triple-negative patients. *CCND1* overexpression has been reported as a favorable prognostic variable for myeloma patients treated with ASCT [9]. Therefore, *CCND1*$^+$ myeloma cases would be the most promising candidates for ASCT. On the other hand, novel drugs did extend OS in the *FGFR3*$^+$/*c-MAF*$^+$ group. Bortezomib has been reported to improve the outcome for patients with t(4;14) myeloma [26], and some studies indicate the use of novel drugs could abrogate the detrimental effect of t(4;14) on survival [24–28]. In the present study, 14 of 19 *FGFR3*$^+$/*c-MAF*$^+$ cases were treated with bortezomib, which may have improved their OS. In contrast, it remains unclear whether thalidomide benefited the *FGFR3*$^+$/*c-MAF*$^+$ patients. In the MRC IX trial, patients with unfavorable cytogenetics including t(4;14) and t(14;16) randomized to thalidomide maintenance showed no prolongation of PFS and OS relative to placebo [32]. However, the use of thalidomide in a salvage setting may benefit these patients. It was recommended that *FGFR3*$^+$/*c-MAF*$^+$ cases were treated with a bortezomib-containing regimen [33]. In this respect, *FGFR3*$^+$/*c-MAF*$^+$ myeloma cases would be the most promising candidates for treatment with novel drugs, especially bortezomib. However, an appropriately stratified protocol for using novel drugs still needs to be established.

There are several limitations to the present study. First, we measured only the expression of the 3 major 14q32 chromosomal translocation-associated proto-oncogenes *CCND1*, *FGFR3* and *c-MAF*. We did not investigate *CCND3* and *MAFB*, but despite the fact that the frequency of *CCND3*$^+$ and *MAFB*$^+$ myeloma cases is very low (only 2% each) [2], these translocations may have specific clinical features and could skew the results. In fact, *MAFB* onco-protein detected by immunohistochemistry was reported as a highly sensitive and specific unfavorable prognostic marker in MM [34]. According to the results of GEP, neither *CCND1*, nor *FGFR3* and *c-MAF* was detected in either *CCND3*$^+$ or *MAFB*$^+$ cases, which would therefore be assigned to the triple-negative group in the present study. A second limitation of our study was that the expression of *MMSET* was not determined and therefore we could not identify all of the t(4;14) cases, because overexpression of *FGFR3* protein occurs in only approximately 70% of patients with the t(4;14) translocation, whereas *MMSET* is overexpressed in all cases [30]. In the present study, two cases with t(4;14) were classified as triple-negative, because they were negative for *FGFR3* but positive for *MMSET*.

5. Conclusions

Global RQ/RT-PCR detecting *CCND1*, *FGFR3* and *c-MAF* will be helpful in selecting patients who will be the best candidates for treatment with the novel agents targeting *CCND1*, *FGFR3* or *c-MAF* currently under development. It is also useful for predicting OS and planning individualized treatment strategies for MM patients. Development of optimal therapies for each patient based on the expression pattern of 14q32 chromosomal translocation-associated proto-oncogenes represents an important objective in personalized treatment of MM.

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Authors' contributions

AI was the principal investigator and takes primary responsibility for the paper; AI, ET, MU, RU and SI conceived and designed the study; AI, ET, MU, HT, YA, HO, AM, TY, FM, AI, HY MR, SK, TK, SK, TI, YH, IH, HK and SI recruited the patients and acquired data; ET, MU, HI and YM performed the laboratory analysis and interpreted the data; AI and SI drafted and wrote the article and revised it critically for important intellectual content. All authors gave their final approval of the version to be submitted.

Conflict of interest

Yasufumi Matsuda is an employee of SRL Inc. The other authors report no potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.leukres.2013.09.026.

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

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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.

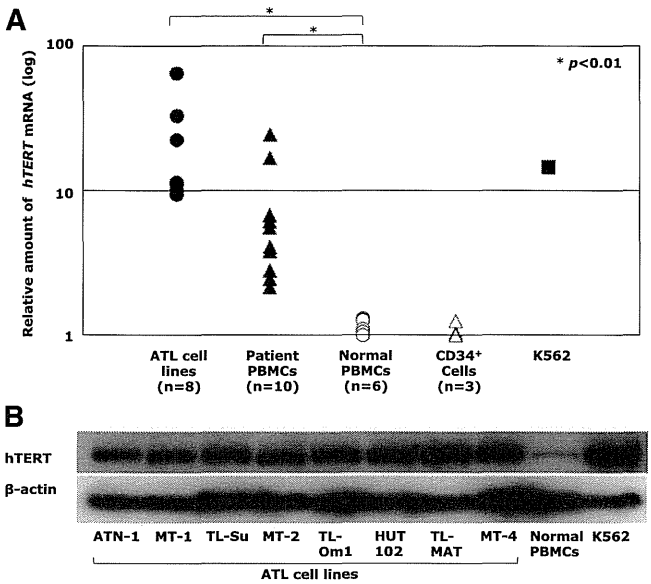

Figure 1. Abundant expression of hTERT in ATL tumor cells. (A) Expression of *hTERT* mRNA in ATL/HTLV-I infected cell lines (■), freshly isolated ATL tumor cells from patients (▲), normal PBMCs (○), and CB-CD34$^+$ cells (△) were examined by qRT-PCR. The level of *hTERT* mRNA expression in the K562 leukemia 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 euthanatized 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

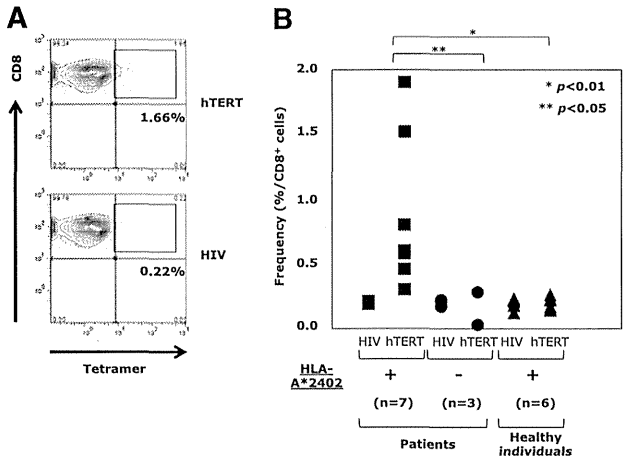

Figure 2. Detection of circulatory hTERT$_{461-469}$-specific CTL precursors in the periphery of ATL patients. (A) hTERT$_{461-469}$-specific CTL precursors in PBMCs repetitively stimulated with hTERT$_{461-469}$ peptide from HLA-A*24:02$^+$ ATL patients were detected by using HLA-A*24:02/hTERT$_{461-469}$ 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)

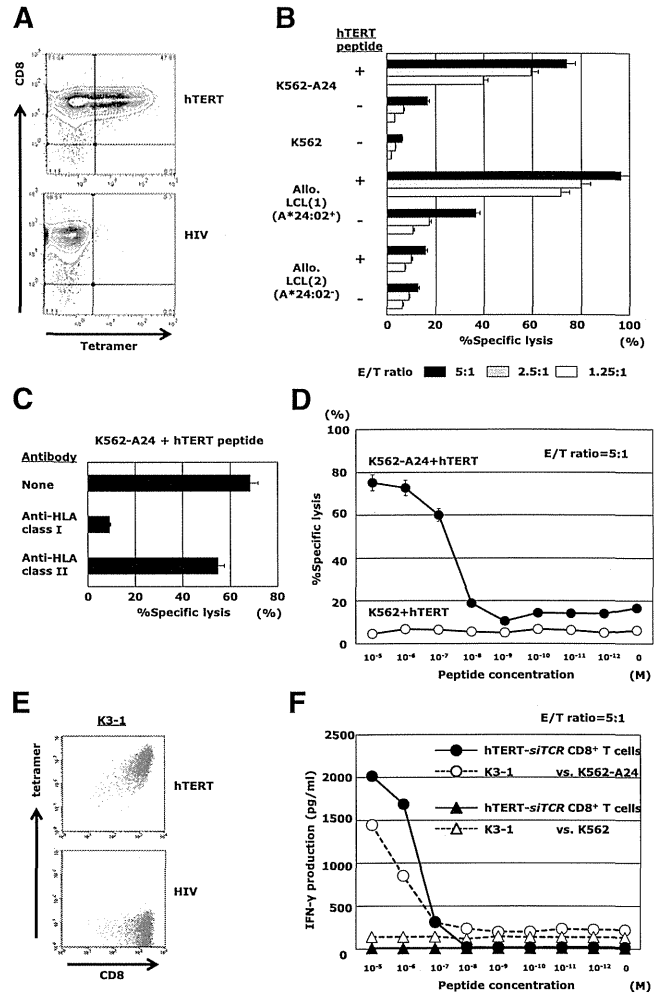

Figure 3. hTERT-*siTCR*–transduced CD8$^+$ T cells display epitope-specific responsiveness. (A) Representative flow cytometry plots showing staining of hTERT-*siTCR*–transduced CD8$^+$ T cells with HLA-A*24:02/hTERT$_{461-469}$ tetramer. HLA-A*24:02/HIV tetramer was used as a negative control. (B) ^{51}Cr-release 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.

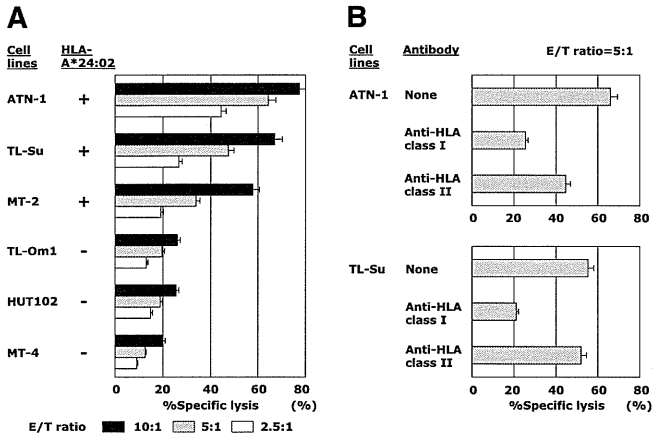

Figure 4. Cytotoxic activity of hTERT-*siTCR*–transduced CD8$^+$ T cells against ATL/HTLV-I–infected cell lines. (A) Cytotoxic activity of hTERT-*siTCR*–transduced CD8$^+$ T cells against HLA-A*24:02$^+$ or HLA-A*24:02$^-$ ATL/HTLV-I–infected cell lines was tested in ^{51}Cr-release assays at the indicated 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

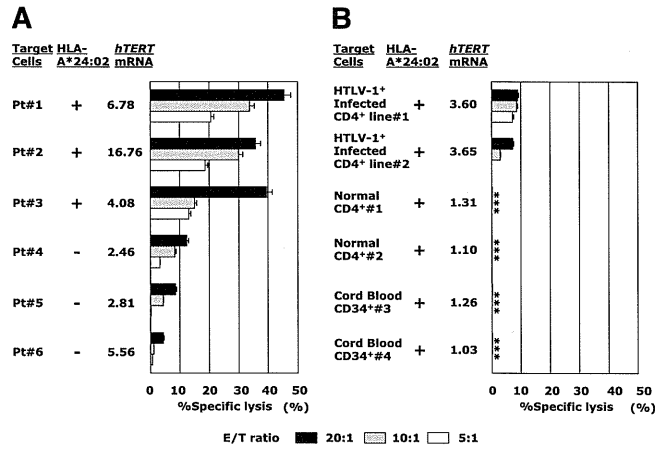

Figure 5. hTERT-*siTCR*–transduced CD8$^+$ T cells kill freshly isolated ATL cells and newly HTLV-I–infected CD4$^+$ T cells, but not normal cells, in vitro. (A) Freshly isolated HLA-A*24:02$^+$ (n = 3) or HLA-A*24:02$^-$ (n = 3) ATL tumor cells overexpressing *hTERT* mRNA were used as targets in ^{51}Cr-release 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).

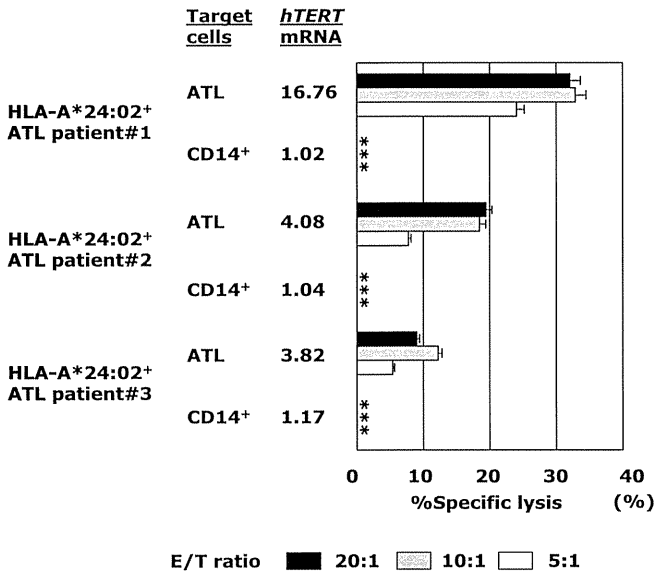

Figure 6. hTERT-*siTCR*–transduced CD8$^+$ T cells kill freshly isolated autologous ATL tumor cells on the basis of hTERT expression levels. Cytotoxic activity of hTERT-*siTCR*–transduced CD8$^+$ T cells obtained from HLA-A*24:02$^+$ ATL patients (n = 3) against autologous freshly isolated ATL tumor 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

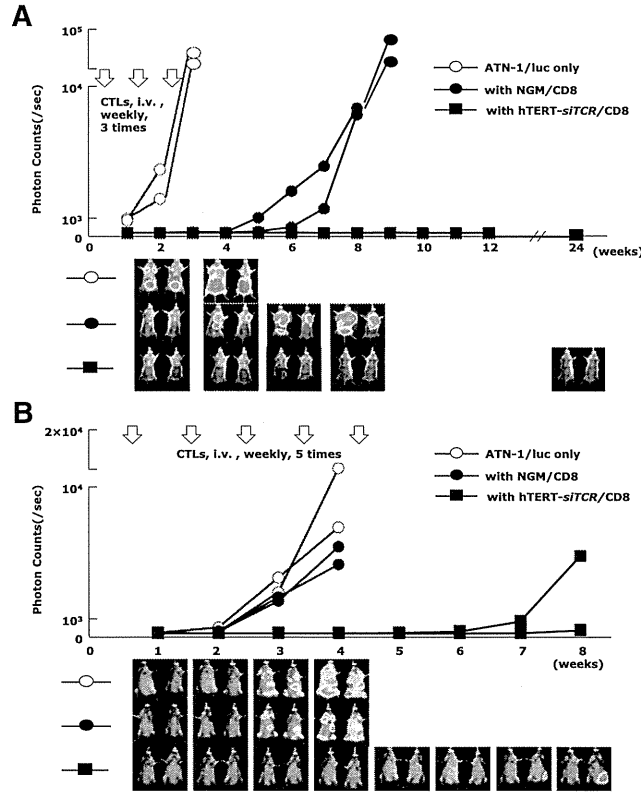

Figure 7. Anti-ATL reactivity of hTERT-*siTCR*–transduced CD8$^+$ T cells in vivo. (A) Winn assay. NOG mice were coinjected with a luciferase-transduced HLA-A*24:02$^+$ ATL cell line (ATN-1/luc) (5×10^5) and either 2.5×10^6 hTERT-*siTCR*–transduced (hTERT-*siTCR*/CD8) or NGM/CD8$^+$ T cells (n = 2 per 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.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Bath-PUVA Therapy Decreases Infiltrating CCR4-Expressing Tumor Cells and Regulatory T Cells in Patients With Mycosis Fungoides

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Abstract

In this study, we analyzed the mechanism that bath-PUVA therapy to CCR4-expressing tumor cells and regulatory T cells (Treg) in patients with mycosis fungoides(MF). The CCR4 positive cell and Treg in patient blood and the skin were analyzed. Both type of cells decreased after bath-PUVA in the skin lesion, in contrast, bath-PUVA did not significantly change the percent circulating Treg. It suggested that bath-PUVA eliminated both pathogenetically relevant cells and Treg and systemic immunosuppression was not induced.

Background: Mycosis fungoides (MF) is a malignant lymphoma characterized by expansion of $CD4^+$ memory T-cell clones. Infiltrating cells express CCR4, which is attracted to CC chemokine ligands 17 and 22 (thymus and activation-regulated chemokine [TARC]/CCL17 and TARC/CCL22). Bath–psoralen plus ultraviolet A (PUVA) is effective against MF. In patients with psoriasis, bath-PUVA induces circulating regulatory T cells (Tregs), which suppress effector T cells. To understand the mechanisms in MF, we analyzed lesion-infiltrating cells before and after bath-PUVA therapy. **Patients and Methods:** Thirteen patients with MF (12 stage IB, 1 stage III; mean age 69.2 years, range 35-87 years; 6 men, 7 women) were recruited. **Results:** Immunohistochemical analysis revealed that lesion CCR4-positive ($CCR4^+$) cells and Tregs significantly decreased from 105.1 ± 164.8 cells/10^{-2} mm^2 to 31.4 ± 39.0 cells/10^{-2} mm^2 and from 78.1 ± 67.8 cells/10^{-2} mm^2 to 24.7 ± 25.0 cells/10^{-2} mm^2, respectively. Serum TARC levels significantly correlated with infiltrating $CD3^+$ (r = 0.997), $CCR4^+$ (r = 0.991), and forkhead box P3–positive ($Foxp3^{+}$) cells (r = 0.843). Circulating Tregs before bath-PUVA therapy were not significantly different from those in healthy volunteers. Bath-PUVA did not significantly change the percentage of circulating Tregs. **Conclusions:** Bath-PUVA decreased $CCR4^+$ cells and Tregs in MF lesions but did not induce circulating Tregs, which might suppress effector T cells. Direct effects through skin lesions might eliminate both pathogenetically relevant cells and Tregs. Systemic immunosuppression was not induced.

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Keywords: Bath-PUVA therapy, CCR4, Mycosis fungoides, Regulatory T cell, Thymus and activation-regulated chemokine (TARC)

Introduction

Mycosis fungoides (MF) is a malignant cutaneous lymphoma with a chronic disease progression.[1] Because erythema and red plaques appear on the patient's whole body at an early stage, it is important to distinguish MF from other skin diseases. Various symptoms are associated with MF, including lymph node enlargement, skin tumors, and ulcer formation in the tumor stage. Symptom onset usually occurs in those older than 60 years of age, but the actual disease onset is earlier.[2] The histologic findings depend on the stage. In the erythema stage (stage I), the characteristic features include epidermal hyperplasia, lymphoid exocytosis, and band-like lymphoid infiltration in the

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Table 1 Patient Characteristics

Patient	Age	Sex	Disease Stage	Irradiation Frequency	Cumulative UV Doses (J/cm^2)
1	82	F	IB	46	170.4
2	68	F	III	42	146.4
3	35	F	IB	43	53.7
4	71	M	IB	20	45.0
5	87	M	IB	33	120.0
6	82	M	IB	5	8.0
7	77	M	IB	29	106.0
8	70	F	IB	37	138.0
9	83	M	IB	38	138.0
10	62	F	IB	14	29.5
11	56	M	IB	30	106.0
12	64	F	IB	42	150.0
13	62	F	IB	25	34.5
Mean ± SD	69.2 ± 14.1			31.1 ± 12.3	95.8 ± 54.5

Abbreviations: SD = standard deviation; UV = ultraviolet.

Figure 1 Immunohistochemical Analysis for Before Bath–Psoralen Plus Ultraviolet A (PUVA) Therapy. Hematoxylin and Eosin Stain (A), Anti-CD3 (B), Anti-CCR4 (C), Anti-Foxp3 (D). A Pautrier Microabscess was Observed in the Epidermis. Many CCR4$^+$ Lymphocytes Were Observed. In Contrast, There Were a Few Foxp3$^+$ Cells. Yellow Triangles Were Some of Positive Cells

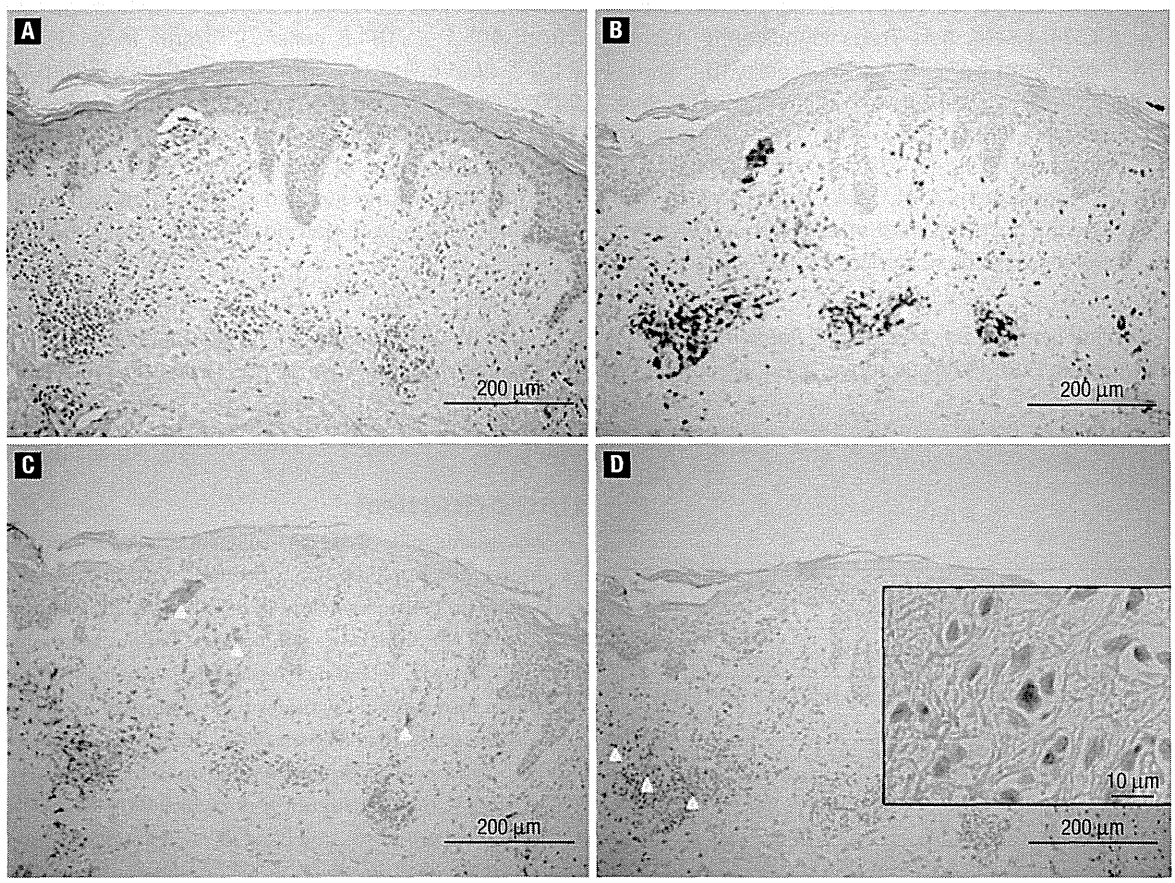
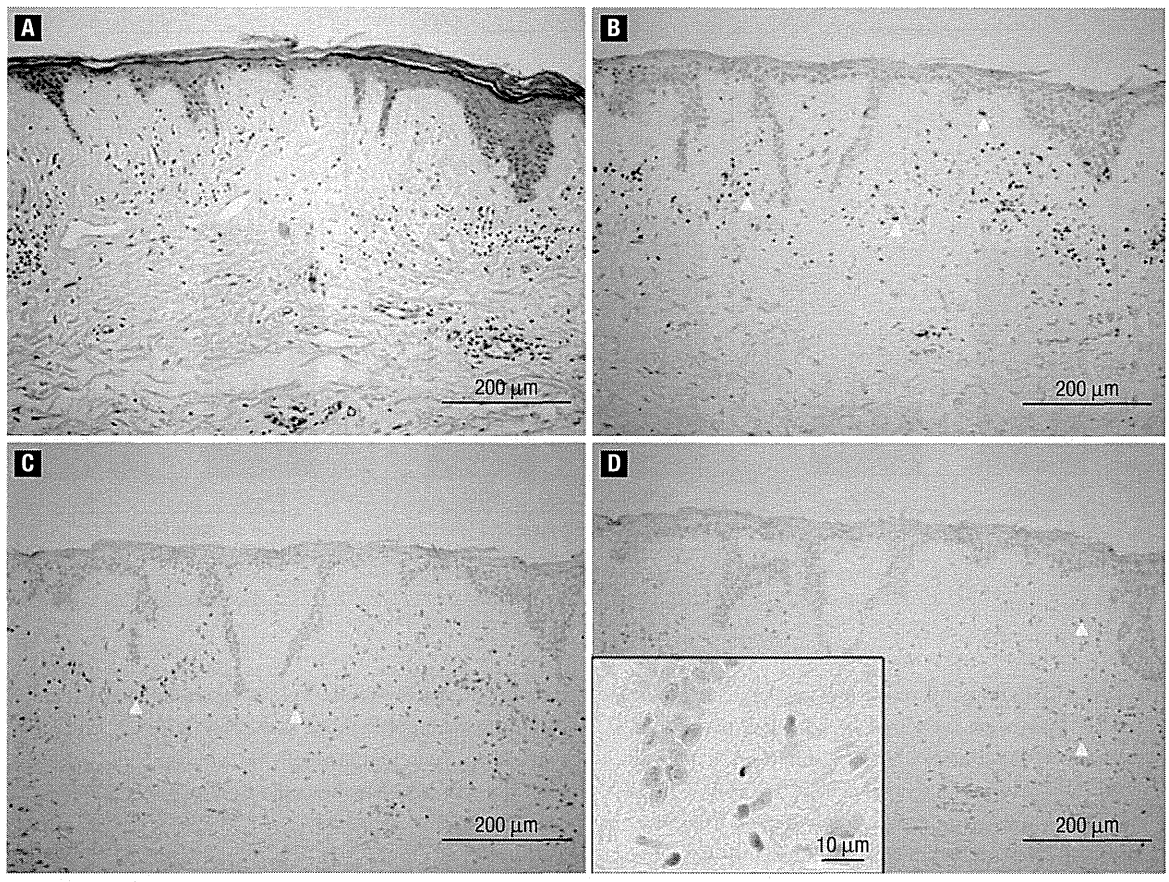


Figure 2 Immunohistochemical Analysis for After Bath-Psoralen Plus Ultraviolet A (PUVA) Therapy. Hematoxylin and Eosin Stain (A), Anti-CD3 (B), Anti-CCR4 (C), and Anti-Foxp3 (D). The Number of Infiltrating Cells was Decreased. Yellow Triangles Were Some of Positive Cells



superficial dermis. In the plaque stage (stage II), Pautrier microabscesses are often observed. In the tumor stage (stage III), tumor cells infiltrate the nodular lesions and proliferate with necrosis, and then ulcers form in the tumorous lesions. In stages I and II, the 5-year survival rate is > 90%, but in stage III the rate drops to approximately 40%.[3]

In the initial stage, topical corticosteroids are used for red plaques.[4] Psoralen ultraviolet A (PUVA) or narrowband UVB is used mainly for stage I.[5,6] More severe cases require radiotherapy and chemotherapy. There are some clinical reports of bath-PUVA therapy for MF.[5] We recently reported that bath-PUVA therapy induces circulating regulatory T cells (Tregs), which suppress effector T cells such as Th17, in patients with psoriasis.[7] The underlying mechanisms of bath-PUVA therapy in MF, however, are unclear. Therefore, we analyzed cells infiltrating the lesions before and after bath-PUVA therapy. Circulating lymphocytes in the peripheral blood were also analyzed.

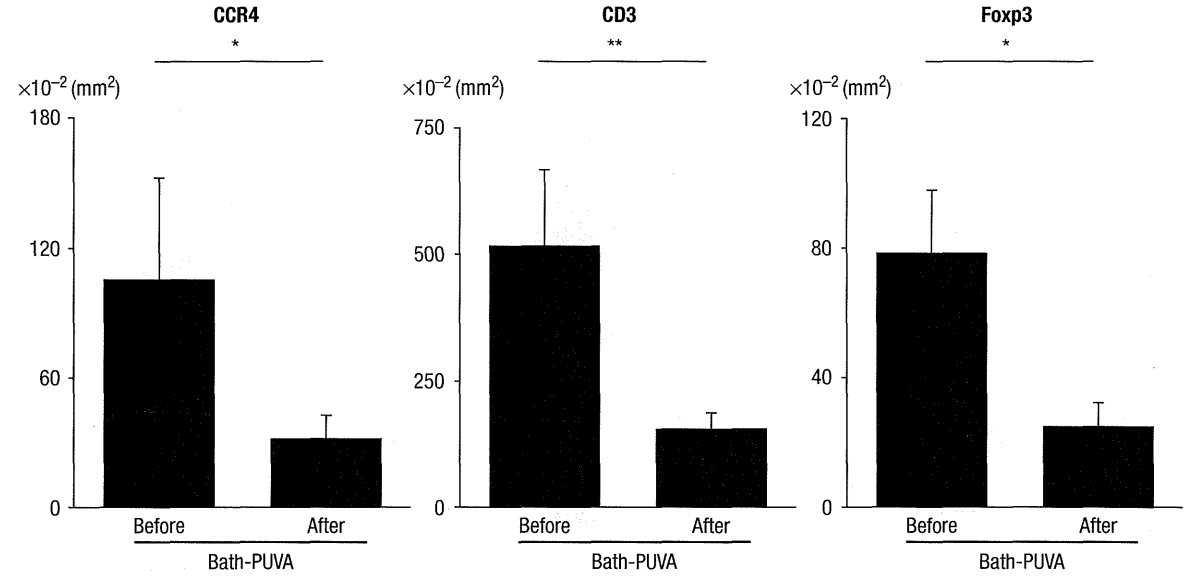
CCR4 is a chemokine receptor expressed on certain types of T-cell neoplasms, including MF and adult T-cell leukemia/lymphoma (ATLL).[8-10] Clinical development of the therapeutic humanized anti-CCR4 monoclonal antibody KW-0761 is in progress.[11] A phase I clinical trial of KW-0761 for patients with relapsed CCR4-positive ($CCR4^+$) T-cell neoplasms including MF was completed.[12] The subsequent phase II clinical trials of KW-0761 against relapsed ATLL (http://ClinicalTrials.gov Identifier: NCT00920790), untreated ATLL (NCT01173887), and relapsed peripheral T-cell lymphoma (NCT01192984) are currently being conducted in Japan. In the United States, a phase I/II clinical trial of KW-0761 against relapsed peripheral T-cell lymphoma has also been conducted (NCT00888927). In the present study, we analyzed the relationship between $CCR4^+$lymphocytes and some parameters from MF patients.

Patients and Methods

Patients

Thirteen patients of Japanese origin diagnosed with MF (mean age, 69.2 years; range, 35-87 years; 7 women and 6 men; 12 patients with stage IB and 1 patient with stage III disease) and 10 healthy controls (mean age, 31.7 years; range, 23-46 years; 6 women and 4 men) were recruited for the study. Serum-soluble interleukin-2

Figure 3 Number of Infiltrating Lymphocytes Before and After Bath–Psoralen Plus Ultraviolet A (PUVA) Therapy in Stage I MF. The Number of Lymphocytes Decreased After Bath-PUVA Therapy (*$P < .05$ by Wilcoxon signed rank test)



receptor (sIL2R) and thymus and activation-regulated chemokine (TARC)/CCL17 levels were measured in 13 and 5 patients, respectively, as part of the clinical blood examination at SRL Inc (Tokyo, Japan). Fluorescence-activated cell sorting (FACS) analysis (FACSCalibur Flow Cytometry System, Becton Dickinson, Franklin Lakes, NJ,) was applied in 6 cases. Punch biopsies of 3 or 4 mm were performed in the same lesion before and after bath-PUVA therapy. In healthy controls, only the peripheral blood was examined. The analysis was conducted with the approval from the Ethics Committee of Nagoya City University. Patient profiles are summarized in Table 1.

Bath-PUVA Therapy

Patients were placed in a 37°C bath containing 0.0001% 8-methoxypsoralen before UVA radiation treatment 5 times per week. A whole-body UVA radiation unit, the Dermaray TS (Eisai-Toshiba, Tokyo, Japan) with FLR100HBL/A/DMR fluorescence tubes, was used for UVA irradiation. The initial dose was 0.5 J/cm^2 with subsequent doses increased by increments of 0.5 J/cm^2 to a maximum dose of 4 J/cm^2. The mean number of irradiation treatments was 25.1. Mean cumulative UVA dose was 79.1 J/cm^2. The patient treatment profiles are summarized in Table 1.

Immunohistochemical Analysis

Staining for CD3, anti-CCR4 antibodies, and forkhead box P3 (Foxp3) was performed as follows. The sections were fixed with 10% neutral-buffered formalin. Formalin-fixed paraffin sections were stained with anti-CCR4 antibody (KM2160, Kyowa Hakko Kirin, Tokyo, Japan), polyclonal rabbit antihuman CD3 antibody (A0452, Dako, Carpinteria, CA), and antimouse monoclonal antibody to Foxp3 (236 A/E7, Abcam, Tokyo, Japan) following standard protocols using diaminobenzidine as the chromogen.[13] Positive cells in all sections were counted manually by 2 independent researchers (HK, CS). The sections were measured and the number of positive cells in each section was calculated.

FACS Analysis of Peripheral Blood Mononuclear Cells

Peripheral blood was obtained from patients before and after bath-PUVA therapy. Peripheral blood mononuclear cells (PBMCs) were obtained by density gradient centrifugation and stained with fluorescence-conjugated antihuman CD4 (MT310; Dako A/S, Glostrup, Denmark), CD25 (ACT-1, Dako A/S), Foxp3 (PCH101, eBioscience, San Diego, CA), and the appropriate isotype control antibodies followed by FACS analysis.

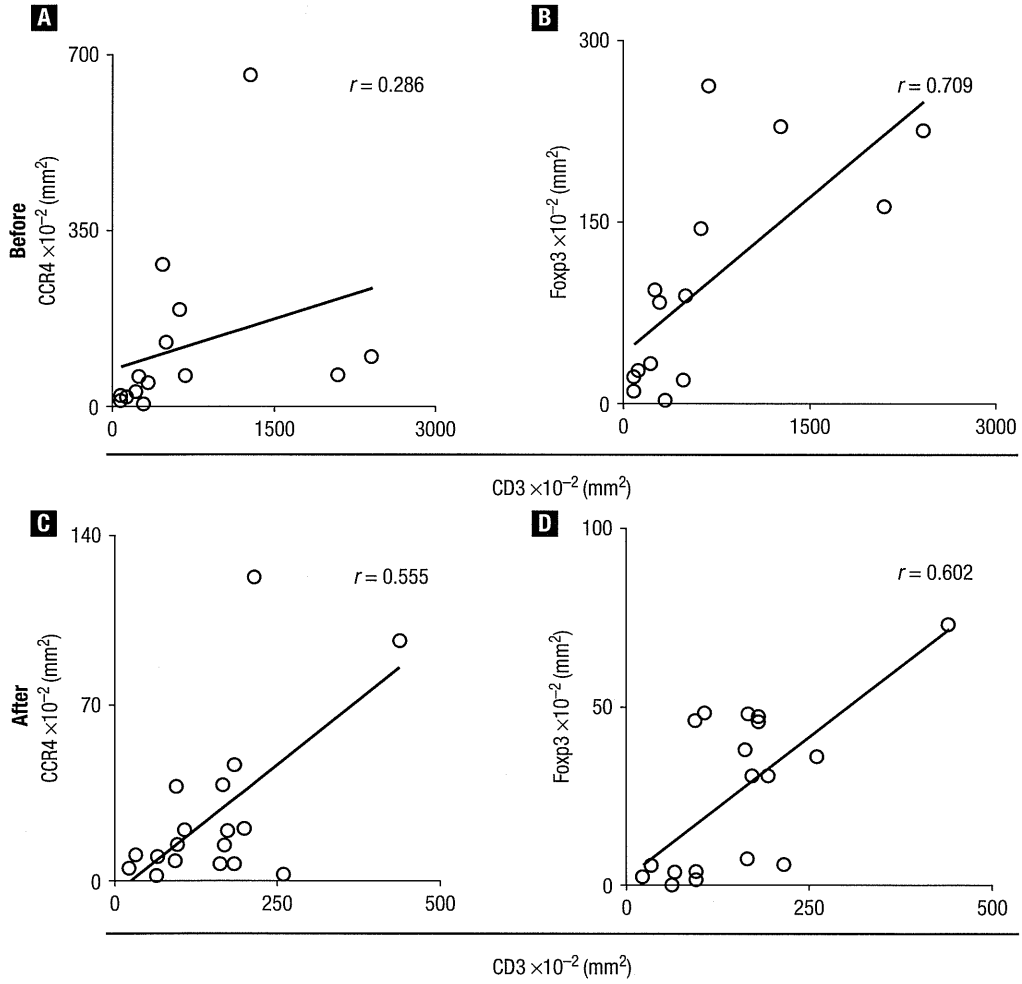
Statistical Analysis

The significance of changes in the variables before and after bath-PUVA therapy was examined using the Wilcoxon signed-rank test. Correlations between 2 variables obtained from patients with MF were assessed using the Spearman rank correlation coefficient. Differences in the variables between the 2 groups were examined with the Wilcoxon rank-sum test. Statistical analyses were performed using the Pharmaco Analyst II software (Human Life, Japan).

Results

Bath-PUVA therapy was well tolerated in all patients enrolled in the study. The red plaques in all patients improved clinically after bath-PUVA therapy. The number of atypical stage IB and stage III MF tumor cells are usually minimal, so it is difficult to clearly identify the tumor cells histologically from many reactive infiltrating

Figure 4 Correlation Between the Lymphocytes Before (A and B) and After (C and D) Bath–Psoralen Plus Ultraviolet A (PUVA) Therapy. There was No Significant Correlation Between CD3 and CCR4 Before Bath-PUVA Therapy, But There was a Positive Correlation After Bath-PUVA Therapy. Correlation Coefficients Were Determined by Spearman Rank Correlation Analyses



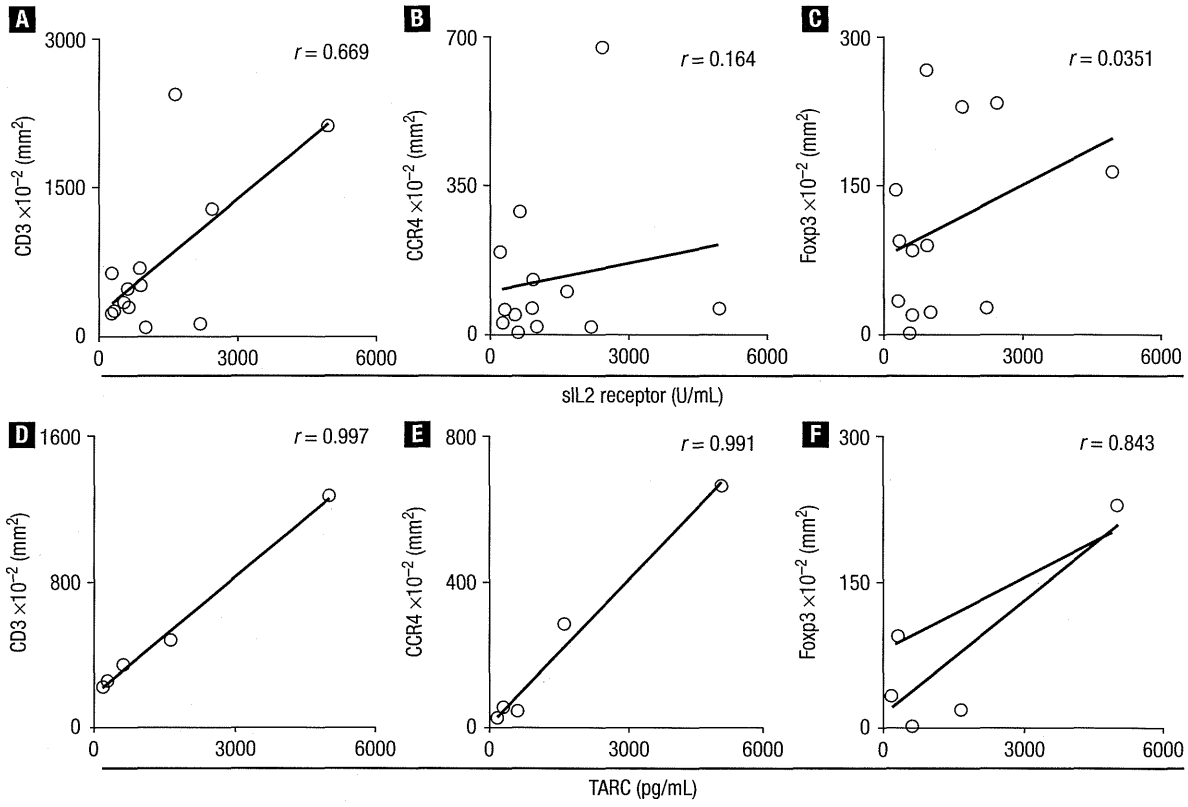
lymphocytes. Therefore we analyzed the numbers of whole stained lymphocytes, including both tumor and reactive cells (Figures 1 and 2). After bath-PUVA therapy, patients with stage I MF had a significant decrease in the number of infiltrating $CD3^+$, $CCR4^+$, and $Foxp3^+$ cells compared with the number of cells before therapy (Figure 3). The number of $CCR4^+$ cells in the lesion significantly decreased from 105.1 ± 164.8 cells/10^{-2} mm^2 to 31.4 ± 39.0 cells/10^{-2} mm^2. Similarly, Tregs in the lesion decreased from 78.1 ± 67.8 cells/10^{-2} mm^2 to 24.7 ± 25.0 cells/10^{-2} mm^2.

Before bath-PUVA, there was no significant correlation between the number of $CD3^+$ cells and $CCR4^+$ cells (r = 0.286) (Figure 4A), but after bath-PUVA, the levels of these cells were positively correlated (r = 0.555) (Figure 4C). The correlations between the number of $CD3^+$ and $Foxp3^+$ cells were significant before and after bath-PUVA therapy (r = 0.709; r = 0.602 (Figures 4B and D).

TARC/CCL17 is a CCR4 ligand used as a disease activity marker in atopic dermatitis,[14] and serum TARC/CCL17 levels are also correlated with MF disease activity.[15] Thus the correlation between the number of each type of infiltrating lymphocyte and sIL2R (n = 13) and TARC/CCL17 (n = 5) was evaluated (Figure 5). sIL2R levels were significantly correlated with the number of infiltrating $CD3^+$ cells (r = 0.669) but not with the number of infiltrating $CCR4^+$ and $Foxp3^+$ cells (r = 0.164 and r = 0.351, respectively). TARC/CCL17 levels were significantly correlated with the numbers of infiltrating $CD3^+$, $CCR4^+$, and $Foxp3^+$ cells (r = 0.997, r = 0.991, and r = 0.843, respectively) (Figure 5).

We then assessed the percentages of $CD4^+CD25^+Foxp3^+$ Tregs in $CD4^+$ T cells in PBMCs obtained from patients with MF and healthy volunteers. There was no significant difference between patients with MF and healthy volunteers. Bath-PUVA

Figure 5 Correlation Between Infiltrating Lymphocytes and Soluble IL-2 Receptor (sIL2) Levels (A-C) and Between Infiltrating Lymphocytes and Thymus and Activation-Regulated Chemokine (TARC) Levels (D-F). Soluble Interleukin-2 Receptor (sIL2) Levels Correlated Significantly With CD3, and TARC Levels Correlated Significantly With CD3, CDR4, and Foxp3. Correlation Coefficients Were Determined by Spearman Rank Correlation Analyses



therapy did not induce any significant change in the percentage of Tregs (Figure 6).

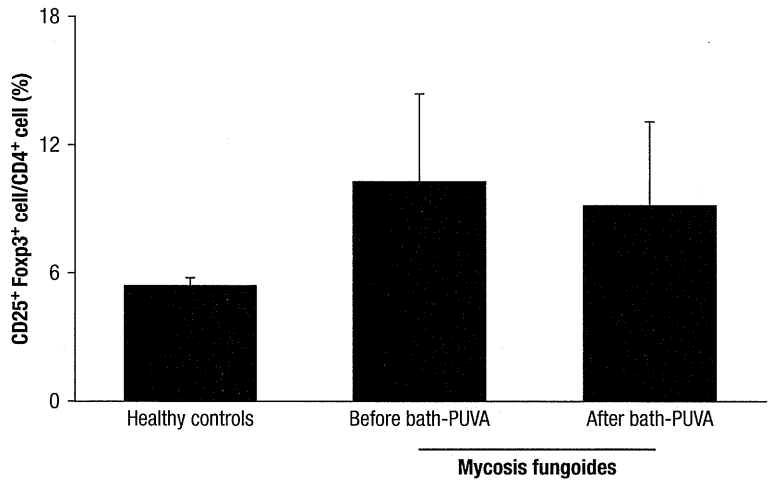
Discussion

The findings of the present study demonstrate that infiltration of both $CCR4^+$ and Tregs is eliminated by bath-PUVA therapy in patients with MF. The mechanism of the effectiveness of phototherapy for MF is not clear. For psoriasis treatment, there are 2 theories underlying the mechanisms of phototherapy: (1) induction of apoptosis in pathogenetically relevant cells[16] and (2) an immunosuppressive mechanism through the induction of Tregs.[7] The former is classified as an induction of apoptosis through oxygen radicals and caspase activation, leading to direct DNA damage. Photophoresis is a unique type of photochemotherapy using extracorporeal circulation and ultraviolet irradiation. In 1987, Edelson reported the efficacy of photophoresis treatment of cutaneous T-cell lymphoma.[17] A growing body of evidence indicates that photophoresis is also effective against other diseases.[18] Photophoresis induces monocytes to activate dendritic cells that express highly costimulatory molecules. The dendritic cells engulf the dying target cells,[19] leading to an immune response. Conversely, photophoresis induces antigen-specific Tregs. Therefore it is unclear how photophoresis regulates the immune responses toward both antiinflammatory and anticancer effects.

Leukocyte trafficking, which is critically regulated by chemokines and their receptors, shares many characteristics with tumor cell infiltration and metastasis. For example, CCR4 is a chemokine receptor selectively expressed on Tregs and Th2 cells[20-22] and also frequently expressed in ATLL cells, and its ligands TARC/CCL17 and macrophage-derived chemokine (MDC)/CCL22 are abundantly present in skin. We previously reported a significant association between the extent of CCR4 expression in ATLL cells and skin involvement.[9] With respect to MF, CCR4 expressed on the tumor cells also has a critical role in tumor formation in the skin.[23]

Generally, sIL2R is used as a tumor marker in cutaneous lymphoma such as in MF,[24] and in the present study sIL2R levels correlated positively with the number of infiltrating $CD3^+$ cells but not with that of infiltrating $CCR4^+$ and $Foxp3^+$ cells. TARC/CCL17 is also reported to be an MF tumor marker[15] and, as expected, serum TARC/CCL17 levels were significantly correlated with the numbers of infiltrating $CD3^+$, $CCR4^+$, and $Foxp3^+$ cells. These findings indicate that the number of the affected skin infiltrating $CD3^+$, $CCR4^+$, and $Foxp3^+$ cells reflect the disease activity of MF.

Figure 6 Fluorescence-Activated Cell Sorting (FACS) Analysis With Peripheral Blood Mononuclear Cells (PBMCs) Obtained From Healthy Volunteers or Patients With MF Before and After Bath–Psoralen Plus Ultraviolet A (PUVA) Therapy. There was No Significant Difference Between Patients With Mycosis Fungoides (MF) Before Bath-PUVA and Healthy Volunteers (Wilcoxon rank-sum test). Bath-PUVA Therapy Did Not Induce Any Significant Change in the Percentage of T-Regulatory Cells (Tregs). The Significance of Changes in the Variables Before and After Bath-PUVA Therapy was Examined Using the Wilcoxon Signed-Rank Test



PUVA is widely used as an effective treatment for cutaneous T-cell lymphoma.[25] MF and Sézary syndrome are the most frequent forms of cutaneous T-cell lymphoma. We previously reported that bath-PUVA therapy induces circulating Tregs in patients with psoriasis.[7] Tregs is a T-cell subset with immune function[26] that is associated with some immune diseases.[27] *FOXP3* is a master regulator gene for the differentiation of Tregs, and Foxp3 is a molecular marker of Tregs.[28] *FOXP3* gene transfection in naive T cells transforms naive T cells into Tregs and, simultaneously, naive T cells acquire CCR4 on their surface.[29]

In the present study, bath-PUVA therapy eliminated $Treg^+$ and $CCR4^+$ cells. Considering that there are some $CCR4^+$ cells among Tregs, the efficacy of bath-PUVA is similar to anti-CCR4 antibody treatment, as previously reported, regarding the decrease in the number of $CCR4^+$ cells.[8,12] In contrast, it was unclear that anti-CCR4 antibody was effective against $Foxp3^+$ cells. Based on the positive correlation between $CCR4^+$ and $CD3^+$cells after bath-PUVA therapy, the other type of T cells may have been eliminated.

Recently, Tregs were divided into naturally occurring Tregs and inducible Tregs.[30] Inducible Tregs are derived from the peripheral blood after antigen stimulation. In MF, Tregs are present in the initial stage, but the number of Tregs decreases in the more advanced stages.[31] This progression suggests a correlation between the number of Tregs and the prognosis of MF. Theoretically, the number of Tregs must be reduced in patients with MF. In psoriasis, bath-PUVA suppresses immunity and concomitantly induces improvement of the lesions. Immunosuppression might lead to an increase of the tumor cells in MF. In the present study, Tregs in the skin decreased after bath-PUVA. Moreover, the number of circulating Tregs in peripheral blood was not changed after bath-PUVA.

Based on these results, systemic immunosuppression is not induced by bath-PUVA therapy in patients with MF. It is generally accepted that increased Tregs in the tumor microenvironment have an important role in tumor escape from host immunity in several different types of cancer. Therefore depletion of Tregs in the tumor vicinity is considered a potential strategy for boosting antitumor immunity. In this context, the bath-PUVA therapy–induced reduction of Tregs observed in the present study may induce antitumor immunity and subsequent tumor elimination in MF skin lesions.

Clinical Practice Points

- MF is a malignant cutaneous lymphoma with a chronic disease progression.
- There are some clinical skin forms in MF according to STAGE. The tumor cells were T lymph cells, especially, it reported that CCR4 was expressed highly in the tumor cells.
- CCR4 is a chemokine receptor expressed on certain types of T cell neoplasms. In ATLL, the subsequent phase II clinical trials targeted CCR4 was started. For MF, some treatments were used such as topical steroid, phototherapy, and chemotherapy. Especially, bath-PUVA therapy was effective for the early STAGE of MF. However, the mechanism of the bath-PUVA therapy for MF was unclear.
- In the present study, bath-PUVA therapy decreased CCR4 positive cells and Treg in MF lesions, but did not induce circulating Treg, which might suppress effector T cells. Direct effects through skin lesions might eliminate both pathogenetically relevant cells and Treg.

- Considering that there are some CCR4-positive cells among Treg, the efficacy of bath-PUVA is similar to anti-CCR4 antibody treatment. Systemic immunosuppression was not induced by bath-PUVA therapy. It is generally accepted that increased Treg in the tumor microenvironment has an important role in tumor escape from host immunity in several different types of cancer.
- Based on these results, bath-PUVA therapy had possibility that widely applied for other disease.

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Disclosure

The authors have stated that they have no conflicts of interest.

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Case Report

Stevens–Johnson Syndrome associated with mogamulizumab treatment of adult T-cell leukemia/lymphoma

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We report an adult T-cell leukemia/lymphoma patient suffering from Stevens–Johnson Syndrome (SJS) during mogamulizumab (humanized anti-CCR4 monoclonal antibody) treatment. There was a durable significant reduction of the $CD4^+CD25^{high}FOXP3^+$ regulatory T (Treg) cell subset in the patient's PBMC, and the affected inflamed skin almost completely lacked FOXP3-positive cells. This implies an association between reduction of the Treg subset by mogamulizimab and occurrence of SJS. The present case should contribute not only to our understanding of human pathology resulting from therapeutic depletion of Treg cells, but also alert us to the possibility of immune-related severe adverse events such as SJS when using mogamulizumab. We are currently conducting a clinical trial of mogamulizumab for CCR4-negative solid cancers (UMIN000010050), specifically aiming to deplete Treg cells. (*Cancer Sci* 2013; 104: 647–650)

Adult T-cell leukemia/lymphoma (ATL) is an aggressive peripheral T-cell neoplasm caused by HTLV-1. The disease is resistant to conventional chemotherapeutic agents, and has a very poor prognosis.[1] Mogamulizumab (KW-0761) is a defucosylated humanized monoclonal antibody targeting CC chemokine receptor 4 (CCR4).[2] A phase I clinical trial for relapsed CCR4-positive peripheral T-cell neoplasms, including ATL, and a phase II study for relapsed ATL have been conducted with mogamulizumab.[3,4] This agent was subsequently approved for the treatment of relapsed or refractory ATL in Japan, the first country in the world to do so, in March 2012. Mogamulizimab went on sale on 29 May 2012. The interim report for the post-marketing surveillance from 29 May to 28 September 2012 revealed skin-related severe adverse events (SAE), as defined by the Medical Dictionary for Regulatory Activities Terminology/Japan, in nine patients. Thus, during only the first 4 months of use, 9 skin-related SAE, including 4 cases of Stevens–Johnson Syndrome (SJS)/toxic epidermal necrolysis (TEN) were reported, with 1 SJS/TEN fatality. These skin-related, potentially fatal SAE are certainly a challenge to the free use of this agent and clearly require investigation. Therefore, here we report an informative ATL patient suffering from SJS on mogamulizumab treatment, focusing on the reduction of the regulatory T (Treg) cell subset ($CD4^+CD25^{high}FOXP3^+$) caused by the antibody.

Case Report

A 71-year old woman was admitted due to elevation of her lymphocyte count. She had been diagnosed as suffering from acute-type ATL nearly 5 months prior to admission. She had received VCAP-AMP-VECP chemotherapy[5] followed by oral sobuzoxane in another hospital, and achieved a transient partial remission. We started mogamulizumab to treat the flare-up of ATL disease (Fig. 1). Grade 1 skin eruptions appeared around her neck after three antibody infusions. Because we were also giving her antibacterial (ciprofloxacin hydrochloride), fungal (itraconazole), pneumocystic (sulfamethoxazole-trimethoprim) and viral (aciclovir) prophylaxes in addition to stomach medicine (lansoprazole), we judged the skin event to be due to drug eruption caused by one of these concomitant drugs. Therefore, we stopped all five, but continued with mogamulizumab. Despite their discontinuation and treatment with topical steroids, the skin rashes continued to worsen. We started the patient on 30 mg oral prednisolone, which improved the skin symptoms. The patient was then able to complete the eight planned infusions, and oral prednisolone was tapered off. She was discharged from hospital 8 days after her eighth infusion (day 65), and thereafter seen as an outpatient. However, she had to be readmitted as an emergency patient at day 75 because of fulminant skin rashes. These included erythemas, scale-like plaques, vesicles, blisters and erosions over many areas of the body. Her lips were swollen and oral mucosa was erosive (Fig. 2a). Skin biopsy revealed marked liquefaction, degeneration and perivascular inflammation with dominant CD8-positive cells but almost complete lack of FOXP3-positive cells (Fig. 2b). We diagnosed her as a SJS, and immediately started steroid pulse therapy (methylprednisolone 500 mg/day $\times$3 days), followed by oral prednisolone. Her skin and mucosal lesions improved gradually, and became inactive. At the same time, her general condition improved. Thus, we again tapered the steroid dose, and she was discharged at day 144. However, she had to come back yet again as an emergency patient on day 151 for the same reason as before, with fulminant skin rashes. We prescribed her mini-steroid pulse therapy (methylprednisolone 125 mg/day $\times$1 day), followed by oral prednisolone. Once more, her skin lesions improved gradually. Over this whole period, complete ATL remission was maintained by mogamulizumab. The HTLV-1 provirus load in PBMC pre-treatment, and at days 121 and 162 was 750.1, 0.0 (under the limit of detection) and 0.8 copies/1000 cells, respectively. These post-treatment values are strikingly low, considering that median HTLV-1

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