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Materials and methods

Patient material

Serum and peripheral blood was collected from cancer patients. All patients were admitted at the University Hospital Zürich and provided written informed consent in accordance with the Declaration of Helsinki. The local ethics committee approved the study.

Memory B cell culture

PBMC were incubated with anti-CD22 coupled to magnetic beads (Miltenyi Biotec), PE-conjugated anti-IgD, and APC-conjugated antibodies to IgM, CD3, CD8, and CD56 (Becton Dickinson). B cells were isolated by positive selection of CD22+ cells using a midi-MACS device and LS columns (Miltenyi Biotec), followed by sorting PE-APC- cells using a MoFlo cell sorter (Beckman Coulter). CD22+ IgD- IgM- memory B cells were incubated with 10% EBV-containing supernatant from B95-8 cells (from European Collection of Cell Cultures, ECACC) in the presence of 2.5 μg/mL CpG 2006 at 37°C for 4 h. Cells were seeded in 96-well U-bottom plates at 10 cells per well plus 3 x 10^4 irradiated allogeneic PBMCs in RPMI 1640 medium supplemented with 10% human serum, antibiotics, 10% supernatant from B95-8 cells, and 2.5 μg/mL CpG 2006. Supernatants were tested for NY-ESO-1-specific antibodies after 2 weeks by ELISA.

Single cell-RT-PCR

B cell cultures were harvested and single cells were deposited into a 96-well PCR plate (Applied Biosystems) using a MoFlo XDP cell sorter (Beckman Coulter). RT-PCR was performed using random hexamer primers for cDNA synthesis and specific primers to amplify the immunoglobulin variable and constant regions. Immunoglobulin heavy and light chain variable regions were amplified using a nested PCR approach as described (36). Primer-encoded amino acid sequences and J-C regions of the antibodies were corrected to represent the authentic amino acid sequence as it occurred in the patient in a subsequent step prior to antibody production.

Antibody production and purification

293-T human embryonic kidney cells were transfected with 25 kDa branched polyethylenimine (PEI, Polysciences, Warrington, PA) plus DNA plasmids (heavy and light chain in equal ratios) in a 1.3:1 ratio and were incubated for 15 min at room temperature. Following transfection, the cells were cultured in serum free Opti-MEM I + GlutaMAX-I (Invitrogen) supplemented with 10 U penicillin-streptomycin (Lonza, Switzerland). After 72 h supernatants were collected and IgG was purified on a protein A column (GE Healthcare, Sweden) using FPLC (GE Healthcare, Sweden).

Biacore analysis

Antibody binding kinetics with NY-ESO-1 proteins derived from *E. coli* (LICR New York Branch) and HEK293 cells (OriGene Technologies, Inc.) were determined by Biacore technology (model Biacore 2000; Biacore AB) using CM5 sensor

chips, EDC-NHS conjugation, and BIAevaluation software. Technical details have been described previously (37).

ELISA

- Protein or peptide ELISA

96-well half-area microtiter plates (Costar, USA) were coated with 30 µL/well of 1 µg/mL recombinant NY-ESO-1 protein, or 10 µg/mL 20-mer peptides spanning the entire NY-ESO-1 protein (Peptides & Elephants, Germany) diluted in PBS overnight at 4°C. After coating, plates were washed with PBS + 0.05% Tween-20 (PBS-T) and blocked for 1 h at room temperature with 2% BSA/PBS (Sigma). B cell-conditioned medium, patient serum, or recombinant antibody was incubated for 2 h at room temperature (RT) at indicated concentrations or dilutions in PBS. Plates were washed with PBS-T and incubated for 1 h at RT with HRPO-conjugated goat-anti-human Fcγ antibody (Jackson ImmunoResearch), diluted 1:4000 in 0.5% BSA/PBS, followed by measurement of the HRPO activity using a TMB substrate solution (Sigma, Buchs, Switzerland). The mouse IgG1 monoclonal anti-NY-ESO-1 antibody E978 (38) and HRPO-conjugated goat-anti-mouse Fcγ antibody (Jackson ImmunoResearch) at 1:4000 dilution in 0.5% BSA/PBS served as positive control.

- Cellular ELISA

4 x 10^4 SK-MEL-37 cells were seeded in a 96-well flat bottom plate and cultured under standard conditions overnight. Cells were fixed in ice-cold ethanol/acetone mix (1:1) for 15 min on ice. After two wash steps with PBS, cells were blocked and permeabilized with 100 µL of PBS + 0.5% BSA + 0.5% Triton X 100 for 2 h at 4°C. B cell-conditioned medium or recombinant antibody was incubated at indicated concentrations for 2 h at 4°C. Bound antibodies were detected after 1 h incubation at 4°C with HRPO-labeled goat anti-human Fc secondary antibody (Jackson ImmunoResearch).

Immunoprecipitation

SK-MEL-37 tumor cells were lysed with Triton X 100/ Glycerol-based lysis buffer for 15 min at 4°C. Cell debris was separated by centrifugation at maximum speed in a table centrifuge and protein concentration of the supernatant analyzed by standard Bradford assay. 300 ng of antibody was used to precipitate NY-ESO-1 from 250 µg of SK-MEL-37 cell lysate in a 16 h incubation at 4°C. The immune complex was isolated by adding magnetic Protein G beads (New England Biolabs, Ipswich, MA) for 1 h at 4°C under constant agitation. Beads were washed, resuspended in NuPAGE LDS sample buffer (Invitrogen) and boiled prior to Gradient SDS Polyacrylamide Gel Electrophoresis (NuPAGE 4-12% Bis-Tris Gel, Invitrogen). NY-ESO-1 protein was detected by Western blot using murine antibody E978 (38).

***In vitro* cross-presentation assay**

Human, HLA-A*0201/NY-ESO-$1_{157-165}$ specific CD8+ T cells were cloned as previously described (39). To generate DCs, CD14+ cells were MACS-purified according to the manufacturer's instructions (Miltenyi Biotech) from PBMC from HLA-A*0201+ healthy donors and cultured at 10^6 cells/mL in serum-free CellGro DC media (CellGenix), supplemented with 800 U/mL GM-CSF and 500 U/mL IL-4 (R&D Systems) to generate DCs. Medium was exchanged the following day and DCs were harvested on d 4 of culture and resuspended at 10^6/mL in Opti-MEM (Gibco). Immune complexes were generated by incubating 20 µg recombinant NY-ESO-1 with 200 µg 12D7 in a total volume of 500 µL Opti-MEM (Gibco) for 30 min at 37°C. Human IgG1 (Sigma Aldrich), 12D7 alone, or NY-ESO-1 alone were used as controls. Alternatively, 200 µg 12D7 was incubated with a lysate of an equivalent of 10^7 NY-ESO-1+ SK-MEL-37 cells in 500 µL Opti-MEM. DCs (5x10^5 in 0.5 mL Opti-MEM) were added to the immune complexes and controls. The mixture was incubated at 37°C for 3 h. DCs were then centrifuged and resuspended in CellGro DC media at 10^6/mL. Hundred µL (10^5 DCs) were cultured in 96-well flatbottom plates at 37°C in the presence or absence of maturation cocktail (1 µg/mL soluble CD40L (sCD40L) trimer (PeproTech) plus TNF-α (25 ng/mL; R&D Systems)). After 36 h, approximately 6 x 10^5 HLA-A*0201/NY-ESO-$1_{157-165}$ specific CD8+ T cells in 100 µL RPMI + 10% human serum + antibiotics + 20 µg/mL Brefeldin A (Sigma Chemicals) were added to the different DC-cultures. After 4 h, cultures were harvested in FACS buffer (PBS + 2% FCS + 2 mM EDTA + 0.05% NaN_3) and surface stained with anti-CD8 followed by intracellular staining for IFN-γ as previously described (39). CD8+ T cells plus unloaded DCs served as negative control, and CD8+ T cells plus DCs with 10^{-6} M of NY-ESO-$1_{157-165}$ (SLLMWITQC, Thermo Fisher Scientific) served as positive control. All cultures were performed at least in duplicates.

Mice and cell lines

BALB/c mice were originally obtained from Jackson Laboratories and were bred and kept under specific pathogen-free conditions in the Institute of Laboratory Animal Sciences (University of Zürich). Age- and sex-matched mice of 9-12 weeks old were used for all experiments. Mice were housed under specific pathogen-free conditions at University Hospital Zürich. All experiments were performed in agreement with the federal and cantonal laws on animal protection.

The colon carcinoma cell line CT26 was transfected to stably express intracellular NY-ESO-1 (40) and was cultured in RPMI + 10% FCS + antibiotics + 10 µg/mL puromycin under standard tissue culture conditions. CT26/NY-ESO-1 and the human melanoma cell line SK-MEL-37 were cultured in RPMI + 10% FCS + antibiotics under standard tissue culture conditions. 293-T cells were cultured in DMEM (Lonza, Switzerland) supplemented with 10% FCS (Linaris) and 10 U penicillin-streptomycin (Lonza, Switzerland) under standard tissue culture conditions.

Treatment of mice

Mice were injected s.c. into the right flank with 10^6 CT26/NY-ESO-1+ cells in 100 µL RPMI. The tumor surface was measured at least twice a week with a calliper. Treatment was started (d 0) when tumors reached a size of approximately 25 mm^2. 5-Fluorouracil (5-FU, TEVA Pharma, Aesch, Switzerland) was diluted in saline and were given i.p. on d 0 and d 7 at 75 mg/kg, respectively. 12D7 (100 µg in 100 µL PBS) was given i.p. on d 2 and d 9. All animal experiments were performed in accordance with the Swiss federal and cantonal law on animal protection.

Flow cytometry

At the end of the experiment (1 week after the last injection of 12D7), mice were injected i.p. with 250 µg Brefeldin A and were euthanized 4 h later. Subsequent processing and staining was performed in the presence of 10 µg/mL Brefeldin A (25). Tumors were cut into small pieces and subsequently digested with 1.5 mg/mL collagenase + 100 µg/mL DNase for 1 h at 37°C followed by filtration through a 50 µm cell strainer. Single cell suspensions were surface stained in FACS buffer (FB, PBS + 2% FCS + 0.03% NaN_3 + 20 mM EDTA) with anti-CD45.2 pacific

blue and anti-CD8bPE. For intracellular staining to detect IFN-γ, cells were permeabilized with permeabilization buffer (PB, FB + 0.1% saponin) and stained intracellularly with anti-IFN-γ APC. All antibodies were obtained from BioLegend, San Diego, CA, USA. Samples were measured with a CyAn ADP9 (Beckman Coulter, Brea, CA, USA) and analyzed using FlowJo Analysis Software (Tree Star Inc., Ashland, OR, USA).

Statistical analysis

Statistics were done using an unpaired Student two-tailed *t*-test. Error bars represent SD. p values less than 0.05 were considered significant.

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Manipulation of human early T lymphopoiesis by coculture on human bone marrow stromal cells: Potential utility for adoptive immunotherapy

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T cell precursors are an attractive target for adoptive immunotherapy. We examined the regulation of human early T lymphopoiesis by human bone marrow stromal cells to explore in vitro manipulation of human T cell precursors in a human-only coculture system. The generation of $CD7^+CD56^-cyCD3^-$ proT cells from human hematopoietic progenitors on telomerized human bone marrow stromal cells was enhanced by stem cell factor, flt3 ligand, and thrombopoietin, but these stimulatory effects were suppressed by interleukin 3. Expression of Notch ligands Delta-1 and -4 on stromal cells additively promoted T cell differentiation into the $CD7^+cyCD3^+$ pre–T cell stage, while cell growth was strongly inhibited. By combining these coculture systems, we found that initial coculture with telomerized stromal cells in the presence of stem cell factor, flt3 ligand, and thrombopoietin, followed by coculture on Delta-1– and -4–coexpressing stromal cells led to a higher percentage and number of pre–T cells. Adoptive immunotherapy using peripheral blood T cells transduced with a tumor antigen-specific T cell receptor (TCR) is a promising strategy but has several limitations, such as the risk of forming a chimeric TCR with the endogenous TCR. We demonstrated that incubation of TCR-transduced hematopoietic progenitors with the combination of coculture systems gave rise to $CD7^+TCR^+CD3^+CD1a^-$ T cell precursors that rapidly proliferated and differentiated under the culture condition to induce mature T cell differentiation. These data show the regulatory mechanism of early T lymphopoiesis on human stromal cells and the potential utility of engineered human stromal cells to manipulate early T cell development for clinical application. © 2013 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.

Adoptive immunotherapy with T cell precursors is considered useful to treat T cell immunodeficiency or enhance immune reconstitution after hematopoietic stem cell transplantation [1–3]. Although the difficulty of in vitro manipulation of T cell precursors from hematopoietic progenitors still hampers their clinical application, the culture system has improved considerably after discovering that the Delta ligand–mediated Notch pathway has a central role in T cell differentiation at various stages [4–9]. In vivo studies of mice show that Notch-1 [4,7,10,11] and Delta-4 [4,7,12,13] are critical for regulation of the B versus T lineage choice of common lymphoid precursors by promoting T cell differentiation, while inhibiting B cell differentiation in the thymus and bone marrow [4–7]. Based on in vitro studies using human hematopoietic progenitors, immobilized forms of Delta-1 ligand or expression of Delta-1 or -4 on murine bone marrow stromal cell lines have been shown to promote T cell differentiation into the pre–T cell stage from human hematopoietic progenitors, while inhibiting B cell differentiation [9,14,15]. However, Delta-1 expression on the OP-9 murine stromal cell line allows generation of $CD4^+CD8^+$ T cell precursors from human hematopoietic progenitors [16,17], although the function of Delta ligand expression on human bone marrow

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stromal cells remains to be elucidated. Moreover, cytokine regulation of human early T lymphopoiesis has been less studied compared with that of early B lymphopoiesis [18–20], because of the lack of an appropriate culture system that supports early T cell differentiation.

Immunotherapy therapy using peripheral T cells engineered to express a tumor antigen–specific T cell receptor (TCR) is a novel and promising strategy [21,22]; however, this strategy presents several challenges. For example, transfer of the TCR into mature T cells has the risk of forming a chimeric TCR of transduced and endogenous TCRs and may exert an unexpected adverse response against other antigens [23–26]. Furthermore, it is uncertain how long the engineered mature T cells persist in vivo. When the TCR gene is transduced into hematopoietic progenitors, formation of the endogenous TCR is prevented in mature T cells derived from the TCR-transduced hematopoietic progenitors [27,28], because of the allelic exclusion mechanism at the TCR-β locus [29]. Nevertheless, gene therapy that targets hematopoietic stem cells has the risk of leukemia development [1].

In this study, we examined cytokine- and Notch-mediated regulation of human early T cell development by coculture with telomerized human bone marrow stromal cells, which support early B and T lymphopoiesis [30], and determined the potential of this coculture system with engineered human stromal cells for clinical application.

Methods

Isolation of $CD34^+$ hematopoietic progenitors

After obtaining informed consent, umbilical cord blood was collected from full-term deliveries according to a protocol approved by the Ethics Committee of Mie University Hospital. $CD34^+$ or $CD34^+CD38^{lo/-}CD7^-CD19^-CD10^-$ hematopoietic progenitor cells were then purified from the mononuclear cells [31].

Recombinant factors

Thrombopoietin (TPO) was a gift from the Kirin Brewery (Tokyo, Japan). Recombinant stem cell factor (SCF), flt3 ligand (Flt3L), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL) 3, IL-7, and IL-15 were purchased from PeproTech (Rocky Hill, NJ, USA). Cytokines were used at the following concentrations: SCF, 10 ng/mL; TPO, 10 ng/mL; Flt3L, 5 or 10 ng/mL; G-CSF, 10 ng/mL; GM-CSF, 10 ng/mL; IL-3, 10 ng/mL; IL-7, 5 ng/mL; IL-15, 10 ng/mL.

Transduction of Delta-1 and -4 genes into telomerized stromal cells

Complementary DNAs (cDNAs) of human Delta-1 in a pMKITneo vector and Delta-4 in a pcDNA3 vector (provided by Dr. Seiji Sakano) were inserted into the *EcoRI/NotI* sites of enhanced green fluorescent protein (EGFP) or Kusabira Orange (KO) retroviral vectors [32,33] to generate pMXs-(Delta1 or Delta4)-IRES-EGFP and pMXs-(Delta1 or Delta4)-IRES–KO vectors. pMXs-IRES-EGFP (GFP mock) and pMXs-IRES-KO (KO mock) vectors were used as controls. Transfection of retroviral vectors into PLAT-A cells [33] was performed as described elsewhere [34], except for the use of 5 mg/mL polybrene (Sigma-Aldrich, St. Louis, MO, USA). Transduced cells were isolated based on GFP or KO expression using a FACSAria (BD Biosciences, San Jose, CA, USA). Delta-1 and -4 expression was confirmed by Western blotting, by applying cell lysates consisting of 2×10^5 cells to an anti-FLAG M2 monoclonal antibody (1:1000; Sigma-Aldrich) and horseradish peroxidase–labeled goat anti-mouse immunoglobulin G (1:5000; Promega, Madison, WI, USA).

Flow cytometric analysis

Immunofluorescence staining was performed as reported previously [30,31] using the following murine monoclonal antibodies: anti-CD4 (BD Pharmingen, San Diego, CA, USA), anti–CD14-FITC (BioLegend, San Diego, CA, USA), anti–CD56-FITC (BD Pharmingen), anti–CD7-PE (Beckman Coulter, Fullerton, CA, USA), anti–CD19-PE (BD Bioscience), anti–T cell α/β receptor-PE (BD Pharmingen), anti–CD34-PE (BD Bioscience), anti–CD1a-APC (BioLegend), anti–CD3-APC (Beckman Coulter), anti–CD7-APC (eBioscience, San Diego, CA, USA), and anti–CD8-APC (BD Pharmingen).

Cocultures

Maintenance and cocultures of human telomerase reverse transcriptase-transduced telomerized stromal cells were performed as described previously [30]. Cocultures of OP9 stromal cells overexpressing Delta-1 (a gift from Dr. Juan Carlos Zúñiga-Pflücker) were performed as described elsewhere [17,30]. Viable cell numbers were determined by trypan blue exclusion.

Transduction of the retroviral vector carrying the TCR into hematopoietic cells

The retroviral vector encoding MAGEA4-specific TCR-α (TRAV8-1) and TCR-β (TRBV7-9) genes (MSbPa retroviral vector) has been described previously [35]. Transduction of the MSbPa retroviral vector into hematopoietic progenitors was performed by culture on RetroNectin (Takara Bio, Shiga, Japan)-coated plates preloaded with retroviral solutions [35,36].

Vβ detection

Total RNA and cDNA were prepared as described previously [30]. cDNA samples were amplified using Vβ-specific primers with a C3′ primer [37] at a final concentration of 0.5 μmol/L for each reaction. Polymerase chain reaction (PCR) was performed with 2.5 U Ex-Taq polymerase (Takara Bio) and a Dice/Takara PCR thermal cycler under the following conditions: 35 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 15 sec. PCR products were separated on 2% agarose gels, and Vβ family genes were identified by Southern blotting using nylon membranes (Roche Diagnostics, Mannheim, Germany) and a probe (5′-gtgttcccacccgaggtcgctgtgtttgagccatcagaa-3′) labeled with Amersham AlkPhos Direct Labeling Reagents (GE Healthcare, Aliso Viejo, CA, USA). Signals were detected using an LAS-1000plus (Fujifilm, Tokyo, Japan). DNA bands at 170–220 bp were Vβ family genes. Vβ of the transduced TCR was 6.1–6.3.

Data analysis

Statistical comparisons were performed using Student t test. Differences were considered significant at $p < 0.05$.

Results

Cytokine-mediated regulation of CD7$^+$CD56$^-$ proT cell generation from human hematopoietic progenitors

We reported previously that human telomerized bone marrow stromal cells support the generation of CD7$^+$CD56$^-$cyCD3$^-$ proT cells from human hematopoietic progenitors, which is enhanced by SCF and TPO in the presence of Flt3L [30]. To elucidate whether pro–T cell generation is further augmented by other cytokines, CD34$^+$CD38$^{lo/-}$CD7$^-$CD19$^-$CD10$^-$ cells were cultured with SCF, Flt3L, TPO, IL-3, IL-6, GM-CSF, G-CSF, and IL-15, some of which have been shown to augment human B or T cell generation [18–20,38], or combinations of these cytokines with SCF, Flt3L, and TPO for 3 weeks. As a single agent, Flt3L considerably enhanced the generation of CD7$^+$CD56$^-$ cells from hematopoietic progenitors, which was further enhanced by combining with SCF and TPO (Fig. 1A) as reported previously [30]. The addition of IL-3 or GM-CSF to cultures with SCF, Flt3L, and TPO (3GF) exerted an inhibitory effect on the generation of CD7$^+$CD56$^-$ T cells. No or few effects were observed by the addition of IL-6, G-CSF, or IL-15 to cultures with 3GF (Fig. 1A). Under all culture conditions, CD7$^+$CD56$^-$ cells were negative for cytoplasmic CD3 (cyCD3; data not shown). Similar effects were observed in the generation of CD19$^+$ proB cells by these cytokines (data not shown). To elucidate whether the inhibitory effect of IL-3 on T cell generation occurs by directly acting on hematopoietic progenitors or by an indirect action via stromal cells, CD34$^+$CD38$^{lo/-}$CD7$^-$CD19$^-$CD10$^-$ cells were cultured either with stromal cells or without stromal cells but supplemented with conditioned medium collected from cultures of stromal cells in the presence of 3GF or 3GF plus IL-3. As shown in Figure 1B, IL-3 addition to cultures with 3GF inhibited the generation of CD7$^+$ and CD19$^+$ cells from hematopoietic progenitors, even without stromal cells, as observed in cultures with stromal cells. Based on the expression profiles of CD34 and CD38, the percentage of cells expressing CD34 in cultures treated with IL-3 was lower than in those without IL-3, and there were no CD34$^+$CD38$^-$ cells in both culture conditions (Supplementary Figure 1, online only, available at www.exphem.org). These data indicate that the suppression of T and B lymphoid differentiation owing to IL-3 is not caused by maintenance of hematopoietic progenitors in an immature state.

Telomerized stromal cells transduced to express Delta-1 or -4 inhibit B cell differentiation and induce pre–T cell differentiation

To examine the effect of high levels of Delta ligand expression on human B and T lymphopoiesis, human Delta-1 and -4 genes were transduced into telomerized human stromal cells by retroviral vectors (Fig. 2A). Although only low levels of Delta-1 and -4 mRNA were detected in telomerized stromal cells transduced with vectors containing GFP or KO alone and in nontransduced stromal cells, which is consistent with a previous report [30]. Significantly high

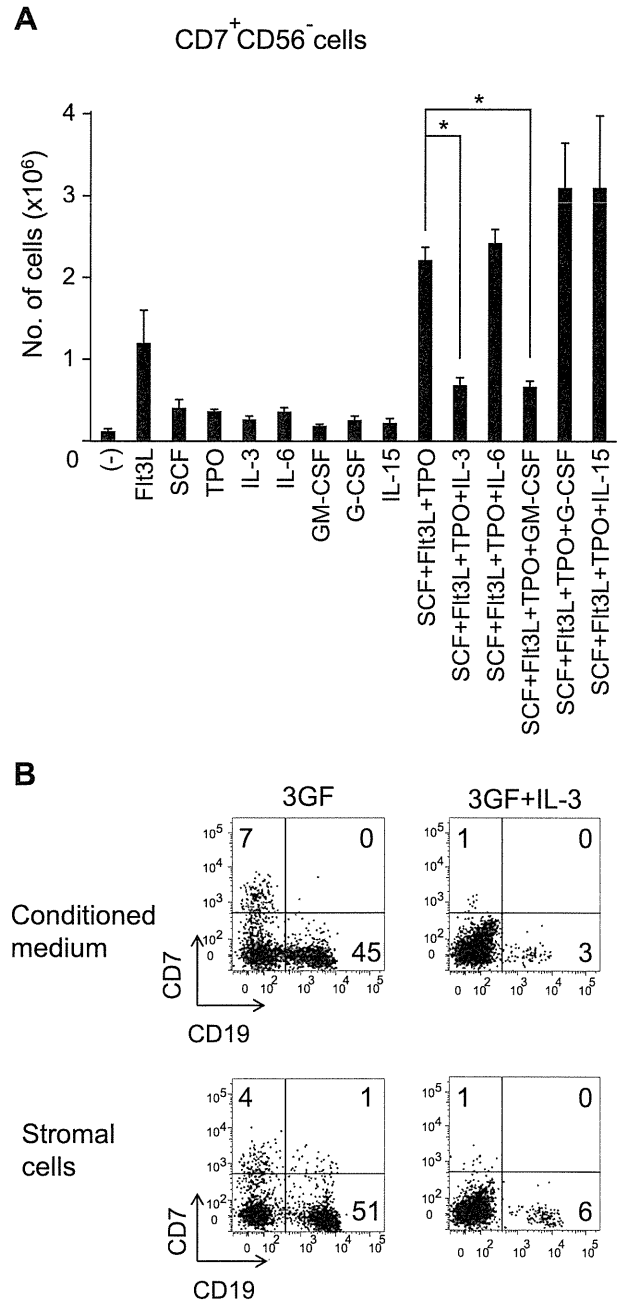


Figure 1. Cytokine regulation of CD7$^+$CD56$^-$ proT cell development. **(A)** CD34$^+$CD38$^{lo/-}$CD7$^-$CD19$^-$CD10$^-$ cells (2×10^4 cells/well) were cultured on telomerized stromal cells in the presence of the indicated cytokines for 21 days, and the number of CD7$^+$CD56$^-$ cells was analyzed. Data are the means ± SD of triplicate cultures and representative of three independent experiments. *$p < 0.05$ compared with cultures containing SCF, Flt3L, and TPO. **(B)** CD34$^+$CD38$^{lo/-}$CD7$^-$CD19$^-$CD10$^-$ cells were cultured with or without stromal cells in the presence or absence of IL-3. In cultures without stromal cells, half of the culture medium was replaced every 3 days with conditioned medium obtained from stromal cell cultures. The phenotypes of cells after exclusion of the CD14$^+$ population in cultures with the indicated cytokines are shown.

levels of Delta-1 or -4 mRNA were detected after transduction of Delta-1–GFP, Delta-1–KO, Delta-4–GFP, or Delta-4–KO genes into stromal cells (Fig. 2B). Expression of Delta-1 and -4 proteins in transduced stromal cells was confirmed by Western blot analysis (Fig. 2C).

We cocultured $CD34^+$ hematopoietic progenitors on GFP-, Delta-1–GFP– or Delta-4–GFP–transduced stromal cells in the presence of 3GF for 21 days, and then analyzed the number and phenotype of cultured cells. After 21 days, coculture with Delta-1–GFP– or Delta-4–GFP–transduced stromal cells strongly inhibited cell proliferation compared with that of GFP-transduced stromal cells (Fig. 3A). Phenotypically, $CD7^+CD34^{lo/-}CD1a^-cyCD3^-$ proT and $CD19^+CD34^{lo/-}$proB cells were generated by coculture with GFP-transduced stromal cells. These findings were similar to those obtained by coculture with nontransduced stromal cells (Supplementary Figure 2, online only, available at www.exphem.org). However, coculture with Delta-1–GFP– or Delta-4–GFP–transduced stromal cells led to a higher percentage of $CD7^+CD34^{+/lo}$ T cell precursors. These cells were negative for CD1a, a phenotype of T lineage-committed precursors [39–41], but expressed cyCD3. The generation of $CD19^+$ proB cells was inhibited by Delta-1– or Delta-4–transduced stromal cells (Fig. 3B). Similar data were obtained by coculture with stromal cells transduced with KO, Delta-1–KO or Delta-4–KO (data not shown). These data suggest that Delta-1 or -4 expression on stromal cells inhibits B cell differentiation and promotes T cell differentiation into the pre–T cell stage.

Coexpression of Delta-1 and -4 on stromal cells

Delta-1 or -4 expression on stromal cells similarly promoted pre–T cell differentiation in vitro. However, it has been suggested that the role of Delta-1 and -4 in T lymphopoiesis is not the same based on in vivo murine studies [42]. We therefore examined whether coexpression of Delta-1 and -4 on stromal cells further augmented T cell differentiation from hematopoietic progenitors. After coculture of $CD34^+$ cells on stromal cells that expressed GFP, Delta-1–GFP, Delta-4–KO or both Delta-1–GFP and Delta-4–KO for 21 days in the presence of 3GF, the percentage and number of $CD7^+cyCD3^+$ pre–T cells were increased by coculture on stromal cells expressing either Delta-1 or -4 as described earlier. Cocultures with stromal cells coexpressing Delta-1 and -4 additively increased the percentage and number of $CD7^+cyCD3^+$ cells (Fig. 4A and 4B). Cells cultured on Delta-1– and Delta-4–coexpressing stromal cells were still negative for CD1a (data not shown). Hairy and enhancer of split homolog-1 (HES-1) is a downstream target gene of the Notch pathway [4]. The expression level of HES-1 was elevated in $CD34^+$ cells cultured on Delta-1– or Delta-4–transduced stromal cells, compared with that of noncultured $CD34^+$ cells or $CD34^+$ cells cultured on GFP-expressing telomerized stromal cells, but an additional increase in the expression levels of HES-1 was not observed by coculture on stromal cells coexpressing Delta-1 and -4 (Supplementary Figure 3, online only, available at www.exphem.org).

Generation of T cell precursors from TCR-transduced hematopoietic progenitors

Because telomerized human bone marrow stromal cells coexpressing Delta-1 and -4 were found to strongly promote T cell differentiation from human hematopoietic progenitors, the clinical utility of this culture system was examined. We examined whether T cell precursors engineered to express a tumor antigen–specific TCR without an endogenous TCR could be efficiently generated from TCR-transduced hematopoietic progenitors in our culture system. In addition, the effect of TCR expression on the proliferation and differentiation of hematopoietic progenitors toward the T cell lineage was studied.

We first attempted to improve the coculture system to generate a higher number of pre–T cells by combining telomerized stromal cells and Delta ligand-transduced stromal cells, because Delta ligand-transduced stromal cells severely inhibited the proliferation of hematopoietic progenitors. The following four culture conditions were tested: (1) coculture on stromal cells that coexpressed Delta-1 and -4 (D1D4 stromal cells) for 21 days; (2) coculture on D1D4 stromal cells for 7 days followed by telomerized stromal cells for 14 days; (3) coculture on telomerized stromal cells for 7 days followed by D1D4 stromal cells for 14 days; and (4) coculture on telomerized stromal cells in the presence of 3GF for 21 days. Cocultures on D1D4 stromal cells for the initial 7 days strongly inhibited cell growth similarly to that in cocultures on D1D4 stromal cells for 21 days. However, growth inhibition was moderate when cocultures started with telomerized stromal cells followed by D1D4 stromal cells, suggesting that cell growth mainly occurred from hematopoietic progenitor to $CD7^+cyCD3^-$ proT cell stages (Fig. 5, left column). The highest number of $CD7^+cyCD3^+$ pre–T precursors was also obtained by coculture with telomerized and then D1D4 stromal cells (Fig. 5, right column).

Based on our data, $CD34^+$ cells were transduced with retroviral vectors carrying the TCR, which specifically recognized the cancer-specific antigen MAGE-A4 [35], on day 2 during the initial 7-day period of coculture on telomerized stromal cells, and then cultured on D1D4 stromal cells for an additional 14 days in the presence of 3GF. At 21 days after coculture on telomerized and then D1D4 stromal cells, a low percentage of $CD7^+TCR^+$ cells was detected. TCR-positive cells were still negative for CD1a, a marker of T-lineage committed precursors [39,43], but CD3 was expressed, presumably in accordance with engineered expression of the TCR (Fig. 6A). Thus, T cell precursors generated from TCR-transduced hematopoietic progenitors by coculture with telomerized followed by D1D4 stromal

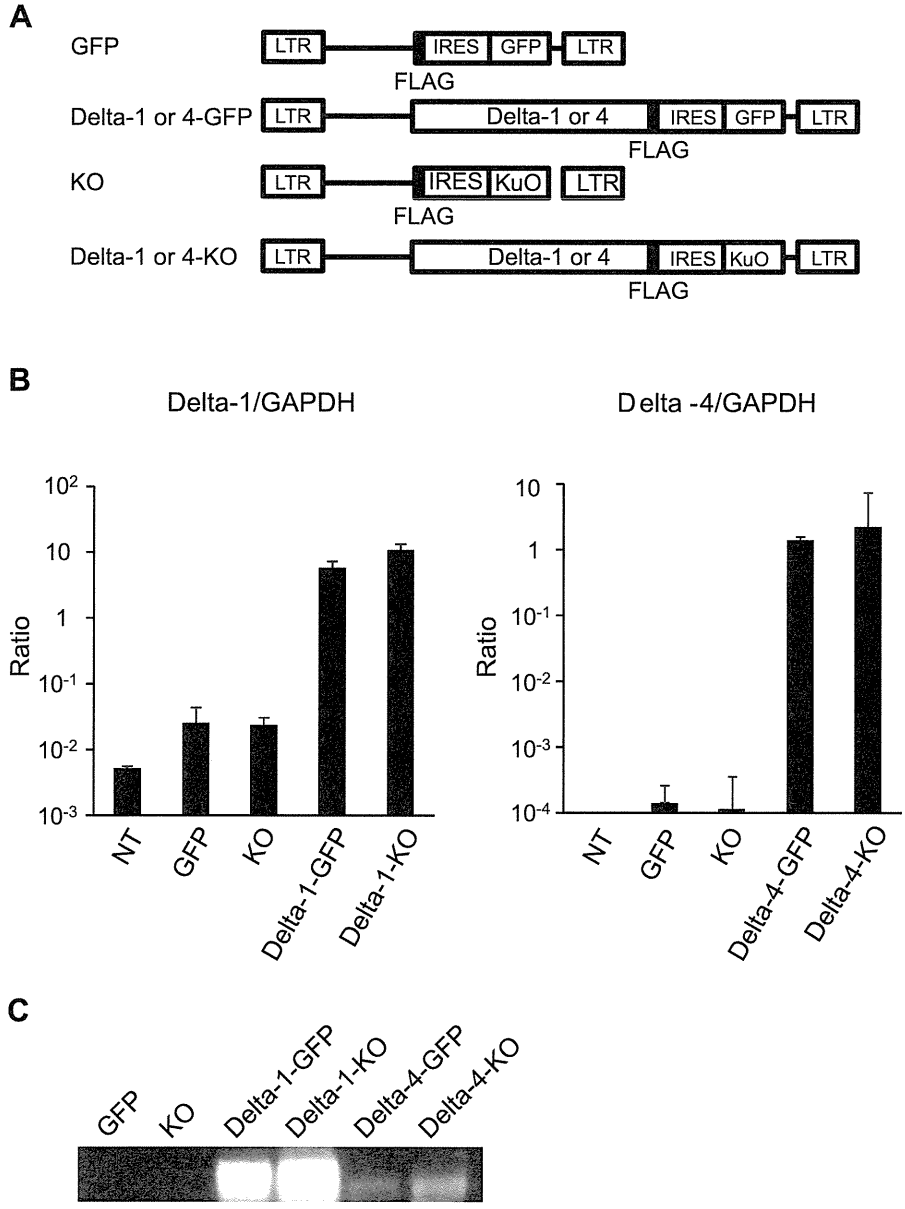


Figure 2. Expression of Delta-1 and -4 in telomerized stromal cells. **(A)** Constructs of GFP, Delta-1–GFP, Delta-4–GFP, KO, Delta-1–KO, and Delta-4–KO. **(B)** Expression of Delta-1 and -4 mRNA in nontransduced stromal cells or stromal cells transduced with GFP, KO, Delta-1–GFP, Delta-1–KO, Delta-4–GFP, or Delta-4–KO vectors. **(C)** Expression of Delta-1 and -4 proteins in stromal cells transduced with GFP, KO, Delta-1–GFP, Delta-1–KO, Delta-4–GFP, or Delta-4–KO vectors.

cells were considered as pre–T cells. To further examine the differentiation and proliferation potentials of the $CD7^+TCR^+$ cells, all cultured cells were recultured on Delta-1–expressing OP9 stromal cells in the presence of Flt3L and IL-7, a condition that supports mature T cell differentiation [16,17]. On days 11–19 after reculture, the percentage and number of $CD7^+TCR^+$ cells rapidly and remarkably increased compared with the number of nontransduced $CD7^+TCR^-$ cells (Fig. 6A and 6B). Phenotypically, a significant proportion of $CD7^+TCR^+$ cells differentiated beyond the $CD1a^+$ stage and coexpressed surface CD3, although only a low percentage of TCR^+ cells became positive for CD8 and CD4 under our culture condition. Most $CD7^+TCR^+$ cells were negative for the NK cell marker CD56 (Fig. 6B). To evaluate whether the TCR, which was expressed by T cell precursors that differentiated on Delta-1–expressing OP-9 stromal cells, was derived from the transduced TCR and not the endogenous TCR, the Vβ repertoire of the TCR was analyzed by reverse transcriptase PCR of Vβ-Cβ transcripts. Almost all types of Vβ chains were detected in normal peripheral blood as expected, but only the Vβ 6.1–6.3 region derived from the transduced TCR was detected in cultured cells (Fig. 6C). These data indicate that coculture of TCR-

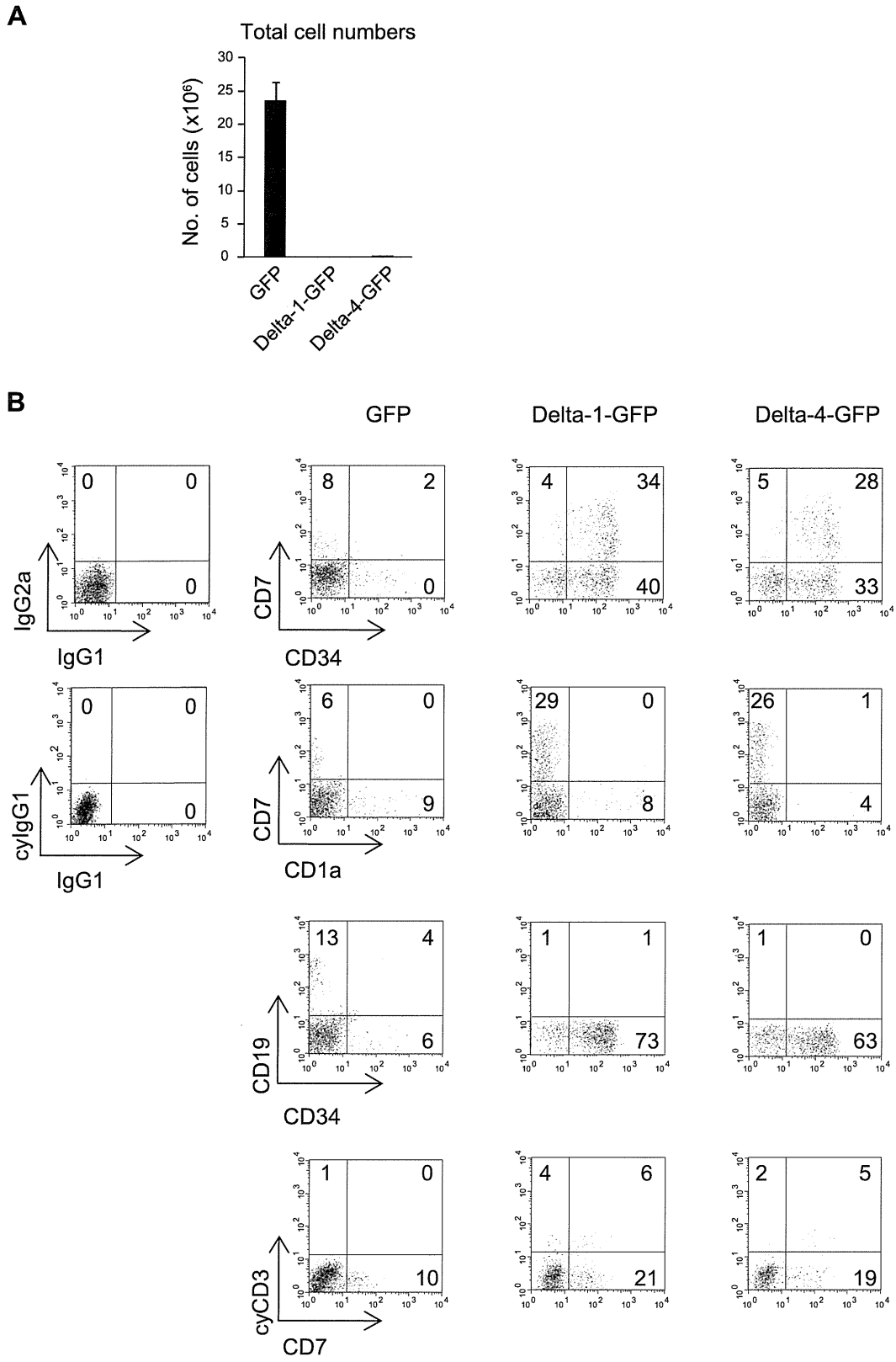
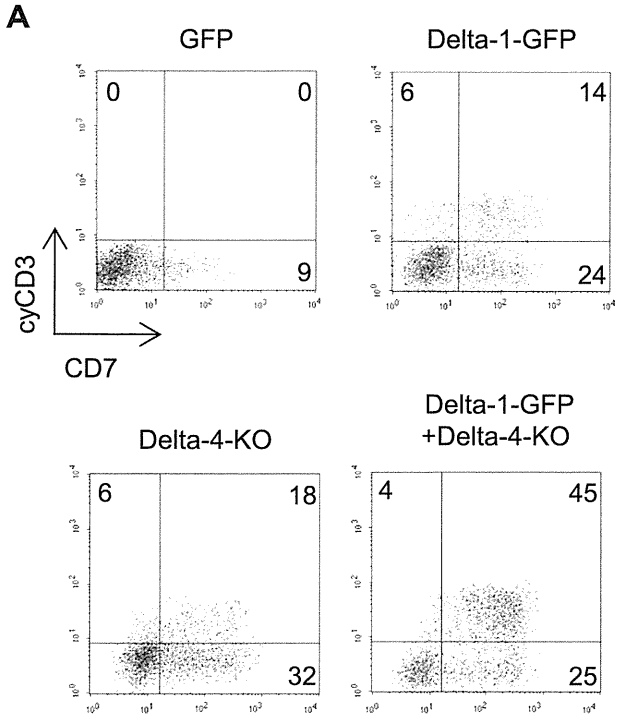


Figure 3. Effect of Delta-1 and -4 expression on T and B cell differentiation. Total cell numbers **(A)** and the phenotype **(B)** of cells after coculture of $CD34^+$ cells (4×10^4 cells/well) on telomerized stromal cells transduced with GFP, Delta-1–GFP or Delta-4–GFP in the presence of 3GF for 3 weeks. Data are the means ± SD of triplicate cultures and are representative of four independent experiments.



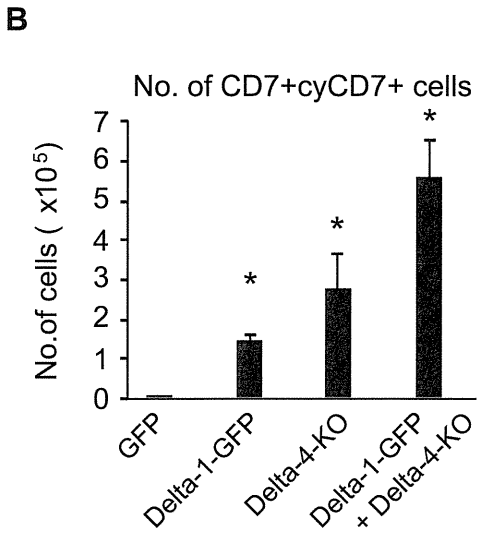


Figure 4. Combinatorial effect of Delta-1 and -4 expression on T cell differentiation. The percentage **(A)** and number **(B)** of $CD7^+cyCD3^+$ T cells after coculture of $CD34^+$ cells (4×10^4 cells/well) on stromal cells expressing GFP, Delta-1–GFP, Delta-4–KO or both Delta-1–GFP and Delta-4–KO with 3GF for 3 weeks are shown. Data are the means ± SD of triplicate cultures. Representative data from three independent experiments are shown. $*p < 0.05$ compared with control cultures.

transduced hematopoietic progenitors on telomerized followed by D1D4 stromal cells can produce pre–T cell precursors that have the potential to proliferate and differentiate under an appropriate culture condition.

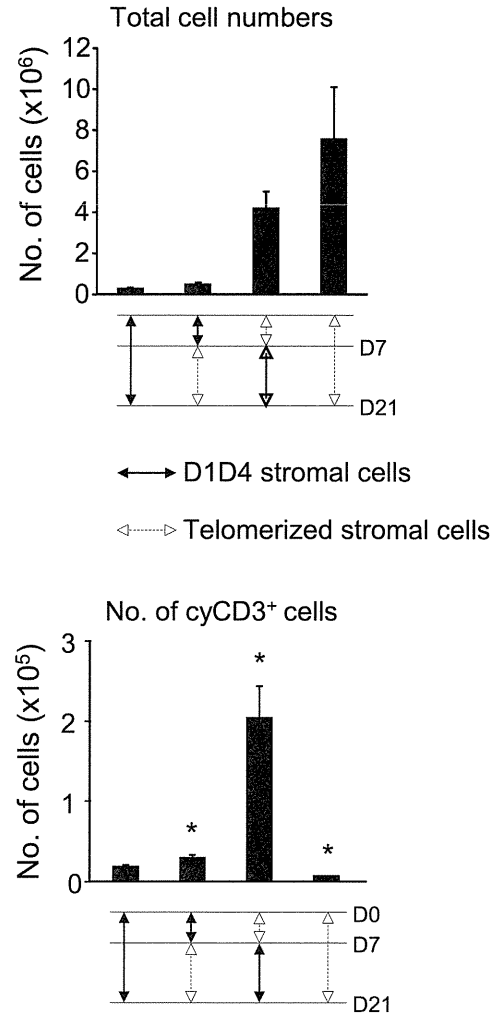


Figure 5. Optimal condition for generation of a higher number of $CD7^+cyCD3^+$ preT cells. $CD34^+$ cells (4×10^4 cells/well) were cocultured on stromal cells transduced with Delta-1 and -4 (D1D4 stromal cells) in the presence of 3GF for 21 days, D1D4 stromal cells for 7 days followed by telomerized stromal cells for 14 days, telomerized stromal cells for 7 days followed by D1D4 stromal cells for 14 days, or telomerized stromal cells for 21 days. Total cell numbers and the percentage and number of $CD7^+cyCD3^+$ preT cells were assessed. Data are the mean of duplicate cultures.

Discussion

In this study, we showed that the generation of early T cell precursors from hematopoietic progenitors was modulated positively or negatively by cytokines, and combinations of SCF, Flt3L, and TPO were best suited to enhance proT cell generation on telomerized stromal cells. Delta-1 and -4 expression on stromal cells additively promoted T cell differentiation into pre–T stages, although cell growth was strongly suppressed. By combining these coculture systems, we showed that a higher percentage and number of pre–T cells can be generated from hematopoietic progenitors, and this culture system could be useful to develop immunotherapy using engineered T cell precursors.

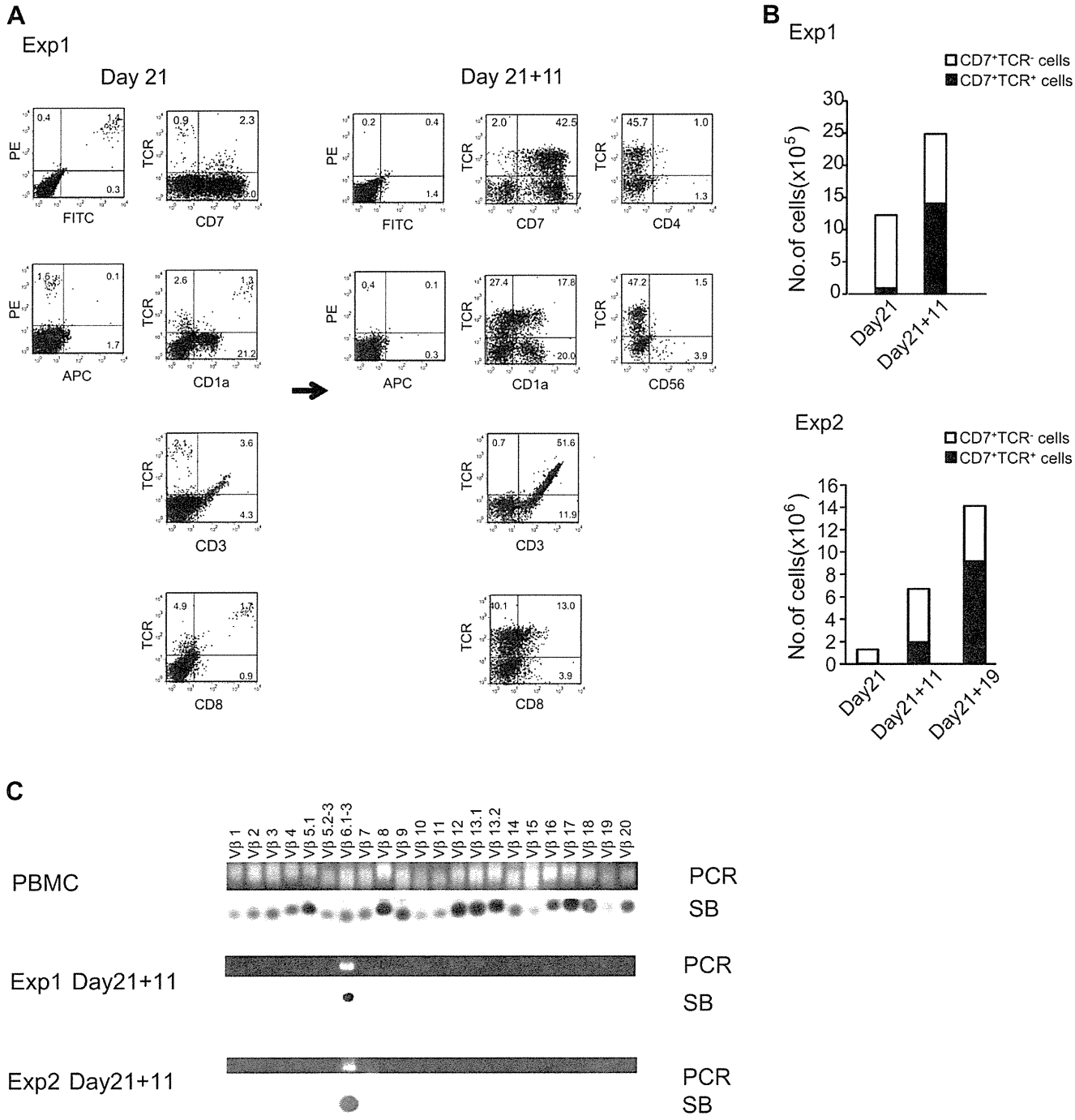


Figure 6. Generation of T cell precursors from TCR-transduced hematopoietic progenitors. $CD34^+$ cells (4×10^4 cells/well) were transduced with the TCR during coculture on telomerized stromal cells for 7 days, and then recultured on D1D4 stromal cells for 14 days in the presence of 3GF. Cultured cells were then recultured on Delta-1–transduced OP-9 stromal cells in the presence of Flt3L (5 ng/mL) and IL-7 (5 ng/mL) for an additional 11 or 19 days. The phenotype **(A)** and number **(B)** of $CD7^+TCR^-$ and $CD7^+TCR^+$ cells were assessed. Data are the mean of duplicate cultures. **(C)** PCR analysis of Vβ in peripheral blood mononuclear cells (PBMCs) or cells cocultured for 11 days on Delta-1–expressing OP9 stromal cells after coculture on telomerized stromal cells and then D1D4 stromal cells for 21 days. PCR products were evaluated using Southern blot (SB) analysis.

Among the cytokines, SCF, Flt3L, and TPO coordinately promoted the generation of proT and proB cells from human hematopoietic progenitors on stromal cells. Conversely, these effects were inhibited by IL-3 and GM-CSF by directly acting on hematopoietic progenitors. Similar effects by cytokines were observed in the generation of plasmacytoid dendritic cells belonging to the lymphoid lineage (data not shown) [44]. An inhibitory effect of IL-3 on B cell development has been suggested by other studies using murine stromal cells [19,45],

but our data are the first to demonstrate that IL-3 suppresses the generation of various types of lymphoid precursors on human bone marrow stromal cells. Because no effect was observed with other cytokines, such as G-CSF, IL-6, and IL-15, a different approach, such as engineered production of Hox B4 protein from stromal cells, would be required to obtain higher numbers of T cell precursors [46].

Delta-1 or -4 expression on stromal cells similarly promoted pre–T cell differentiation, and their coexpression additively promoted preT cell differentiation. It remains uncertain whether Delta-1 and -4 ligands bind to distinct Notch receptors or identical Notch receptors with different affinities [7,11–13,42,47]. The mechanism of the additive effect of Delta-1 and -4 cannot be explained by HES-1 gene expression. However, our study suggests that coexpression of Delta-1 and -4 on stromal cells induces a higher percentage of hematopoietic progenitors to differentiate into pre–T cells. Notably, although even bone marrow stromal cells transduced with Delta-1 and -4 did not support T cell differentiation into the $CD1a^+$ cell stage, Delta ligand expression on human thymic stromal cells promotes differentiation into $CD7^+CD1a^+$ cells that are detectable in the thymus [48]. These data imply that not only Delta ligand-mediated Notch signaling, but also unknown signals from thymic stromal cells are required for T cell differentiation into the $CD7^+CD1a^+$ stage.

Transduction of the TCR into hematopoietic progenitors followed by coculture on Delta-transduced human bone marrow stromal cells led to the generation of pre-T cells expressing the TCR, although the transduction efficiency of the TCR into hematopoietic progenitors appeared remarkably lower than that in previous studies targeting mature T cells [35]. Nonetheless, these TCR-transduced T cell precursors, upon coculture with Delta-1–expressing OP-9 murine stromal cells, promptly and remarkably proliferated and differentiated toward $CD8^+$ cells, relative to that of nontransduced T cell precursors. Although it has been speculated that TCR-transduced T lymphoid precursors differentiated toward $CD8^+$ cells rather than $CD4^+$ cells, presumably because of a lack of human leukocyte antigen class II expression on OP-9 stromal cells [49], it is interesting to note that similar rapid growth has been observed in other studies by coculture of TCR-transduced hematopoietic progenitors on Delta-1–expressing OP-9 murine stromal cells from the beginning of cultures [27,28,49]. Further investigation is required to elucidate whether such rapid proliferation of TCR-transduced T cell precursors occurs at or beyond the $CD1a^+$ stage, or by a specific interaction with OP-9 murine stromal cells. Nevertheless, these studies will contribute to our understanding of the regulation of human early T lymphopoiesis on bone marrow stromal cells and to the development of novel therapies with T cell precursors.

Acknowledgments

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Conflict of interest disclosure

H.S. received research funding from Takara Bio. No other financial interest/relationships with financial interest relating to the topic of this article have been declared.

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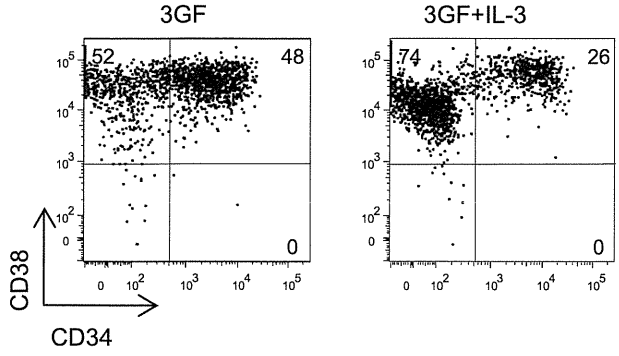
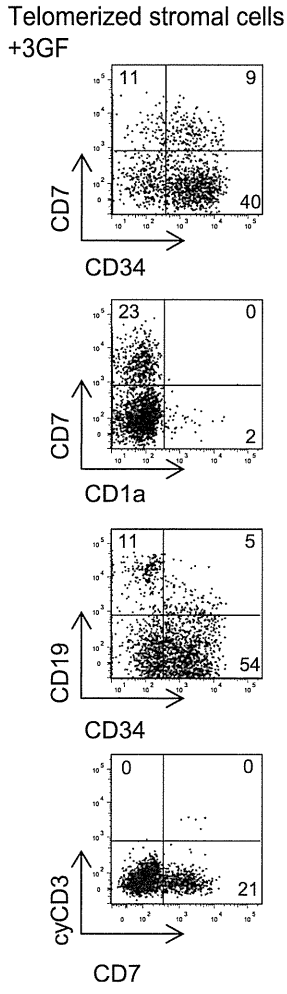
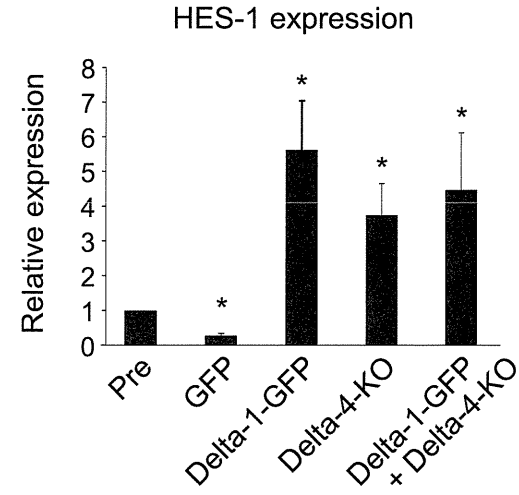


Supplementary Figure 1. Effect of IL-3 on the differentiation of hematopoietic progenitors. $CD34^{+}CD38^{lo/-}CD7^{-}CD19^{-}CD10^{-}$ cells were cultured with telomerized stromal cells and 3GF in the presence or absence of IL-3. The expression of CD34 and CD38 after exclusion of the $CD14^{+}$ population is shown.



Supplementary Figure 2. T and B cell differentiation on nontransduced telomerized stromal cells. The phenotypes of $CD34^{+}$ cells (4×10^{4} cells/well) after coculture on nontransduced telomerized stromal cells in the presence of 3GF for 3 weeks are shown.



Supplementary Figure 3. Comparison of HES-1 expression. Relative expression of HES-1 mRNA in uncultured $CD34^{+}$ cells and cells generated by coculture of $CD34^{+}$ cells on stromal cells transduced with GFP, Delta-1–GFP, Delta-4–KO or Delta-1–GFP plus Delta-4–KO vectors in the presence of 3GF for 24 hours are shown. Relative gene expression was calculated as the fold induction compared with untreated $CD34^{+}$ cells. Data are the means ± SD of triplicate cultures. Quantitative reverse transcriptase PCR was performed by modification of a previously published method [33]. Hairy and enhancer of split homolog-1 (HES-1) primers were obtained from Assays on-Demand (Assay ID: Hs00172878_m1; Applied Biosystems, Foster, CA, USA). PCR conditions were as follows: initial denaturation at 95°C for 15 min, and then 50 cycles of denaturation at 94°C for 1 min, and annealing and extension at 60°C for 1 min. Transcript quantification was performed in triplicate for each sample. Gene expression was normalized to that of endogenous glyceraldehyde-3-phosphate dehydrogenase as an internal standard (Pre-Developed TaqMan Assay Reagents, 4326317E; Applied Biosystems). Relative gene expression was calculated as a fold induction compared with that in untreated $CD34^{+}$ cells.

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ORIGINAL REPORT

Defucosylated Anti-CCR4 Monoclonal Antibody (KW-0761) for Relapsed Adult T-Cell Leukemia-Lymphoma: A Multicenter Phase II Study

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ABSTRACT

Purpose
Adult T-cell leukemia-lymphoma (ATL) is usually resistant to conventional chemotherapies, and there are few other treatment options. Because CC chemokine receptor 4 (CCR4) is expressed on tumor cells from most patients with ATL, KW-0761, a humanized anti-CCR4 monoclonal antibody, which markedly enhances antibody-dependent cellular cytotoxicity, was evaluated in the treatment of patients with relapsed ATL.

Patients and Methods
A multicenter phase II study of KW-0761 for patients with relapsed, aggressive CCR4-positive ATL was conducted to evaluate efficacy, pharmacokinetic profile, and safety. The primary end point was overall response rate, and secondary end points included progression-free and overall survival from the first dose of KW-0761. Patients received intravenous infusions of KW-0761 once per week for 8 weeks at a dose of 1.0 mg/kg.

Results
Of 28 patients enrolled onto the study, 27 received at least one infusion of KW-0761. Objective responses were noted in 13 of 26 evaluable patients, including eight complete responses, with an overall response rate of 50% (95% CI, 30% to 70%). Median progression-free and overall survival were 5.2 and 13.7 months, respectively. The mean half-life period after the eighth infusion was 422 ± 147 hours (± standard deviation). The most common adverse events were infusion reactions (89%) and skin rashes (63%), which were manageable and reversible in all cases.

Conclusion
KW-0761 demonstrated clinically meaningful antitumor activity in patients with relapsed ATL, with an acceptable toxicity profile. Further investigation of KW-0761 for treatment of ATL and other T-cell neoplasms is warranted.

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INTRODUCTION

Adult T-cell leukemia-lymphoma (ATL) is an aggressive peripheral T-cell neoplasm caused by human T-cell lymphotropic virus type I. The disease is resistant to conventional chemotherapeutic agents, and there currently exist limited treatment options; thus, it has a poor prognosis.[1-4] A recent phase III trial for previously untreated patients with aggressive ATL (acute, lymphoma, or unfavorable chronic type) age 33 to 69 years demonstrated that a dose-intensified multidrug regimen, VCAP-AMP-VECP (vincristine, cyclophosphamide, doxorubicin, and prednisone; doxorubicin, ranimustine, and prednisone; and vindesine, etoposide, carboplatin, and prednisone), resulted in median progression-free (PFS) and overall survival (OS) of 7.0 and 12.7 months, respectively.[5] This remains unsatisfactory compared with responses in other hematologic malignancies. Allogeneic hematopoietic stem-cell transplantation has evolved into a potential approach to treating patients with ATL over the last decade. However, only a small fraction of patients with ATL have the opportunity to benefit from transplantation, such as those who are younger, have achieved sufficient disease control, and have an appropriate stem-cell source.[6,7] Therefore, the development of alternative treatment strategies for patients with ATL is an urgent issue.

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Because CC chemokine receptor 4 (CCR4) is expressed on tumor cells from most patients with ATL,[8,9] we postulated that it might represent a novel molecular target for immunotherapy. Accordingly, KW-0761, a next-generation humanized anti-CCR4 immunoglobulin G1 (IgG1) monoclonal antibody (mAb) with a defucosylated Fc region, which markedly enhances antibody-dependent cellular cytotoxicity (ADCC), was developed.[10,11] We demonstrated that robust ADCC by the defucosylated anti-CCR4 mAb against primary tumor cells from patients with ATL mediated by autologous effector cells was triggered both in vitro and in a humanized mouse model in vivo.[11-13] These promising preclinical results prompted us to conduct a phase I clinical trial of KW-0761 for patients with relapsed CCR4-positive peripheral T-cell lymphoma (PTCL), including ATL. This study demonstrated good tolerability, predictable pharmacokinetics, and preliminary evidence of potent antitumor activity and resulted in a recommended dose of 1.0 mg/kg for subsequent clinical trials.[14] Herein, we report the results of a multicenter phase II study designed to assess the efficacy, pharmacokinetic profile, and safety of KW-0761 monotherapy in patients with relapsed CCR4-positive aggressive ATL.

PATIENTS AND METHODS

Patients

Patients 20 years of age or older with CCR4-positive aggressive ATL (acute, lymphoma, or unfavorable chronic type)[1,4] who had relapsed after at least one prior chemotherapy regimen were eligible. The unfavorable chronic type of ATL was defined by the presence of at least one of the following three factors: low serum albumin, high lactate dehydrogenase, or high blood urea nitrogen concentration.[5] CCR4 expression was determined by immunohistochemistry or flow cytometry using a mouse anti-CCR4 mAb (KM2160)[8,14] and confirmed by a central review committee. All patients were required to have an Eastern Cooperative Oncology Group performance status of 0 to 2. Eligibility criteria also included the following laboratory values: absolute neutrophil count $\geq$ 1500/μL, platelet count $\geq$ 50,000/μL, hemoglobin $\geq$ 8.0 g/dL, AST $\leq$ 2.5 $\times$ the upper limit of the normal range (UNL), ALT [lteuq] 2.5 $\times$ UNL, total bilirubin $\leq$ 1.5 $\times$ UNL, serum creatinine $\leq$ 1.5 $\times$ UNL, corrected serum calcium $\leq$ 11.0 mg/dL, and arterial partial oxygen pressure $\geq$ 65 mmHg or arterial blood oxygen saturation $\geq$ 93%. Patients were excluded if they had an active infection, a history of organ transplantation, active concurrent cancers, CNS involvement, a bulky mass requiring emergent radiotherapy, or seropositivity for hepatitis B virus antigen, hepatitis C virus antibody, or HIV antibody.

Study Design

This study was a multicenter, single-arm, phase II trial. Objectives of the study were to evaluate the efficacy, pharmacokinetic profile, and safety of KW-0761 monotherapy. Patients received intravenous infusions of KW-0761 once per week for 8 weeks at a dose of 1.0 mg/kg.[14] Oral antihistamine and acetaminophen were administered before each KW-0761 infusion to prevent infusion reactions. The primary end point was overall response rate (ORR), and secondary end points included the best response by disease site, PFS, and OS. Objective responses were assessed after the fourth and eighth infusions of KW-0761 by an independent efficacy assessment committee according to the modified response criteria for ATL.[4] It was estimated that 25 patients would be required to detect a lower limit of the 95% CI exceeding the 5% threshold of ORR based on the assumptions that the minimum required ORR for a new drug for relapsed, aggressive ATL is 5%,[15] with an expected ORR for KW-0761 of 30%[14] with 90% power. Adverse events (AEs) were graded according to the National Cancer Institute Common Terminology Criteria for AEs, version 3.0. The presence of human anti-KW-0761 antibodies in the patients' plasma was examined using enzyme-linked immunosorbent assay. Blood samples collected at times strictly in accordance with the protocol were employed for the pharmacokinetic analysis. Samples were obtained from patients who had received at least one dose of KW-0761 up to all eight doses. When any event resulted in an alteration in the infusion protocol, only those samples taken before the alteration were used for the analysis. The following parameters were calculated for plasma KW-0761: maximum drug concentration and trough drug concentration of each KW-0761 administration, area under the blood concentration time curve from 0 to 7 days after the first and eighth doses, and half-life period ($t_{1/2}$) after the eighth dose. As an additional research parameter, we investigated blood T-cell subset distribution during and after KW-0761 treatment and compared these values with those of 10 healthy donors as controls (five men, five women; median age, 45 years; range, 41 to 57 years).

Statistical Analysis

Survival estimates were calculated using the Kaplan-Meier method. PFS was defined as the time from the first dose of KW-0761 to progression, relapse, or death resulting from any cause, whichever occurred first. OS was measured from the day of the first dose to death resulting from any cause. Regarding T-cell subset analysis, differences between the patients' values before KW-0761 treatment and those of the controls were examined using the Mann-Whitney U-test. Differences between KW-0761 pretreatment values and those at each time point after KW-0761 treatment were examined using the Wilcoxon signed-rank test. All analyses were performed with SPSS Statistics 17.0 (SPSS, Chicago, IL). In this study, $P < .05$ was considered significant.

Study Oversight

The study was sponsored by Kyowa Hakko Kirin Company (Tokyo, Japan). The academic investigators and the sponsor were jointly responsible for the study design. The protocol was approved by the institutional review board at each participating site, and all patients and controls provided written informed consent before enrollment according to the Declaration of Helsinki.

RESULTS

Patients

Of the 28 patients enrolled onto the study, 27 (12 men, 15 women) received at least one infusion of KW-0761. One patient was withdrawn for aggravation of the general condition before the administration of KW-0761. Demographics and clinical characteristics of the 27 patients are summarized in Table 1. Median age was 64 years (range, 49 to 83). The disease subtypes included 14 acute, six lymphoma, and seven unfavorable chronic type ATL. Of these 27 patients, 14 (52%) completed the schedule of eight planned infusions. Of the remaining 13 patients, 11 (41%) discontinued treatment because of disease progression, one (4%) because of skin rash, and another (4%) because of concurrent colon cancer, for which this patient was excluded from the efficacy evaluation.

Efficacy of KW-0761

Of 26 patients evaluable for efficacy, objective responses were noted in 13 patients (ORR, 50%; 95% CI, 30% to 70%), including eight complete responses (CRs). Responses according to disease site were 100% (13 of 13; all CRs) for blood, 63% (five of eight) for skin, and 25% (three of 12) for nodal and extranodal lesions. Responses according to disease subtype were 43% (six of 14) for acute, 33% (two of six) for lymphoma, and 83% (five of six) for unfavorable chronic type ATL. Responses according to number of prior chemotherapy regimens were 48% (10 of 21) in those who had one prior regimen and 60% (three of five) for those who had two or three prior regimens. Median PFS and OS were 5.2 and 13.7 months, respectively (Figs 1A, 1B).

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Table 1. Patient Demographics and Clinical Characteristics (n = 27)*

Characteristic	No.	%
Age, years		
Median	64	
Range	49-83	
≥ 65	13	48
Sex		
Male	12	44
Female	15	56
ECOG performance status†		
0	15	56
1	7	26
2	5	19
Disease subtype		
Acute	14	52
Lymphoma	6	22
Chronic	7	26
Prior chemotherapy regimens, No.		
1	22	82
2	3	11
3	2	7

Abbreviation: ECOG, Eastern Cooperative Oncology Group.
*Of 28 patients enrolled, 27 received at least one infusion of KW-0761.
†ECOG performance status scores range from 0 (normal activity) to 5 (death), with higher scores indicating more severe disability.

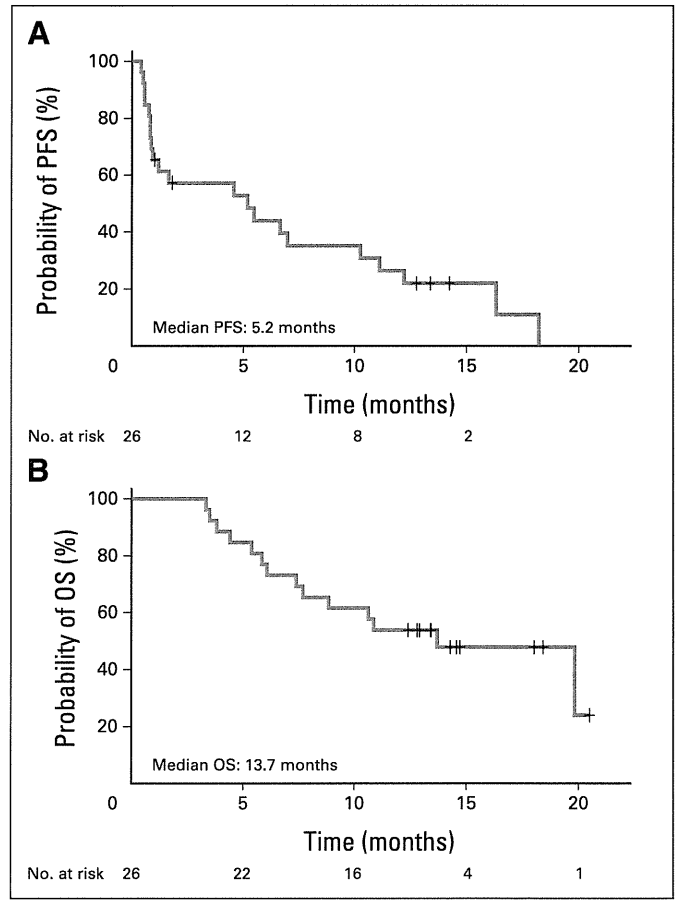

Fig 1. Kaplan-Meier curves of estimated (A) progression-free survival (PFS; median, 5.2 months) and (B) overall survival (OS; median, 13.7 months).

Pharmacokinetics

KW-0761 plasma concentrations over eight infusions, once per week, at 1.0 mg/kg are shown in Figure 2. Mean maximum drug concentration and trough drug concentration (± standard deviation) of the eighth infusion were 42.9 ± 14.2 μg/mL and 33.6 ± 10.6 μg/mL, respectively. Mean area under the blood concentration time curve from 0 to 7 days after the eighth infusion was 6,297 ± 1,812 μg × hours/mL. The mean $t_{1/2}$ after the eighth infusion was 422 ± 147 hours.

AEs

Table 2 lists AEs that occurred in at least 15% of patients or at grades 3 to 4, which were determined as possibly, probably, or definitely KW-0761 related. The most common nonhematologic AE was an infusion reaction (89%). In addition, 80% or more of the following recorded AEs occurred along with an infusion reaction: fever, chills, tachycardia, hypertension, nausea, and hypoxemia (Table 2). These events occurred primarily at the first infusion, becoming less frequent with subsequent treatments. The infusion reactions and component events were transient, and all patients recovered, although some needed systemic steroids. Skin rashes were observed as another frequent nonhematologic AE (63%), mostly occurring after the fourth or subsequent infusions. Of the 14 patients who developed grade 2 or higher skin rashes, objective responses were noted in 13 patients (93%), including eight CRs. On the other hand, of the 12 patients who developed no or grade 1 skin rashes, no objective responses were observed. A typical clinical course of the rash is depicted in Appendix Figures A1A and A1B (online only). The skin rash observed in this patient appeared after the seventh infusion, and the corresponding skin biopsy revealed mild perivascular CD8-positive cells dominating an inflammatory reaction, with an absence of ATL cells. The skin rash recovered on application of topical steroid. Of the 17 patients who developed skin rashes, one developed Stevens-Johnson syndrome, which was determined as possibly KW-0761 related, although that patient also received trimethoprim/sulfamethoxazole, fluconazole, and acyclovir for prevention of infection according to the protocol. This patient stopped those preventive agents and was treated with

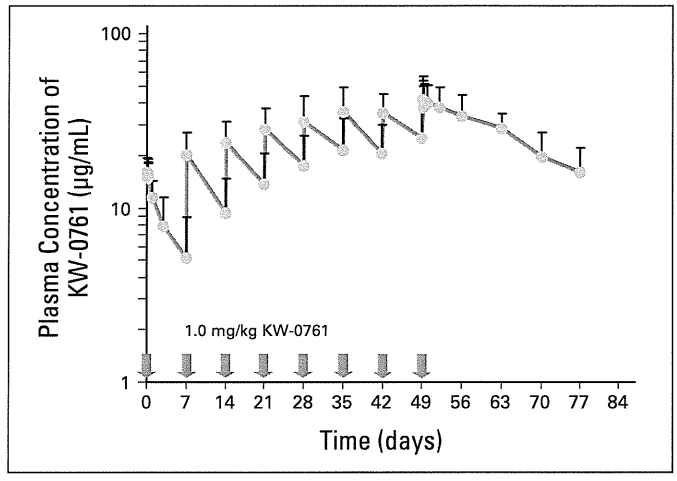

Fig 2. Pharmacokinetics of KW-0761. Mean KW-0761 plasma concentrations during and after 1.0 mg/kg KW-0761 infusions once per week for 8 weeks. Bar indicates upper limit of standard deviation.

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Table 2. Adverse Events (n = 27)*

Adverse Event	Grade (No. of patients) 1	2	3	4	All Grades No. of Patients	%	Infusion Reaction Related (No. of patients) All Grades	≥ Grade 2
Nonhematologic								
Infusion reaction	1	22	1	0	24	89		
Fever	20	2	0	0	22	82	18	2
Rash	3	9	5	0	17	63	1	0
Chills	14	2	0	0	16	59	16	2
ALT	5	4	2	0	11	41		
AST	3	5	2	0	10	37		
Tachycardia	9	0	0	0	9	33	9	0
Hypertension	6	2	0	0	8	30	8	1
Albuminemia	7	1	0	0	8	30		
ALP	4	2	0	0	6	22		
Weight gain	5	0	0	0	5	19		
Nausea	4	1	0	0	5	19	5	1
Hyponatremia	5	0	0	0	5	19		
Hypoxemia	0	2	3	0	5	19	4	4
Hypotension	2	2	0	0	4	15	3	1
Pruritus	0	3	1	0	4	15		
γ-GTP	0	1	3	0	4	15		
Hypophosphatemia	0	4	0	0	4	15		
Hyperuricemia	4	0	0	0	4	15		
Hypercalcemia	1	1	0	1	3	11		
Hypokalemia	1	0	2	0	3	11		
Erythema multiforme†	0	0	1	0	1	4		
Hyperglycemia	0	0	1	0	1	4		
Tumor lysis syndrome	0	0	1	0	1	4		
Metabolic/laboratory, other‡	4	7	3	0	14	52		
Hematologic								
Lymphopenia§	0	6	9	11	26	96		
Leukocytopenia	3	7	8	0	18	67		
Thrombocytopenia	7	2	3	2	14	52		
Neutropenia	5	4	5	0	14	52		
Hemoglobin	4	3	1	0	8	30		

Abbreviations: ALP, alkaline phosphatase; BUN, blood urea nitrogen; CRP, C-reactive protein; GTP, glutamyl transpeptidase.

*Of 28 patients enrolled, 27 received at least one infusion of KW-0761. Listed are adverse events determined as possibly, probably, or definitely KW-0761 related that occurred in at least 15% of patients or were of grade 3 to 4 severity.

†One patient diagnosed as having Stevens-Johnson syndrome.

‡Other metabolic and laboratory test abnormalities included hypoproteinemia, BUN elevation, CRP, glycosuria, hypochloremia, and hyperammoniemia.

§Lymphopenia included decrease of abnormal lymphocytes.

systemic steroids, but improvement required the passage of 4 months. Lymphopenia, including a decrease in the number of ATL cells, occurred in 26 (96%) of the 27 patients. Grades 3 to 4 thrombocytopenia was observed in five patients (19%) but was not associated with bleeding, and grade 3 neutropenia also occurred in five patients but did not lead to a febrile episode. The latter two hematologic AEs improved in all patients. None of the patients developed detectable anti–KW-0761 antibody.

T-Cell Subset Analysis

The numbers of circulating blood CD4+ CCR4+, CD4+ CD25+ FOXP3+, CD4+ CCR4−, and CD4− CD8+ cells from KW-0761–treated patients and those from the 10 controls are presented as box and whisker plots in each graph (Appendix Figs A2A to A2D, online only). The numbers of CD4+ CCR4+ and CD4+ CD25+ FOXP3+ cells in patients with ATL before treatment were significantly higher than those in the controls but were significantly reduced after the first KW-0761 infusion. The reduction lasted for at least 4 months after the eighth infusion (Appendix Figs A2A, A2B; online only). The numbers of CD4+ CCR4−, and CD4− CD8+ cells in patients with untreated ATL were significantly lower than those in the controls. KW-0761 treatment led to a transient further reduction of those cells; however, recovery took place by the fifth infusion (Appendix Figs A2C, A2D; online only).

DISCUSSION

In the present multicenter phase II study, KW-0761 monotherapy demonstrated significant responses in patients with relapsed ATL with an acceptable toxicity profile. An ORR of 50% and median PFS and OS values of 5.2 and 13.7 months, respectively, were observed. Because the lower limit for an ORR with a 95% CI was 30%, this study met the primary end point. These results suggest an improvement over what has been achieved with other agents in relapsed ATL.[15] Cladribine was associated with an ORR of 7% (one of 15 patients),[16] and irinotecan hydrochloride treatment had an ORR of 38% (five of 13 patients) with a median duration of response of 31 days.[17] Antiviral therapy consisting of a combination of zidovudine and interferon, which has been proposed as a standard first-line therapy in leukemic subtypes of ATL,[18] was initially reported as having a median OS of 3.0 months in 19 patients with acute or lymphoma type ATL.[19] In addition, White et al[20] reported three objective responses lasting longer than 1 month with zidovudine plus interferon in 18 patients with ATL, of whom 15 had received prior therapy. Those observations collectively suggest that KW-0761 may offer an advantage over or provide an additional therapeutic option to the currently available therapy for relapsed ATL, although there were no direct comparisons.

On examining the results of ATL treatment according to disease site, disease in blood seemed to be more sensitive to KW-0761 than at other disease sites. Currently, we are unable to fully explain this difference; however, factors such as the KW-0761 delivery or the amount of ADCC effector cells such as natural killer (NK) cells and monocytes/macrophages in each disease site may be important.

Pharmacokinetic analyses demonstrated that the $t_{1/2}$ after the eighth administration of KW-0761 was nearly the same as that of circulating endogenous human IgG1, indicating good stability of this antibody in vivo. In addition, no anti-KW-0761 antibody was detected, suggesting that the antigenicity of this novel defucosylated mAb is not likely to be a problem clinically, consistent with findings in our preceding phase I study.[14]

The infusion reactions observed in the present study may also provide novel insights into problems associated with antibody therapy. It is generally recognized that complement plays a major role in infusion reactions,[21] but this mechanism cannot apply to KW-0761, because the agent is unable to mediate complement-dependent cytotoxicity.[11] Therefore, the infusion reactions observed here may have a different mechanism compared with those of other antibody therapies, such as rituximab. KW-0761 has a defucosylated Fc region, which markedly enhances ADCC because of increased binding affinity to the

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Fcγ receptor on effector cells. Defucosylated IgG1 is a more potent activator of NK cells than nondefucosylated IgG1 during ADCC.[22] We surmise that the infusion reactions to KW-0761 were mainly induced by cytokines and related cytotoxic molecules released from highly activated NK cells.

The present study demonstrated that compared with the levels in the controls, KW-0761 led to a significant and lasting decrease in the number of CD4+ CCR4+ but not CD4+ CCR4− or CD4− CD8+ cells in patients with ATL. Consistent with the fact that CCR4 is expressed not only on T-helper type 2 cells but also on regulatory T (Treg) cells,[23-26] KW-0761 treatment also resulted in a significant and lasting decrease in CD4+ CD25+ FOXP3+ cells, including both ATL cells and endogenous non-ATL Treg cells.[27-29] Reduction or suppression of Treg cells is expected to be a potentially promising strategy for boosting antitumor immunity in patients with cancer, as observed in studies with ipilimumab,[30-33] although ipilimumab and KW-0761 have different targets; the former suppresses Treg cell function, and the latter decreases their number. Hence, KW-0761 could also lead to activation of antitumor immunity, which might also contribute to its potent anti-ATL response. Because ipilimumab causes immune-related AEs such as diarrhea and colitis, we were especially vigilant in monitoring for this type of AE. Because CCR4 contributes to lymphocyte skin-specific homing,[34] it was not surprising that skin rashes, which could be an immune-related AE, were frequently observed in the present KW-0761 study. Skin rashes, including the most severe case of Stevens-Johnson syndrome, the causal association of which with concomitant medications other than KW-0761 could not be excluded, proved to be manageable, and patients improved in all cases, although some needed systemic or topical steroid treatment. The observed better responses to KW-0761 in patients with grade 2 or higher skin rashes were highly impressive. However, the underlying mechanisms for this finding are not clear; thus, further detailed investigation is warranted. All of the 14 patients who developed grade 2 or higher skin rashes received five or more KW-0761 infusions according to the protocol, whereas only three of the 12 patients who developed no or grade 1 skin rashes received five or more KW-0761 infusions. This suggests the possibility that skin rashes were associated with the number of KW-0761 infusions. The Cochran-Mantel-Haenszel test stratified by the number of KW-0761 infusions ($\leq$ four $v \geq$ five) indicated a significant association between clinical response and skin rashes (no or grade 1 v grades 2 to 4; $P = .009$). However, the sample size is insufficient to draw such a conclusion.

Following on a phase III study (JCOG9801 [Japan Clinical Oncology Group 9801]) for untreated aggressive ATL,[5] the present promising results for KW-0761 monotherapy prompted us to conduct a subsequent randomized trial of VCAP-AMP-VECP chemotherapy with or without KW-0761 for previously untreated ATL (Clinicaltrials.gov: NCT01173887). CCR4 is also expressed on tumor cells from a subgroup of PTCL other than ATL, which also has an unfavorable prognosis.[2,35,36] Thus, we are currently conducting a phase II study of KW-0761 monotherapy for relapsed CCR4-positive PTCL (Clinicaltrials.gov:NCT01192984). In addition, Duvic et al[37] recently reported a phase I/II study of KW-0761 for refractory cutaneous T-cell lymphoma. They found that KW-0761 was well tolerated at doses of 0.1 to 1.0 mg/kg, and a promising ORR of 39% (15 of 38 patients) was achieved, although expression of CCR4 on lymphoma cells was not included as one of the eligibility criteria (Clinicaltrials-.gov: NCT00888927). Furthermore, clinical trials of KW-0761 for patients with Hodgkin's lymphoma may be worth trying, because it has been reported that Hodgkin's lymphoma tumor cells produce CCR4 ligand molecules, and migratory CCR4-expressing Treg cells prevent a host immune attack on tumor cells, thereby creating an immunologically favorable environment for the tumor cells.[38]

Although this phase II study offers a novel promising treatment option (KW-0761) for patients with relapsed ATL, some limitations should be discussed. First, the present phase II study was relatively small, with consequent limitations on drawing definitive conclusions about the efficacy and safety profile of KW-0761. Second, patients received different prior systemic chemotherapy regimens, which could affect the results of the present study. Finally, the enrolled patients all had aggressive ATL, but three clinical subtypes (acute, lymphoma, and unfavorable chronic type) were included. Although there may be no significant differences in susceptibility to conventional chemotherapies between these subtypes, the heterogeneity of the enrolled patients might have affected the results.

In conclusion, this multicenter phase II study demonstrated that KW-0761 monotherapy showed clinically meaningful antitumor activity in patients with relapsed ATL, with an acceptable toxicity profile. Further investigation of KW-0761 for ATL and other T-cell neoplasms is warranted on the basis of the present results.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

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