88 Matsuzaki J, Gnjatic S, Mhawech-Fauceglia P *et al.* Tumor-infiltrating NY-ESO-1-specific CD8$^+$ T cells are negatively regulated by LAG-3 and PD-1 in human ovarian cancer. *Proc. Natl Acad. Sci. USA* 107(17), 7875–7880 (2010).

89 Nishimura H, Nose M, Hiai H *et al.* Development of lupus-like autoimmune diseases by disruption of the *PD-1* gene encoding an ITIM motif-carrying immunoreceptor. *Immunity* 11(2), 141–151 (1999).

90 Nishimura H, Okazaki T, Tanaka Y *et al.* Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. *Science* 291(5502), 319–322 (2001).

91 Okazaki T, Tanaka Y, Nishio R *et al.* Autoantibodies against cardiac troponin I are responsible for dilated cardiomyopathy in *PD-1*-deficient mice. *Nat. Med.* 9(12), 1477–1483 (2003).

92 Zou W, Chen L. Inhibitory B7-family molecules in the tumour microenvironment. *Nat. Rev. Immunol.* 8(6), 467–477 (2008).

93 Dong H, Strome SE, Salomao DR *et al.* Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat. Med.* 8(8), 793–800 (2002).

94 Iwai Y, Terawaki S, Honjo T. PD-1 blockade inhibits hematogenous spread of poorly immunogenic tumor cells by enhanced recruitment of effector T cells. *Int. Immunol.* 17(2), 133–144 (2005).

95 Iwai Y, Ishida M, Tanaka Y, Okazaki T, Honjo T, Minato N. Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. *Proc. Natl Acad. Sci. USA* 99(19), 12293–12297 (2002).

96 Brahmer JR, Drake CG, Wollner I *et al.* Phase I study of single-agent anti-programmed death-1 (MDX-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates. *J. Clin. Oncol.* 28(19), 3167–3175 (2010).

97 Topalian SL, Hodi FS, Brahmer JR *et al.* Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N. Engl. J. Med.* 366(26), 2443–2454 (2012).

98 Brahmer JR, Tykodi SS, Chow LQ *et al.* Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *N. Engl. J. Med.* 366(26), 2455–2465 (2012).

99 Mitsui J, Nishikawa H, Muraoka D *et al.* Two distinct mechanisms of augmented antitumor activity by modulation of immunostimulatory/inhibitory signals. *Clin. Cancer Res.* 16(10), 2781–2791 (2010).

100 Verma S, Miles D, Gianni L *et al.* Trastuzumab emtansine for HER2-positive advanced breast cancer. *N. Engl. J. Med.* 367(19), 1783–1791 (2012).

101 White AL, Chan HT, Roghanian A *et al.* Interaction with FcγRIIB is critical for the agonistic activity of anti-CD40 monoclonal antibody. *J. Immunol.* 187(4), 1754–1763 (2011).

102 Li F, Ravetch JV. Inhibitory Fcγ receptor engagement drives adjuvant and antitumor activities of agonistic CD40 antibodies. *Science* 333(6045), 1030–1034 (2011).

103 Wilson NS, Yang B, Yang A *et al.* An Fcγ receptor-dependent mechanism drives antibody-mediated target-receptor signaling in cancer cells. *Cancer Cell* 19(1), 101–113 (2011).

104 Galon J, Costes A, Sanchez-Cabo F *et al.* Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* 313(5795), 1960–1964 (2006).

105 Pages F, Berger A, Camus M *et al.* Effector memory T cells, early metastasis, and survival in colorectal cancer. *N. Engl. J. Med.* 353(25), 2654–2666 (2005).

Websites

201 Study of adecatumumab relative to FOLFOX after R0 resection of colorectal liver metastases (MT201-204). http://clinicaltrials.gov/show/NCT00866944

202 ClinicalTrials.gov. http://clinicaltrials.gov

ELSEVIER

Experimental Hematology

Experimental Hematology 2013;41:367–376

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

Bing Liu[a], Kohshi Ohishi[b], Yuki Orito[c], Yoshiki Nakamori[a], Hiroyoshi Nishikawa[c], Kazuko Ino[a], Kei Suzuki[a], Takeshi Matsumoto[b], Masahiro Masuya[a], Hirofumi Hamada[d], Junichi Mineno[e], Ryoichi Ono[f], Tetsuya Nosaka[f], Hiroshi Shiku[c], and Naoyuki Katayama[a]

[a]*Hematology and Oncology, Mie University Graduate School of Medicine, Tsu, Mie, Japan;* [b]*Blood Transfusion Service, Mie University Hospital, Tsu, Mie, Japan;* [c]*Department of Cancer Vaccine, Mie University Graduate School of Medicine, Tsu, Mie, Japan;* [d]*Department of Life Science, Tokyo University of Pharmacy and Life Sciences, Hachioji, Tokyo, Japan;* [e]*Center for Cell and Gene Therapy, Takara Bio, Shiga, Japan;* [f]*Department of Microbiology and Molecular Genetics, Mie University Graduate School of Medicine, Tsu, Mie, Japan*

(Received 14 September 2012; revised 6 December 2012; accepted 7 December 2012)

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

Offprint requests to: Kohshi Ohishi, M.D., Ph.D., Blood Transfusion Service, Mie University Hospital, 2-174 Edobashi, Tsu, Mie 514-8507, Japan; E-mail: koishi@clin.medic.mie-u.ac.jp

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.exphem.2012.12.001.

http://dx.doi.org/10.1016/j.exphem.2012.12.001

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

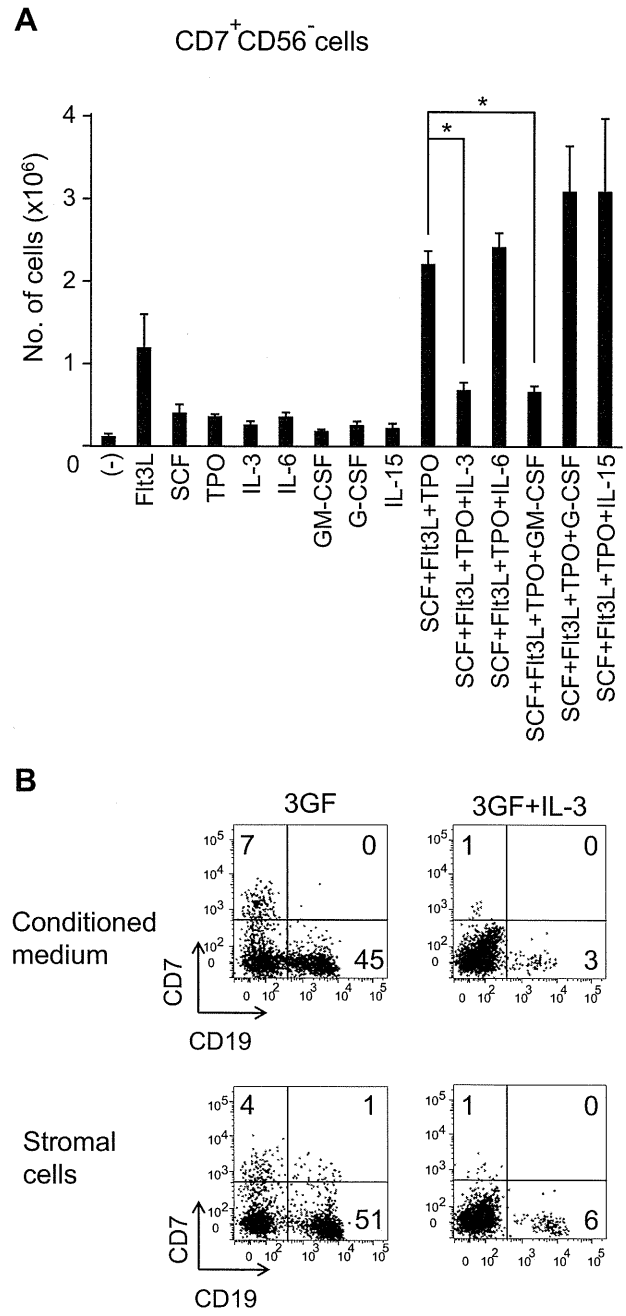


Figure 1. Cytokine regulation of CD7$^+$CD56$^-$ proT cell development. **(A)** CD34$^+$CD38$^{lo/-}$CD7$^-$CD19$^-$CD10$^-$ cells (2×10^4 cells/well) were cultured on telomerized stromal cells in the presence of the indicated cytokines for 21 days, and the number of CD7$^+$CD56$^-$ cells was analyzed. 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

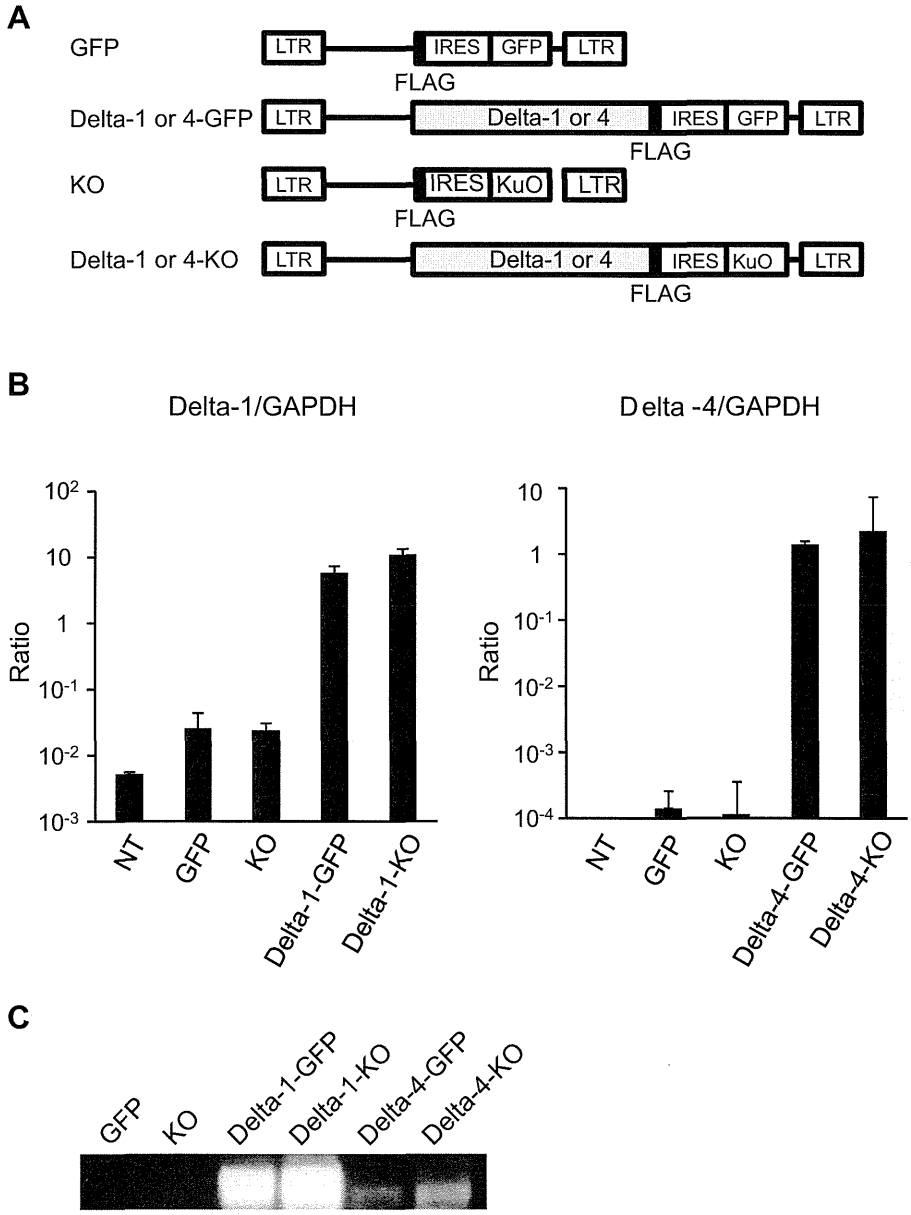


Figure 2. Expression of Delta-1 and -4 in telomerized stromal cells. **(A)** Constructs of GFP, Delta-1–GFP, Delta-4–GFP, KO, Delta-1–KO, and Delta-4–KO. **(B)** Expression of Delta-1 and -4 mRNA in nontransduced stromal cells or stromal cells transduced with GFP, KO, Delta-1–GFP, Delta-1–KO, Delta-4–GFP,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

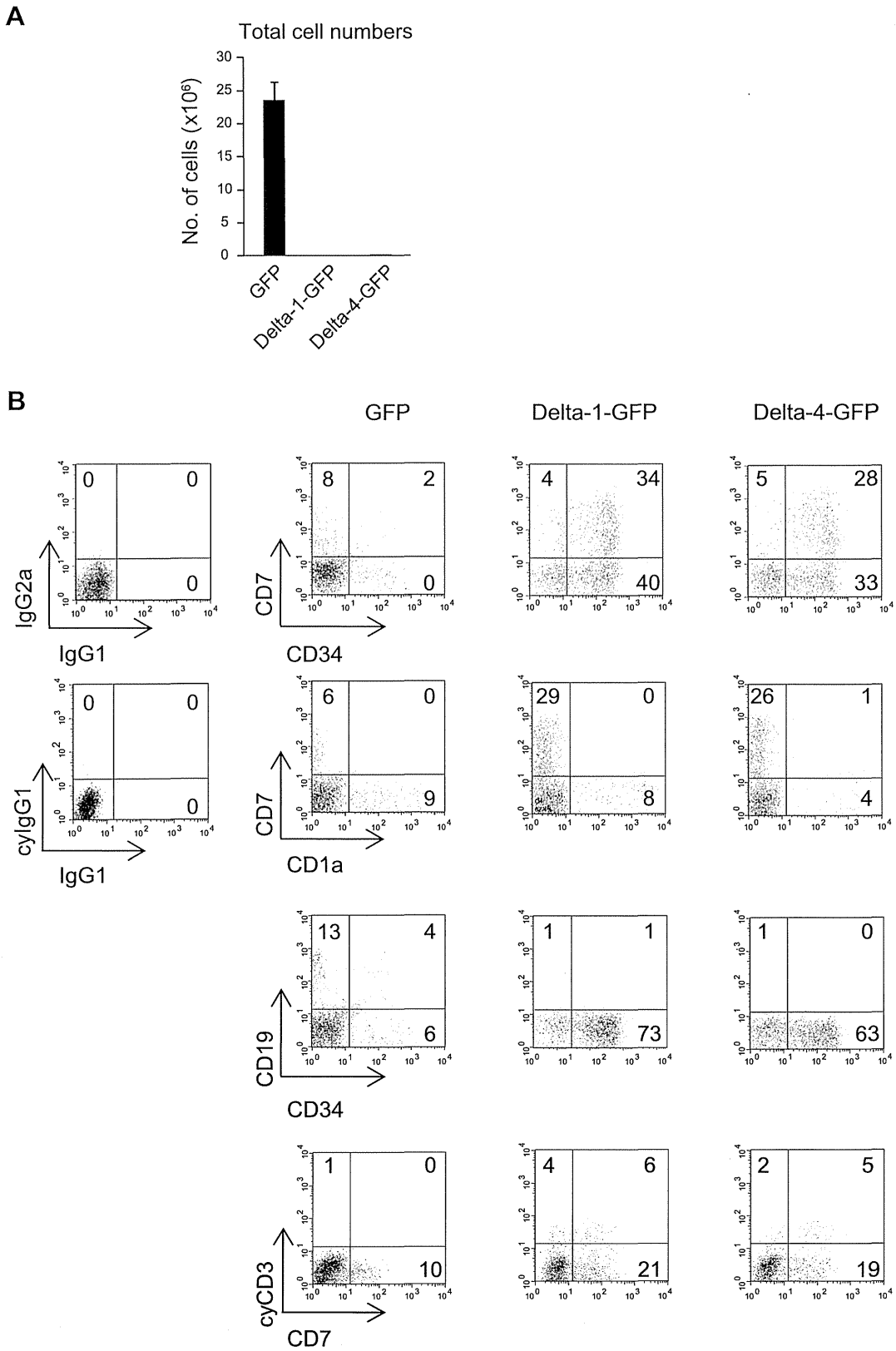
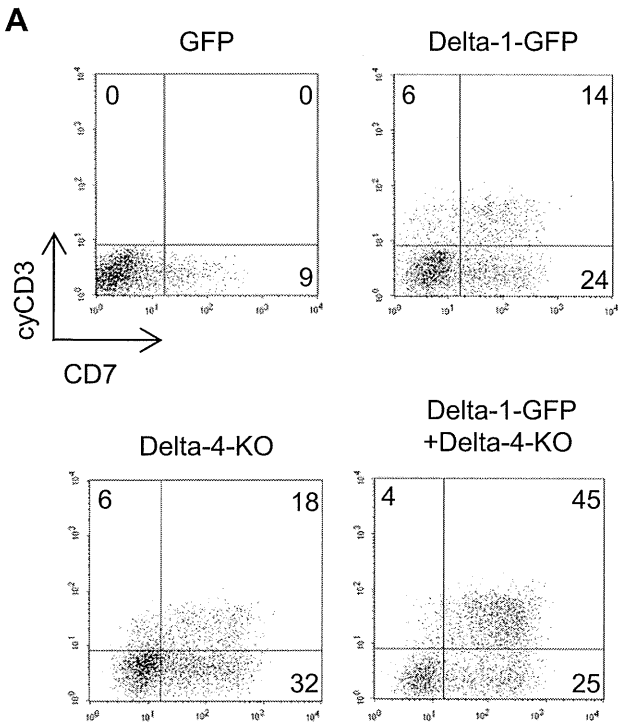


Figure 3. Effect of Delta-1 and -4 expression on T and B cell differentiation. Total cell numbers **(A)** and the phenotype **(B)** of cells after coculture of $CD34^+$ cells (4×10^4 cells/well) on telomerized stromal cells transduced with GFP, Delta-1–GFP or Delta-4–GFP in the presence of 3GF for 3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



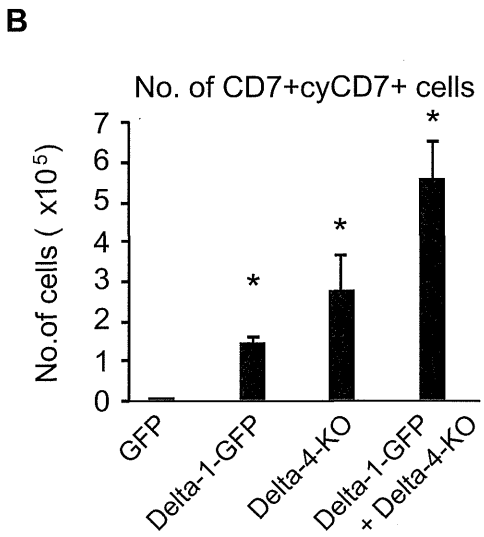


Figure 4. Combinatorial effect of Delta-1 and -4 expression on T cell differentiation. The percentage **(A)** and number **(B)** of $CD7^+cyCD3^+$ T cells after coculture of $CD34^+$ cells (4×10^4 cells/well) on stromal cells expressing GFP, Delta-1–GFP, Delta-4–KO or both Delta-1–GFP and Delta-4–KO 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.

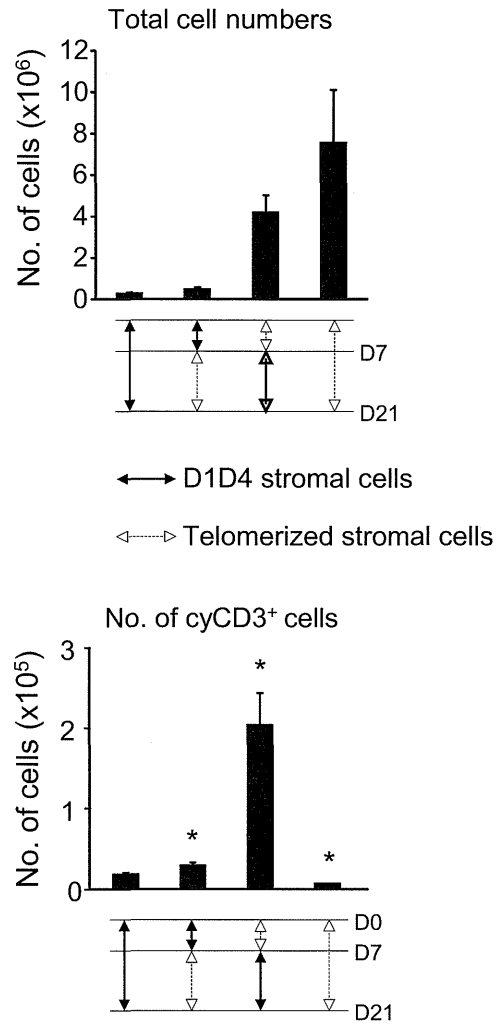


Figure 5. Optimal condition for generation of a higher number of $CD7^+cyCD3^+$ preT cells. $CD34^+$ cells (4×10^4 cells/well) were cocultured on stromal cells transduced with Delta-1 and -4 (D1D4 stromal cells) in the presence of 3GF for 21 days, D1D4 stromal cells for 7 days followed by telomerized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

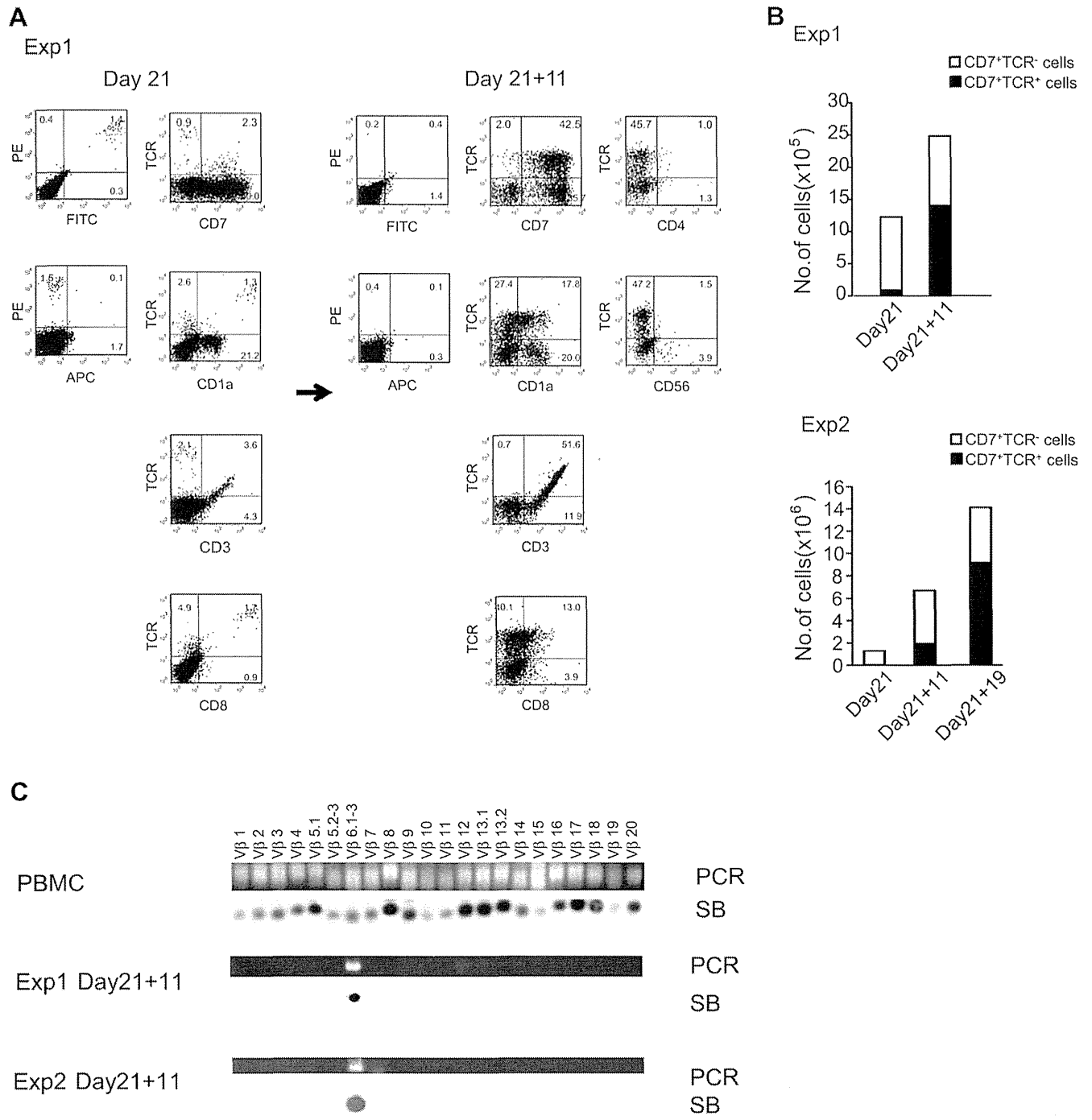


Figure 6. Generation of T cell precursors from TCR-transduced hematopoietic progenitors. $CD34^+$ cells (4×10^4 cells/well) were transduced with the TCR during coculture on telomerized stromal cells for 7 days, and then recultured on D1D4 stromal cells for 14 days in the presence of 3GF. Cultured cells 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

We thank Dr. Juan Carlos Zúñiga-Pflücker (Department of Immunology, University of Toronto, Toronto, Ontario, Canada) for providing the OP9 stromal cells expressing Delta-1, Dr Toshio Kitamura (Division of Cellular Therapy, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan) for providing pMXs-IRES-EGFP and PLAT-A cells, Dr. Seiji Sakano (Advanced Medical Device Center, Asahi Kasei Corporation, Tokyo, Japan) for providing the cDNAs for Delta-1 and -4 in pMKITneo and pcDNA3 vectors, and Chisaki Amaike and Sahoko Hori (Department of Immuno-Gene Therapy, Mie University Graduate School of Medicine, Tsu, Mie, Japan) for technical assistance with TCR transduction and flow cytometric analysis, respectively. This study was supported, in part, by a Japan Society for the Promotion of Science Grant-in-Aid for Scientific Research (21591240).

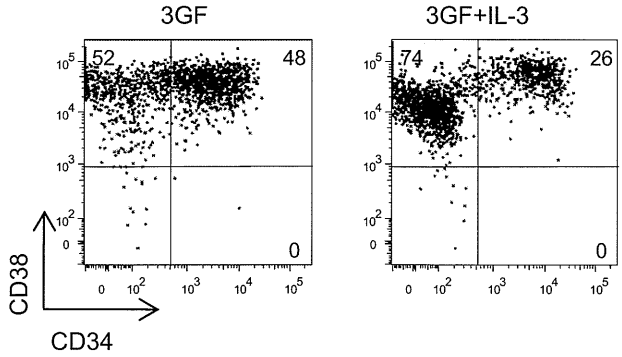
Conflict of interest disclosure

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.

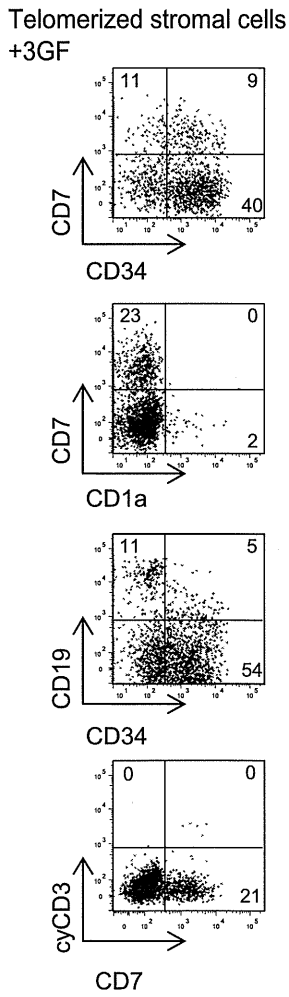
References

1. Aiuti A, Roncarolo MG. Ten years of gene therapy for primary immune deficiencies. Hematology Am Soc Hematol Educ Program. 2009;682–689.
2. Liuba K, Pronk CJ, Stott SR, Jacobsen SE. Polyclonal T-cell reconstitution of X-SCID recipients after in utero transplantation of lymphoid-primed multipotent progenitors. Blood. 2009;113: 4790–4798.
3. Holland AM, Zakrzewski JL, Goldberg GL, Ghosh A, van den Brink MR. Adoptive precursor cell therapy to enhance immune reconstitution after hematopoietic stem cell transplantation in mouse and man. Semin Immunopathol. 2008;30:479–487.
4. Radtke F, Fasnacht N, Macdonald HR. Notch signaling in the immune system. Immunity. 2010;32:14–27.
5. Thompson PK, Zuniga-Pflucker JC. On becoming a T cell, a convergence of factors kick it up a Notch along the way. Semin Immunol. 2011;23:350–359.
6. Tanigaki K, Honjo T. Regulation of lymphocyte development by Notch signaling. Nat Immunol. 2007;8:451–456.
7. Sultana DA, Bell JJ, Zlotoff DA, De Obaldia ME, Bhandoola A. Eliciting the T cell fate with Notch. Semin Immunol. 2010;22: 254–260.
8. Delaney C, Heimfeld S, Brashem-Stein C, Voorhies H, Manger RL, Bernstein ID. Notch-mediated expansion of human cord blood progenitor cells capable of rapid myeloid reconstitution. Nat Med. 2010;16: 232–236.
9. Ohishi K, Varnum-Finney B, Bernstein ID. Delta-1 enhances marrow and thymus repopulating ability of human CD34(+)CD38(-) cord blood cells. J Clin Invest. 2002;110:1165–1174.
10. Pui JC, Allman D, Xu L, et al. Notch1 expression in early lymphopoiesis influences B versus T lineage determination. Immunity. 1999;11: 299–308.
11. Wilson A, MacDonald HR, Radtke F. Notch 1-deficient common lymphoid precursors adopt a B cell fate in the thymus. J Exp Med. 2001;194:1003–1012.
12. Koch U, Fiorini E, Benedito R, et al. Delta-like 4 is the essential, nonredundant ligand for Notch1 during thymic T cell lineage commitment. J Exp Med. 2008;205:2515–2523.

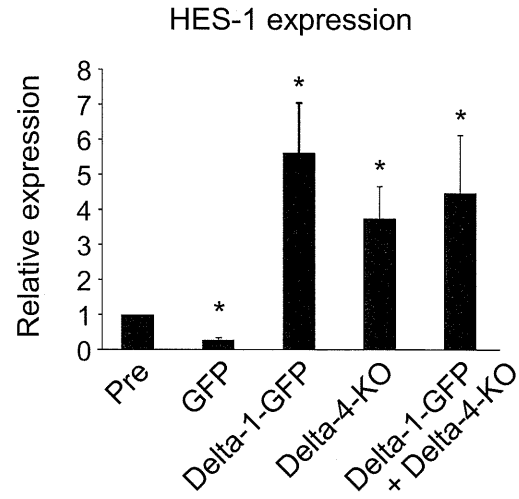
13. Hozumi K, Mailhos C, Negishi N, et al. Delta-like 4 is indispensable in thymic environment specific for T cell development. J Exp Med. 2008;205:2507–2513.
14. Lefort N, Benne C, Lelievre JD, et al. Short exposure to Notch ligand Delta-4 is sufficient to induce T-cell differentiation program and to increase the T cell potential of primary human CD34+ cells. Exp Hematol. 2006;34:1720–1729.
15. Jaleco AC, Neves H, Hooijberg E, et al. Differential effects of Notch ligands Delta-1 and Jagged-1 in human lymphoid differentiation. J Exp Med. 2001;194:991–1002.
16. La Motte-Mohs RN, Herer E, Zuniga-Pflucker JC. Induction of T-cell development from human cord blood hematopoietic stem cells by Delta-like 1 in vitro. Blood. 2005;105:1431–1439.
17. Schmitt TM, Zuniga-Pflucker JC. Induction of T cell development from hematopoietic progenitor cells by delta-like-1 in vitro. Immunity. 2002;17:749–756.
18. Nishihara M, Wada Y, Ogami K, et al. A combination of stem cell factor and granulocyte colony-stimulating factor enhances the growth of human progenitor B cells supported by murine stromal cell line MS-5. Eur J Immunol. 1998;28:855–864.
19. Crooks GM, Hao QL, Petersen D, Barsky LW, Bockstoce D. IL-3 increases production of B lymphoid progenitors from human CD34+CD38- cells. J Immunol. 2000;165:2382–2389.
20. Yoshikawa Y, Hirayama F, Kanai M, et al. Stromal cell-independent differentiation of human cord blood CD34+CD38- lymphohematopoietic progenitors toward B cell lineage. Leukemia. 2000;14:727–734.
21. Park TS, Rosenberg SA, Morgan RA. Treating cancer with genetically engineered T cells. Trends Biotechnol. 2011;29:550–557.
22. Morgan RA, Dudley ME, Wunderlich JR, et al. Cancer regression in patients after transfer of genetically engineered lymphocytes. Science. 2006;314:126–129.
23. Schmitt TM, Ragnarsson GB, Greenberg PD. T cell receptor gene therapy for cancer. Hum Gene Ther. 2009;20:1240–1248.
24. Merhavi-Shoham E, Haga-Friedman A, Cohen CJ. Genetically modulating T-cell function to target cancer. Semin Cancer Biol. 2012;22:14–22.
25. Jorritsma A, Schotte R, Coccoris M, de Witte MA, Schumacher TN. Prospects and limitations of T cell receptor gene therapy. Curr Gene Ther. 2011;11:276–287.
26. Bendle GM, Linnemann C, Hooijkaas AI, et al. Lethal graft-versus-host disease in mouse models of T cell receptor gene therapy. Nat Med. 2010;16:565–570, 561p following 570.
27. Zhao Y, Parkhurst MR, Zheng Z, et al. Extrathymic generation of tumor-specific T cells from genetically engineered human hematopoietic stem cells via Notch signaling. Cancer Res. 2007;67:2425–2429.
28. van Lent AU, Nagasawa M, van Loenen MM, et al. Functional human antigen-specific T cells produced in vitro using retroviral T cell receptor transfer into hematopoietic progenitors. J Immunol. 2007;179:4959–4968.
29. Khor B, Sleckman BP. Allelic exclusion at the TCRbeta locus. Curr Opin Immunol. 2002;14:230–234.
30. Nakamori Y, Liu B, Ohishi K, et al. Human bone marrow stromal cells simultaneously support B and T/NK lineage development from human haematopoietic progenitors: a principal role for flt3 ligand in lymphopoiesis. Br J Haematol. 2012;157:674–686.
31. Liu B, Ohishi K, Yamamura K, et al. A potential activity of valproic acid in the stimulation of interleukin-3-mediated megakaryopoiesis and erythropoiesis. Exp Hematol. 2010;38:685–695.
32. Ono R, Kumagai H, Nakajima H, et al. Mixed-lineage-leukemia (MLL) fusion protein collaborates with Ras to induce acute leukemia through aberrant Hox expression and Raf activation. Leukemia. 2009;23:2197–2209.
33. Kitamura T, Koshino Y, Shibata F, et al. Retrovirus-mediated gene transfer and expression cloning: powerful tools in functional genomics. Exp Hematol. 2003;31:1007–1014.
34. Suzuki K, Ono R, Ohishi K, et al. IKAROS isoform 6 enhances BCR-ABL1-mediated proliferation of human CD34+ hematopoietic cells on stromal cells. Int J Oncol. 2011;40:53–62.
35. Hiasa A, Hirayama M, Nishikawa H, et al. Long-term phenotypic, functional and genetic stability of cancer-specific T-cell receptor (TCR) alphabeta genes transduced to CD8+ T cells. Gene Ther. 2008;15:695–699.
36. Shirakura Y, Mizuno Y, Wang L, et al. T-cell receptor gene therapy targeting melanoma-associated antigen-A4 inhibits human tumor growth in non-obese diabetic/SCID/gammacnull mice. Cancer Sci. 2012;103:17–25.
37. Choi YW, Kotzin B, Herron L, Callahan J, Marrack P, Kappler J. Interaction of Staphylococcus aureus toxin "superantigens" with human T cells. Proc Natl Acad Sci U S A. 1989;86:8941–8945.
38. Huntington ND, Alves NL, Legrand N, et al. Autonomous and extrinsic regulation of thymopoiesis in human immune system (HIS) mice. Eur J Immunol. 2011;41:2883–2893.
39. Blom B, Spits H. Development of human lymphoid cells. Annu Rev Immunol. 2006;24:287–320.
40. Weerkamp F, Baert MR, Brugman MH, et al. Human thymus contains multipotent progenitors with T/B lymphoid, myeloid, and erythroid lineage potential. Blood. 2006;107:3131–3137.
41. Dik WA, Pike-Overzet K, Weerkamp F, et al. New insights on human T cell development by quantitative T cell receptor gene rearrangement studies and gene expression profiling. J Exp Med. 2005;201:1715–1723.
42. de La Coste A, Freitas AA. Notch signaling: distinct ligands induce specific signals during lymphocyte development and maturation. Immunol Lett. 2006;102:1–9.
43. Spits H, Lanier LL, Phillips JH. Development of human T and natural killer cells. Blood. 1995;85:2654–2670.
44. Reizis B. Regulation of plasmacytoid dendritic cell development. Curr Opin Immunol. 2010;22:206–211.
45. Miyamoto K, Tsuji K, Maekawa T, Asano S, Nakahata T. Inhibitory effect of interleukin 3 on early development of human B-lymphopoiesis. Br J Haematol. 2001;114:690–697.
46. Lawrence HJ, Sauvageau G, Humphries RK, Largman C. The role of HOX homeobox genes in normal and leukemic hematopoiesis. Stem Cells. 1996;14:281–291.
47. Hozumi K, Negishi N, Suzuki D, et al. Delta-like 1 is necessary for the generation of marginal zone B cells but not T cells in vivo. Nat Immunol. 2004;5:638–644.
48. Beaudette-Zlatanova BC, Knight KL, Zhang S, Stiff PJ, Zuniga-Pflucker JC, Le PT. A human thymic epithelial cell culture system for the promotion of lymphopoiesis from hematopoietic stem cells. Exp Hematol. 2011;39:570–579.
49. Dai B, Wang P. In vitro differentiation of adult bone marrow progenitors into antigen-specific CD4 helper T cells using engineered stromal cells expressing a notch ligand and a major histocompatibility complex class II protein. Stem Cells Dev. 2009;18:235–245.



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.

INTERNAL MEDICINE

□ CASE REPORT □

Amelanotic Malignant Melanoma of Unknown Primary Origin Metastasizing to the Bone Marrow: A Case Report and Review of the Literature

Tomotaka Suzuki[1], Shigeru Kusumoto[2], Shinsuke Iida[2], Toyohiro Tada[3] and Fumiko Mori[1]

Abstract

We herein describe the case of a 77-year-old Japanese man who presented with progressive thrombocytopenia. No lymphadenopathies, bone lesions, hepatosplenomegaly or masses within any internal organs were detectable. Bone marrow smears revealed diffuse infiltration of large atypical cells morphologically resembling mature lymphoid neoplasms. A flow cytometric analysis showed that the tumor cells strongly expressed CD56 without myeloid or lymphoid antigens, suggesting that they were non-hematologic in origin. Ultimately, amelanotic malignant melanoma of unknown primary origin was diagnosed based on positive immunostaining for S100 proteins, HMB-45 and Melan-A. This case illustrates the usefulness of flow cytometric analyses for making such diagnoses. We also review the available literature on similar cases.

Key words: malignant melanoma, unknown primary origin, bone marrow, amelanotic, CD56

(Intern Med 53: 325-328, 2014)
(DOI: 10.2169/internalmedicine.53.1412)

Introduction

Although more than 90% of malignant melanomas have a cutaneous origin, melanomas may present metastatically in the absence of an identified primary lesion (1); such tumors are designated "melanomas of unknown primary origin" (MUP). Furthermore, some malignant melanomas are "amelanotic," meaning that they do not contain histopathologically detectable melanin pigment, which often makes diagnosing MUP difficult (2, 3). We herein report a case of bone marrow metastasis of amelanotic MUP and describe the usefulness of flow cytometric analyses for diagnosing this disease, with a review of the literature.

Case Report

A 77-year-old Japanese man was referred to our hospital due to progressive thrombocytopenia that had developed over the previous two months. His medical history included myocardial infarction, diabetes mellitus and early gastric cancer that was completely resected via endoscopy. On a physical examination, the patient exhibited general weakness and significant emaciation (body mass index: 17 kg/m^2) without other significant findings, including superficial lymphadenopathies or skin lesions. He also had no fever or night sweats.

Peripheral blood tests showed thrombocytopenia (platelet count: 2.6×10^4/μL), normocytic anemia (a hemoglobin level of 10.3 g/dL with an absolute reticulocyte count of 4.5×10^4/μL) and mild leukocytosis (1.1×10^4/μL with 10% myelocytes, 4% metamyelocytes, 74% neutrophils, 9% lymphocytes, 2% monocytes, 1% eosinocytes and 4% erythroblasts). Additional laboratory tests showed an elevated FDP level of 153 μg/dL, suggesting the presence of coexisting disseminated intravascular coagulation (DIC). No lymphadenopathies, bone sclerotic or lytic lesions, hepatomegaly, splenomegaly or masses within any internal organs were detectable on contrast-enhanced computed tomography.

Bone marrow aspiration indicated hypercellular marrow with diffuse involvement of tumor cells and strongly suppressed hematopoiesis, as revealed on May-Giemsa staining.

[1]Department of Hematology, Toyokawa City Hospital, Japan, [2]Department of Medical Oncology and Immunology, Nagoya City University Graduate School of Medical Sciences, Japan and [3]Department of Pathology, Toyokawa City Hospital, Japan
Received for publication July 22, 2013; Accepted for publication September 2, 2013
Correspondence to Dr. Tomotaka Suzuki, let.me.be.92@gmail.com

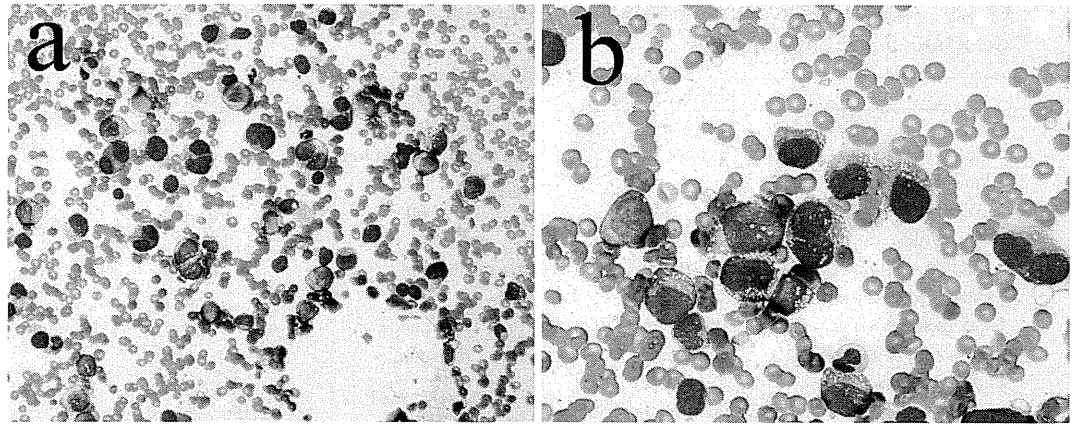


Figure 1. Bone marrow aspiration smear showing infiltration of large atypical cells. The cells had vacuoles in the cytoplasm (a: original magnification ×200, b: ×400 with May-Giemsa staining).

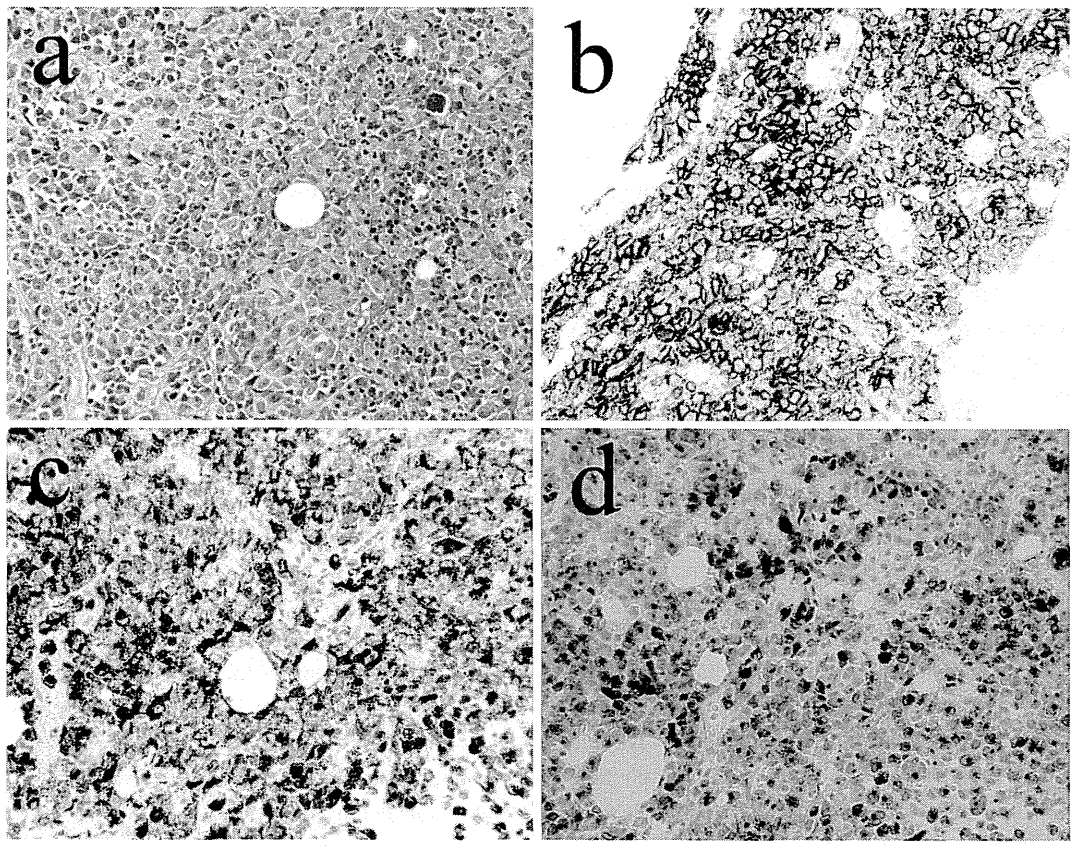


Figure 2. Bone marrow biopsy specimens showing diffuse infiltration of tumor cells with some clear nucleoli (a: ×400 with Hematoxylin and Eosin staining). The cells were positive for CD56 (b: ×400), HMB-45 (c: ×400) and Melan-A (d: ×400).

The tumor cells were non-cohesive and large with abundant vacuoles in basophilic cytoplasm. More than two nuclei were evident in 5% to 10% of infiltrating cells. The tumor cells were negative for myeloperoxidase staining and, based on their morphology, were suspected of being derived from mature lymphoid neoplasms (Fig. 1a, b).

However, an immunophenotypic analysis of bone marrow aspiration using flow cytometry (FCM) showed a strong CD56 expression in the tumor cells; however, myeloid and lymphoid antigens, including CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD11c, CD16, CD19, CD20, CD23, CD25, CD30, CD33, CD34, CD43 and CD45, were absent.

The bone marrow biopsy specimen showed diffuse infiltration of large, rounded neoplastic cells with clear nucleoli without evidence of melanin pigmentation (Fig. 2a). An immunohistochemical examination demonstrated tumor cells positive for CD56 (Fig. 2b), S100 proteins, HMB-45 (Fig. 2c) and Melan-A (Fig. 2d) and negative for CD3, granzyme, keratin, synaptophysin and chromogranin A. These results confirmed that the patient had metastasis of

Table. Three Case Reports of Malignant Melanoma of Unknown Primary Origin with Metastasis to the Bone Marrow

	Reference No.9	Reference No.2	current case
Age/sex	34/male	22/male	77/male
Chief complaint	axillary mass	weakness, weight loss	thrombocytopenia
Blood examination			
WBC (/μL)	2,900	7,800	11,400
Hb (g/dL)	7.1	5.5	10.2
Plt (/μL)	20,000	65,000	20,000
Leukoerythroblastosis	Yes	Yes	Yes
Other metastasis sites	lymph node	lymph node	none
Amelanotic/melanotic	melanotic	melanotic	amelanotic
Flow cytometric analysis	performed CD56 was not described	not performed	performed CD56 was positive
Positive immunostains	S 100 proteins, HMB-45	S 100 proteins, HMB-45	S 100 proteins, HMB-45, Melan-A
Prognosis	died 3 weeks after diagnosis	not available	died 1 week after diagnosis

malignant melanoma to the bone marrow.

We carefully reexamined the patient after making the diagnosis, although no evidence of concomitant cutaneous or mucosal primary lesions was identified and no previous history of skin lesions or surgery was reported. Although the patient had been periodically examined by an ophthalmologist due to his diabetic retinopathy, uveal melanoma had not been detected. He experienced continuous nasal and gastrointestinal bleeding as a result of rapidly aggravating DIC and died approximately one week after the diagnosis on palliative treatment.

Discussion

The incidence of MUP varies from 4% to 8% of patients with malignant melanoma (4-7). Standard criteria for diagnosing MUP have been described by Das Gupta (8); the present patient met these diagnostic criteria. Baab et al. reported the metastatic site at diagnosis in 98 cases of MUP to be as follows: lymph node metastases only (55%), subcutaneous metastases with or without lymph node involvement (13%) and visceral metastases with or without lymph node involvement (32%). Among the visceral cases, metastasis to the bone was observed in only two patients (2% of the MUP cases) although little information was available regarding bone marrow metastasis of MUP (4). Therefore, MUP with metastasis to the bone marrow is rare. To the best of our knowledge, there are only two case reports of bone marrow metastasis of MUP (2, 9) (Table).

The histopathological findings of MUP suggest that the tumor cells are comprised of polygonal or spindle cells with prominent nucleoli (2). On the other hand, they may also exhibit varying cellular morphology with high-grade anaplastic cellular features (10). Because making the morphological diagnosis of MUP is often difficult, approximately 10% of MUP lymph node biopsies may be misdiagnosed as anaplastic carcinoma instead of MUP (5). Importantly, most melanoma cells have melanin pigment in their cytoplasm, which is a major clue to suspect MUP; however, the diagnosis of MUP is established based on immunostaining for markers such as HMB-45, Melan-A and S-100 proteins (11, 12).

However, if the MUP cells are amelanotic, the tumor cells will lack melanin pigment, as observed in the present case, and it becomes difficult to identify malignant melanoma based on histopathology (10). Because there are multiple differential diagnoses to be considered in such cases, the diagnostic process is complicated. Giuliano et al. reported that 50 of 2,881 melanoma patients (1.7%) had amelanotic malignant melanoma, including 29 amelanotic primary sites and 21 amelanotic metastases from a melanotic primary site. Only three patients presented with an absence of pigment in both the primary and metastatic sites (3). Interestingly, amelanotic melanoma has been reported to be associated with an even more aggressive clinical course and a high incidence of metastasis (13-15), similar to the fulminant course observed in the present case.

FCM is an essential tool for diagnosing hematological malignancies in order to confirm the immunophenotype of leukemia and lymphoma (16). However, there is little evidence regarding the clinical significance of FCM for diagnosing non-hematological malignancies. In the presented case, the tumor cells were strongly positive for CD56 but expressed no T-cell, B-cell or myeloid antigens, suggesting that they were more likely non-hematological in origin. CD56 is a cell adhesion protein called NCAM1 (neural cell adhesion molecule 1) that is known to be expressed in hematological malignancies, such as NK/T cell neoplasms, malignant myeloma and some cases of acute leukemia. However, CD56 is also expressed in some non-hematological malignancies, such as melanoma (17), small cell carcinoma (18), rhabdomyosarcoma (19), Merkel cell carcinoma (20), neuroblastoma (21) and neuroendocrine tumors/carcinoma (22).

Farionla et al. evaluated the CD56 expression in neuroendocrine tumors obtained from fine-needle aspiration biopsy specimens and reported the utility of the CD56 expression in the absence of lymphoid antigens for specifically diag-

nosing neuroendocrine tumors (22). On the other hand, few reports regarding the usefulness of FCM in diagnosing malignant melanoma have been published. Bhagwati et al. performed FCM on samples of bone marrow metastasis obtained from a patient with MUP. However, the authors did not test for CD56 positivity and obtained little information for diagnosing malignant melanoma using FCM (9).

We herein reported a case of amelanotic MUP with metastasis exclusively to the bone marrow and demonstrated the usefulness of FCM for diagnosing malignant melanoma, especially when the tumor cells at the biopsy site are amelanotic.

The authors state that they have no Conflict of Interest (COI).

References

1. Katz KA, Jonasch E, Hodi FS, et al. Melanoma of unknown primary: experience at Massachusetts General Hospital and Dana-Farber Cancer Institute. Melanoma Res **15**: 77-82, 2005.
2. Jain D, Singh T, Kumar N, Daga MK. Metastatic malignant melanoma in bone marrow with occult primary site: a case report with review of literature. Diagn Pathol **2**: 38, 2007.
3. Giuliano AE, Cochran AJ, Morton DL. Melanoma from unknown primary site and amelanotic melanoma. Semin Oncol **9**: 442-447, 1982.
4. Baab GH, McBride CM. Malignant melanoma: the patient with an unknown site of primary origin. Arch Surg **110**: 896-900, 1975.
5. Lopez R, Holyoke ED, Moore RH, Karakousis CP. Malignant melanoma with unknown primary site. J Surg Oncol **19**: 151-154, 1982.
6. Chang P, Knapper WH. Metastatic melanoma of unknown primary. Cancer **49**: 1106-1111, 1982.
7. Reintgen DS, McCarty KS, Woodard B, Cox E, Seigler HF. Metastatic malignant melanoma with an unknown primary. Surg Gynecol Obstet **156**: 335-340, 1983.
8. Dasgupta T, Bowden L, Berg JW. Malignant melanoma of unknown primary origin. Surg Gynecol Obstet **117**: 341-345, 1963.
9. Bhagwati N, Seno R, Dutcher JP, Oleksowicz L. Fulminant metastatic melanoma complicated by a microangiopathic hemolytic anemia. Hematopathol Mol Hematol **11**: 101-108, 1998.
10. Roy S, Dhingra K, Mandal S, Khurana N. Unusual presentation of metastatic amelanotic melanoma of unknown primary origin as a solitary breast lump. Melanoma Res **18**: 447-450, 2008.
11. Uesawa M, Sato K, Ozaki K, Nagai T, Muroi K, Ozawa K. Bone marrow metastasis of malignant melanoma. Intern Med **45**: 341, 2006.
12. Serrier C, Lesesve JF. Metastatic malignant melanoma in the bone marrow. Blood **121**: 721, 2013.
13. Gibson LE, Goellner JR. Amelanotic melanoma: cases studied by Fontana stain, S-100 immunostain, and ultrastructural examination. Mayo Clin Proc **63**: 777-782, 1988.
14. Villarrubia J, de Misa RF, Escribano L, Bellas C, Velasco JL. Amelanotic bone marrow infiltration secondary to pigmented malignant melanoma. J Dermatol **22**: 620-622, 1995.
15. Ariel IM. Amelanotic melanomas: an analysis of 77 patients. Curr Surg **38**: 151-155, 1981.
16. Orfao A, Ruiz-Arguelles A, Lacombe F, Ault K, Basso G, Danova M. Flow cytometry: its applications in hematology. Haematologica **80**: 69-81, 1995.
17. Hirata Y, Muroi K, Ozawa T, Matsuhara D. Picture in clinical hematology no.47: case with lymphatic metastasis of malignant melanoma. Rinsho Ketsueki (Jpn J Clin Hematol) **51**: 587, 2010 (in Japanese).
18. Zheng G, Ettinger DS, Maleki Z. Utility of the quantitative Ki-67 proliferation Index and CD56 together in the cytologic diagnosis of small cell lung carcinoma and other lung neuroendocrine tumors. Acta Cytol **57**: 281-290, 2013.
19. Fadare O, Bonvicino A, Martel M, Renshaw IL, Azodi M, Parkash V. Pleomorphic rhabdomyosarcoma of the uterine corpus: a clinicopathologic study of 4 cases and a review of the literature. Int J Gynecol Pathol **29**: 122-134, 2010.
20. Kurokawa M, Nabeshima K, Akiyama Y, et al. CD56: a useful marker for diagnosing Merkel cell carcinoma. J Dermatol Sci **31**: 219-224, 2003.
21. Park SJ, Park CJ, Kim S, et al. Detection of bone marrow metastases of neuroblastoma with immunohistochemical staining of CD56, chromogranin A, and synaptophysin. Appl Immunohistochem Mol Morphol **18**: 348-352, 2010.
22. Farinola MA, Weir EG, Ali SZ. CD56 expression of neuroendocrine neoplasms on immunophenotyping by flow cytometry: a novel diagnostic approach to fine-needle aspiration biopsy. Cancer **99**: 240-246, 2003.

JOURNAL OF TRANSLATIONAL MEDICINE

RESEARCH Open Access

Dose-dependent effects of NY-ESO-1 protein vaccine complexed with cholesteryl pullulan (CHP-NY-ESO-1) on immune responses and survival benefits of esophageal cancer patients

Shinichi Kageyama[1*†], Hisashi Wada[2†], Kei Muro[3], Yasumasa Niwa[4], Shugo Ueda[5], Hiroshi Miyata[2], Shuji Takiguchi[2], Sahoko H Sugino[1], Yoshihiro Miyahara[1], Hiroaki Ikeda[1], Naoko Imai[1], Eiichi Sato[6], Tomomi Yamada[7], Masaharu Osako[8], Mami Ohnishi[9], Naozumi Harada[9], Tadashi Hishida[9], Yuichiro Doki[2] and Hiroshi Shiku[1]

Abstract

Background: Cholesteryl pullulan (CHP) is a novel antigen delivery system for cancer vaccines. This study evaluated the safety, immune responses and clinical outcomes of patients who received the CHP-NY-ESO-1 complex vaccine, Drug code: IMF-001.

Methods: Patients with advanced/metastatic esophageal cancer were enrolled and subcutaneously vaccinated with either 100 μg or 200 μg of NY-ESO-1 protein complexed with CHP. The primary endpoints were safety and humoral immune responses, and the secondary endpoint was clinical efficacy.

Results: A total of 25 patients were enrolled. Thirteen and twelve patients were repeatedly vaccinated with 100 μg or 200 μg of CHP-NY-ESO-1 with a median of 8 or 9.5 doses, respectively. No serious adverse events related to the vaccine were observed. Three out of 13 patients in the 100-μg cohort and 7 out of 12 patients in the 200-μg cohort were positive for anti-NY-ESO-1 antibodies at baseline. In the 100-μg cohort, an antibody response was observed in 5 out of 10 pre-antibody-negatives patients, and the antibody levels were augmented in 2 pre-antibody-positive patients after vaccination. In the 200-μg cohort, all 5 pre-antibody-negative patients became seropositive, and the antibody level was amplified in all 7 pre-antibody-positive patients. No tumor shrinkage was observed. The patients who received 200 μg of CHP-NY-ESO-1 survived longer than patients receiving 100 μg of CHP-NY-ESO-1, even those who exhibited unresponsiveness to previous therapies or had higher tumor burdens.

Conclusions: The safety and immunogenicity of CHP-NY-ESO-1 vaccine were confirmed. The 200 μg dose more efficiently induced immune responses and suggested better survival benefits. (Clinical trial registration number NCT01003808).

Keywords: Esophageal cancer, Cancer vaccine, NY-ESO-1, Cholesteryl pullulan (CHP)

* Correspondence: kageyama@clin.medic.mie-u.ac.jp
†Equal contributors
[1]Departments of Immuno-Gene Therapy and Cancer Vaccine, Mie University Graduate School of Medicine, 2-174, Edobashi, Tsu, Mie 514-8507, Japan
Full list of author information is available at the end of the article

Background

Complexes of cholesteryl pullulan (CHP) nano-particles that contain a tumor antigen are a new type of cancer vaccine with a novel antigen delivery system that presents multiple epitope peptides to both the MHC class I and class II pathways [1-4]. We have been developing CHP-protein human cancer vaccines that efficiently induce immune responses against multiple T cell epitopes for various HLA types. Previous clinical studies using CHP-HER2 and CHP-NY-ESO-1 vaccines showed that these vaccines could be administered repeatedly without serious adverse effects, and both vaccines induced antigen-specific $CD4^+$ and $CD8^+$ T cell immunity as well as humoral immunity [5-7].

Because the NY-ESO-1 antigen is a cancer-testis antigen that is exclusively expressed in the tumor tissue, aside from expression in the normal testis and placenta, this antigen is considered an ideal target for cancer immunotherapy [8,9].

The appropriate dose for NY-ESO-1 protein vaccine has not been determined, although doses up to 100 μg have been examined, in which a higher dose was more immunogenic compared to lower doses of 10 μg and 30 μg [10].

We conducted a dose-escalating trial with CHP-NY-ESO-1 vaccine doses of 100 μg and 200 μg for esophageal cancer patients who were resistant to standard therapies. We evaluated the safety and immune responses to the NY-ESO-1 antigen over the vaccination period, and explored the clinical impact on esophageal cancer patients with a poor prognosis.

In this study, we analyzed IgG antibody responses as antigen-specific immune responses. Although T cells that are induced by a cancer vaccine should be evaluated as an immune-monitoring marker, T cells can be difficult to detect directly and quantitatively assess, whereas IgG titers measured by ELISA could act as a suitable immune-monitoring marker. Analyzing antibody responses induced by CHP-NY-ESO-1 vaccine, the 200 μg-dose more efficiently induced immune responses and suggested better survival benefits.

Materials and methods

Preparation of CHP-NY-ESO-1 complex vaccine

CHP-NY-ESO-1 complex vaccine (Drug code: IMF-001) was provided by ImmunoFrontier, Inc. (Tokyo, Japan). All processes were performed following current Good Manufacturing Practices (cGMP) conditions. The toxicity of the drug products was assessed using animal models, and stability was monitored during the clinical trial using representative samples of the investigational drug product.

Study design

This study was a phase 1, open-label, multi-institutional, dose-escalating clinical trial of the CHP-NY-ESO-1 complex vaccine administered subcutaneously to patients with unresectable, advanced, or refractory esophageal tumors that expressed the NY-ESO-1 antigen. The primary objective was to determine the maximum tolerated dose (MTD) and the biological recommended dose, and the secondary objective was to assess clinical efficacy.

Patients were eligible for entry, if they had a performance status of 0, 1, or 2, were at least 20 years old, had a life expectancy of 4 months or more, and did not have impaired organ function. Patients were ineligible if they were positive for HIV antibody, had multiple cancers, autoimmune disease, serious allergy history, or active brain metastasis, or received previous chemotherapy, systemic steroid or immunosuppressive therapy within less than 4 weeks.

The patients were divided into the following two cohorts of 10 patients each: Cohort 1, 100 μg of the NY-ESO-1 protein every two weeks, and Cohort 2, 200 μg of the NY-ESO-1 protein every two weeks. When a patient withdrew from the trial within three vaccinations, they were replaced with an additional patient.

Clinical responses were assessed according to the Response Evaluation Criteria in Solid Tumors (RECIST ver1.1) [11] and its modified version. The modified version is based on immune-related Response Criteria (ir-RC) [12] and includes the following: Tumor responses were assessed every 6 weeks. Even if disease progression was observed within the first 12 weeks, PD (progressive disease) was not judged. When disease progression was observed after 18 weeks, PD was determined.

Each patient received 6 administrations. However, the treatment could be continued beyond this period if the patient wished to maintain treatment and met the following criteria: 1) no evidence of tumor progression or worsening of performance status (PS), and 2) an anti-NY-ESO-1 antibody response was confirmed. Safety was evaluated according to the National Cancer Institute Common Terminology Criteria for Adverse Events ver.3.0 (NCI-CTCAE ver.3.0) [13]. All the safety information was collected and evaluated, and dose escalation was judged by the Independent Data and Safety Committee.

The study was performed in accordance with the current version of the Declaration of Helsinki and Good Clinical Practice. Written informed consent was obtained from all patients participating in this study. The protocol was approved by the institutional review board at each site. The clinical trial was sponsored by ImmunoFrontier, Inc. (Tokyo, Japan), and registered as ID: NCT01003808 of ClinicalTrials.Gov.

Expression of NY-ESO-1 antigen

NY-ESO-1 expression was assessed by immunohistochemistry with the monoclonal antibody, E978 (Sigma-Aldrich, Saint Louis, MO), [9] or quantitative RealTime-PCR (qRT-PCR) using specific primers [14].

Serum samples

To analyze antigen-specific antibody responses, sera were collected at baseline and two weeks after each vaccination. All sera were stored at −80°C until analysis.

Antibody responses to NY-ESO-1 antigen

NY-ESO-1-specific antibodies in the sera were measured by ELISA as described previously [15]. Briefly, recombinant NY-ESO-1 proteins (His-tag and GST-tag) and NY-ESO-1 peptides were absorbed onto immunoplates (442404; Nunc, Roskilde, Denmark) at a concentration of 10 ng/50 μL/well at 4°C. The collected serum samples were diluted from 1:400 to 1:102,400. After washing and blocking the plate, the sera were added and incubated for 10 h. After washing, goat anti-human IgG (H + L chain) (MBL, Nagoya, Japan) conjugated with peroxidase (The Binding Site, San Diego, CA) was added. After adding the TMB substrate (Pierce, Rockford, IL), the plate was read using a Microplate Reader (model 550; Bio-Rad, Hercules, CA).

Serum samples for 80 healthy volunteers were evaluated to determine a cut-off level for the anti-NY-ESO-1 antibody based on the optical density $(OD)_{450-550}$ absorption value. The cut-off level of anti-NY-ESO-1 IgG was 0.182. A sample was considered to be positive for anti-NY-ESO-1 antibodies if the optical density $(OD)_{450-550}$ absorption value in the ELISA was at the cut-off level or higher at a serum dilution of 1:400. The immune responses of patients with pre-existing anti-NY-ESO-1 antibodies were judged as augmentation if the serum diluted 4-fold or more remained positive.

Statistical analysis

Rates of the immune responses between the patients in Cohort 1 and Cohort 2 were compared by Fisher's exact test, and the survival curve was estimated using the Kaplan–Meier method and compared by the log-rank test. In order to adjust the confounding factors, Cox proportional hazards model was applied. All analyses were done using SAS 9.2 (SAS Institute Inc., Cary, NC).

Results and discussion

Patient characteristics and clinical safety

A total of 25 patients were enrolled in the clinical trial. All patients had unresectable, advanced, or refractory esophageal cancers. The tumor cells in all of these patients were NY-ESO-1-positive, in which the positivity was determined by immunohistochemistry and qRT-PCR for 24 patients and one patient, respectively. All patients received standard chemotherapy and/or other cancer therapies including radiotherapy and surgery, which were ultimately ineffective (Table 1).

Cohort 1 consisted of 13 patients who were given 100 μg of the vaccine; Cohort 2 consisted of 12 patients who were given 200 μg of the vaccine. The patients in Cohort 1 and Cohort 2 received 2 to 27 vaccinations with a median of 8 doses and 3 to 21 vaccinations with a median of 9.5 doses, respectively (Table 1). No dose-limiting toxicity (DLT) was observed. All the patients except one developed transient, grade 1 skin reactions at the injection sites. Other adverse events included swallowing disturbance (n = 8), diarrhea (n = 3), and fever (n = 2), in which events of grade 3 or 4 were included. These events were considered unrelated to the CHP-NY-ESO-1 vaccination. Based on the laboratory data, decreased lymphocyte counts were observed (n = 10), which were all grade 3. These patients had lymphopenia at baseline, probably due to the previous chemotherapies. During the course of the vaccinations, they developed grade 3 lymphopenia, which were shifted from the other grade of the pre-vaccine lymphopenia. Other changes included decreased Na levels (n = 4), decreased hemoglobin levels (n = 3), elevated transaminase levels (n = 2) and elevated uric acid (n = 2) (Table 2). These adverse events were changed from the decreased or elevated levels at baseline. They did not affect the vaccine continuation. Therefore, the changes were considered not related or unlikely related to the vaccination.

Table 1 Patients demographics

	100 μg	200 μg
No. patients enrolled	13	12
Sex		
Male	13	11
Female	0	1
Age		
Median	69	64.5
Range	49-72	53-79
Prior therapy		
Surgery	6	5
Radiotherapy	11	7
Chemotherapy	13	12
Pre-existing antibody to NY-ESO-1 antigen	3	7
No. vaccinations		
Median	8	9.5
Range	2-27	3-21

Immune responses to NY-ESO-1 protein

As shown Table 3, 3 out of the 13 patients, and 7 out of 12 patients had pre-existing antibodies to NY-ESO-1, while the remaining 10 and 5 patients did not have this reactivity in Cohort 1 and Cohort 2, respectively.

To evaluate the antibody responses after vaccination, serum samples collected at the serial vaccinations were analyzed using an antigen-specific IgG ELISA. In three patients of 100–02, 100–3 and 200–7 who were vaccinated three times, the serum samples from 1st and 2nd

Table 2 Adverse events during CHP-NY-ESO-1 vaccinations

Adverse event	100 μg(n = 13)						200 μg(n = 12)						Total
	Grade						Grade						
	1	2	3	4	5	Subtotal	1	2	3	4	5	Subtotal	
Skin reaction	12	0	0	0	0	12	12	0	0	0	0	12	24
Swallowing disturbance	0	0	3	0	0	3	0	0	4	1	0	5	8
Diarrhea	0	0	2	0	0	2	1	0	0	0	0	1	3
Fever	2	0	0	0	0	2	0	0	0	0	0	0	2
Decreased lymphocytes count	0	0	7	0	0	7	0	0	3	0	0	3	10
Decreased Na level	0	0	2	0	0	2	0	0	2	0	0	2	4
Decreased Hb level	0	0	3	0	0	3	0	0	0	0	0	0	3
Elevated ALT/AST level	0	0	2	0	0	2	0	0	0	0	0	0	2
Elevated uric acid level	0	0	1	1	0	2	0	0	0	0	0	0	2

NOTE: Events occurring more than once are listed. Events of disease progression are not listed.

vaccination were assayed. In Cohort 1, out of 10 pre-antibody-negative patients, 5 became seropositive. Two out of 3 pre-antibody-positive patients had augmented antibody responses. In total, 7 of 13 (53.8%) patients exhibited immune responses. Five pre-antibody-negative and 7 pre-antibody-positive patients in Cohort 2 became positive or were augmented, yielding 12 out of 12 or 100% responsiveness. The 200-μg dose was more immunogenic than the 100-μg dose ($p = 0.015$, Fisher's exact test). In Cohort 1, immune reactions were observed after a median of 2 cycles, with a range of 1 to 4 vaccine cycles. In Cohort 2, the immune responses were also evident after a median of 2 cycles with a range of 1 to 5 cycles (Table 3). The chronological appearance of the immune responses and antibody titers are shown in Figure 1. The antibody intensities appeared more quickly and at a higher titer in patients in Cohort 2 (200 μg) than those in Cohort 1(100 μg). In addition to His-tag NY-ESO-1 protein, we tested serum reactivities to GST-tag NY-ESO-1 protein and NY-ESO-1 peptides. We confirmed specific reactions to NY-ESO-1 antigen in these sera.

Clinical responses and long-term follow-up

There were no cases of tumor shrinkage with partial response (PR) or complete response (CR) in any of the 25 patients. At the assessment that occurred every 6 weeks after vaccination, stable disease (SD) was observed in 3 patients in Cohort 1 and 6 patients in Cohort 2 (Table 4). There was no discordance in the evaluations between RECIST ver1.1 [11] and its modified version [12].

Table 3 Antibody responses in patients vaccinated with 100 μg or 200 μg of CHP-NY-ESO-1

100 μg				200 μg			
pt No.	Vaccination cycle	Baseline (dilution titer)	Antibody response (cycle*)	pt No.	Vaccination cycle	Baseline (dilution titer)	Antibody response (cycle*)
100-01	9	negative	responded(4)	200-01	15	negative	responded(2)
100-02	3	negative	no response**	200-02	9	negative	responded(2)
100-03	3	negative	no response**	200-03	8	positive (x1,600)	responded(5)
100-04	7	negative	no response	200-04	21	negative	responded(2)
100-05	2	negative	no response	200-05	3	negative	responded(2)
100-06	16	positive (x6,400)	responded(1)	200-06	10	positive (x400)	responded(1)
100-07	9	positive (x25,600)	no response	200-07	3	positive (x25,600)	responded(2)**
100-08	10	negative	responded(1)	200-08	11	positive (x400)	responded(1)
100-09	5	negative	no response	200-09	18	positive (x400)	responded(3)
100-10	27	positive (x400)	responded(3)	200-10	11	positive (x400)	responded(2)
100-11	8	negative	responded(2)	200-11	3	positive (x400)	responded(2)
100-12	8	negative	responded(2)	200-12	9	negative	responded(1)
100-13	26	negative	responded(2)				
antibody response rate			53.8%***			100%***	

*vaccine cycles with which antibody responses appeared. **antibody responses assayed after two vaccinations.***p = 0.015(Fisher's exact test).