml rhIL-2. Cells were stained with HLA/Tax tetramer-PE, CD3-FITC, and CD8-PE-Cy5 or CD4-PE-Cy5 and then analyzed by flow cytometry.

Statistic analysis

Statistical significance was evaluated with the unpaired t test using Graphpad Prism 5 (Graphpad Software, La Jolla, CA). In all cases, two-tailed p values <0.05 were considered significant.

Results

Tax-specific T cell responses in ATL patients who received allo-HSCT with RIC

We previously reported that Tax-specific $CD8^+$ T cells were induced in some ATL patients after allo-HSCT with RIC from HLA-identical sibling donors (10). In this study, we examined the Tax-specific T cell response in a larger number of ATL patients who received allo-HSCT with RIC. Table I provides a summary of the

Table I. Clinical information and summary for Tax-specific $CD8^+$ T cells in 18 ATL patients at 180 d post–allo-HSCT with RIC

ID (Age, Sex)	ATL Subtype	Type of Donor	Donor-HLA	Donor HTLV-1 Sero Status	Chimerism (%)[a]	Tetramer (%)[b]	Proviral Load[c]
239 (55, M)	Lymphoma	r-PB	A 26/33, DR 4/13	(−)	<5	NT	0.1
241 (61, F)	Acute	r-PB	A 2/26, DR 10/18	(−)	<5	0.00	0.1
247 (52, F)	Lymphoma	r-PB	A 24/−, DR 9/15	(−)	<5	0.07	0.1
270 (57, M)	Lymphoma	r-PB	A 24/33, DR 13/15	(−)	<5	0.00	0.0
300 (53, F)	Lymphoma	r-PB	A 24/26, DR 4/15	(+)	<5	1.34	4.8
301 (57, F)	Acute	ur-BM	A 24/33, DR 13/15	(−)	<5	0.72	0.0
307 (68, F)	Acute	r-PB	A 2/11, DR 14/15	(+)	<5	0.10	5.4
317 (60, M)	Acute	ur-BM	A 2/24, DR 14/15	(−)	<5	0.92	0.0
328 (62, M)	Acute	ur-BM	A 11/24, DR 8/9	(−)	<5	0.75	NT
340 (50, M)	Acute	r-PB	A 2/24, DR 4/8	(−)	<5	1.40	0.7
341 (61, F)	Acute	ur-BM	A 24/33, DR 1/15	(−)	<5	0.45	0.1
344 (58, M)	Lymphoma	ur-BM	A 2/24, DR 4/−	(−)	<5	0.44	0.0
349 (53, M)	Acute	r-PB	A 24/−, DR 8/15	(+)	<5	0.00	0.0
350 (60, F)	Acute	ur-BM	A 24/26, DR 1/14	(−)	<5	0.59	0.6
351 (57, F)	Acute	ur-BM	A 24/26, DR 9/12	(−)	<5	0.45	0.0
358 (63, F)	Lymphoma	r-PB	A 2/11, DR 4/14	(−)	<5	0.42	0.0
352 (61, M)	Acute	ur-BM	A 11/26, DR 8/15	(−)	<5	0.14	0.0
364 (52, M)	Acute	r-PB	A 24/26, DR 1/−	(−)	<5	0.11	0.0

[a]Indicates percentage of recipient-derived T cell chimerism.
[b]Indicates percentage of tetramer$^+$ cells among $CD8^+$ T cells in PBMCs.
[c]Indicates copy number per 1000 PBMCs.
F, Female; M, male; NT, not tested; r-PB, related donor-derived peripheral blood stem cell; ur-BM, unrelated donor-derived bone marrow cell.