bjh research paper

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

Yoshiki Nakamori,[1†] Bing Liu,[1†] Kohshi Ohishi,[2] Kei Suzuki,[1] Kazuko Ino,[1] Takeshi Matsumoto,[2] Masahiro Masuya,[1] Hiroyoshi Nishikawa,[3] Hiroshi Shiku,[3] Hirofumi Hamada[4] and Naoyuki Katayama[1]

[1]Haematology and Oncology, Mie University Graduate School of Medicine, Tsu, Mie, Japan, [2]Blood Transfusion Service, Mie University Hospital, Tsu, Mie, Japan and [3]Department of Cancer Vaccine, Mie University Graduate School of Medicine, Tsu, Mie, Japan and [4]Department of Life Science, Tokyo University of Pharmacy and Life Sciences, Hachioji, Tokyo, Japan

Received 07 November 2011; accepted for publication 25 February 2012

Correspondence: Kohshi Ohishi, Blood Transfusion Service, Mie University Hospital, 2-174 Edobashi, Tsu, Mie 514-8507, Japan. E-mail: koishi@clin.medic.mie-u.ac.jp and Naoyuki Katayama, Haematology and Oncology, Mie University Graduate School of Medicine, 2-174 Edobashi, Tsu, Mie 514-8507, Japan. E-mail: n-kata@clin.medic.mie-u.ac.jp

[†]These authors contributed equally to this study.

Summary

The regulation of human early lymphopoiesis remains unclear. B- and T-lineage cells cannot develop simultaneously with conventional stromal cultures. Here we show that telomerized human bone marrow stromal cells supported simultaneous generation of $CD19^+CD34^{lo/-}CD10^+cyCD79a^+CD20^{+/-}$ $VpreB^-$ pro-B cells and $CD7^+CD34^+$ $CD45RA^+CD56^-cyCD3^-$ early T/Natural Killer (NK) cell precursors from human haematopoietic progenitors, and the generation of both lymphoid precursors was promoted by flt3 ligand (flt3L). On the other hand, stem cell factor or thrombopoietin had little or no effect when used alone. However, both acted synergistically with flt3L to augment the generation of both lymphoid precursors. Characteristics of these lymphoid precursors were evaluated by gene expression profiles, rearrangements of IgH genes, or replating assays. Similar findings were observed with primary human bone marrow stromal cells. Notably, these two lymphoid-lineage precursors were generated without direct contact with stromal cells, indicating that early B and T/NK development can occur, at least in part, by stromal cell-derived humoral factors. In serum-free cultures, flt3L elicited similar effects and appeared particularly important for B cell development. The findings of this study identified the potential of human bone marrow stromal cells to support human early B and T lymphopoiesis and a principal role for flt3L during early lymphopoiesis.

Keywords: stromal cells, B lymphopoiesis, T lymphopoiesis, haematopoietic progenitors, Flt3 ligand.

Early lymphoid differentiation from haematopoietic stem cells takes place in the bone marrow (LeBien, 2000; Zlotoff *et al*, 2008). *Ex vivo* and *in vivo* murine studies have led to significant advances in the understanding of lymphoid differentiation pathways and the regulatory mechanisms involved (Akashi *et al*, 2000; Rothenberg, 2010). Within the murine bone marrow, specific stromal or osteoblastic cells are thought to be crucial for B lymphopoiesis (Tokoyoda *et al*, 2004; Zhu *et al*, 2007; Wu *et al*, 2009). In mice, besides stem cell factor (SCF) and interleukin (IL)-7 (Kang & Der, 2004), flt3 ligand (flt3L) plays an important role in the differentiation of common lymphoid precursors from haematopoietic stem cells (Sitnicka *et al*, 2002) and in T and B cell reconstitution after bone marrow transplantation (Buza-Vidas *et al*, 2007). However, the cytokine-mediated regulation of human early lymphopoiesis remains uncertain (Blom & Spits, 2006). The roles of flt3L and IL-7 in haematopoiesis differ between humans and mice (LeBien, 2000; Blom & Spits, 2006). For example, flt3 is expressed on human but not murine haematopoietic stem cells (Sitnicka *et al*, 2003). Furthermore, IL-7 signalling is essential for murine B cell development (Peschon *et al*, 1994; von Freeden-Jeffry *et al*, 1995), while congenital immunodeficiency patients lacking expression of the common γ chain or IL-7-specific α chain of the IL-7 receptor have normal or even elevated numbers of peripheral blood B cells (LeBien, 2000; Blom & Spits, 2006).

First published online 30 March 2012
doi: 10.1111/j.1365-2141.2012.09109.x

Human B cell development from haematopoietic progenitors is generally examined by coculture with murine stromal cell lines, such as MS-5, or human bone marrow stromal cells (LeBien, 2000). Although it is difficult to assess human T lymphopoiesis *in vitro*, we and others previously showed that the Notch ligand DLL1 induces the differentiation of *multipotent* haematopoietic progenitors into $CD7^+CD34^+CD45RA^+$ early T cell precursors (Jaleco *et al*, 2001; Ohishi *et al*, 2002). Thereafter, DLL1 or DLL4, engineered to be expressed in murine OP9 bone marrow or thymic stromal cell lines, was shown to induce the differentiation of human *multipotent* haematopoietic progenitors into $CD4^+CD8^+$ or $CD1a^+CD5^+$ T cell precursors (Schmitt & Zuniga-Pflucker, 2002; Awong *et al*, 2007; Meek *et al*, 2010). Nevertheless, the potential utility of these *in vitro* coculture assays for studying B and T lymphopoiesis remains limited because it has been thought that the differentiation of haematopoietic progenitors into T and B cell lineages cannot be analysed simultaneously in culture systems.

In this study, we found for the first time that telomerized (Kawano *et al*, 2003; Matsunaga *et al*, 2006; Fujimi *et al*, 2008) and primary human bone marrow stromal cells support simultaneous development of early B and T/NK lymphoid precursors from human haematopoietic progenitors. This coculture system led us to identify a principal role for flt3L in human early B and T/NK cell lymphopoiesis.

Methods

Isolation of haematopoietic progenitors

After obtaining informed consent, human umbilical cord blood was obtained from full-term deliveries according to a protocol approved by the Ethics Committee of Mie University Hospital. $CD34^+$ cells were isolated from mononuclear cells using CD34 immunomagnetic beads (MACS; Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's instructions as previously described (Ohishi *et al*, 2002; Liu *et al*, 2010). $CD34^+$ cells were stained with anti-CD10-fluorescein isothiocyanate (FITC; BD Biosciences, San Jose, CA, USA), anti-CD19-phycoerythrin (PE; Beckman Coulter, Fullerton, CA, USA), anti-CD34-peridinin chlorophyll cyanin 5·5 (PerCPCy5·5; BD Biosciences), anti-CD38-PECy7 (BioLegend, San Diego, CA, USA), and anti-CD7-allophycocyanin (APC; Bay Bioscience, Kobe, Japan) mouse monoclonal antibodies and sorted using a FACSAria flow cytometer (BD Biosciences).

Recombinant factors

Recombinant human thrombopoietin (TPO) was a gift from Kirin Brewery (Tokyo, Japan). Recombinant SCF and flt3L were purchased from R&D Systems (Minneapolis, MN, USA). IL-7 and IL-15 were purchased from PeproTech (Rocky Hill, NJ, USA). All cytokines were used at the following concentrations unless otherwise stated: SCF, 10 ng/ml; TPO, 10 ng/ml; flt3L, 5 or 10 ng/ml; IL-7, 5 ng/ml; IL-15, 10 ng/ml.

Flow cytometric analysis

Immunofluorescence staining was performed as previously described (Ohishi *et al*, 2002; Liu *et al*, 2010), using the following murine monoclonal antibodies: anti-CD1a-FITC (Dako Japan, Kyoto, Japan), anti-CD5-FITC, anti-CD10-FITC (both from BD Biosciences), anti-CD14-FITC (BioLegend), anti-CD45RA-FITC, anti-HLA-DR-FITC (BD Pharmingen, San Diego, CA, USA), anti-CD2-PE, anti-CD19-PE, anti-CD20-PE (all from BD Biosciences), anti-CD7-PE, anti-CD56-PE, anti-VpreB-PE (all from Beckman Coulter), Anti-CD123 (Interleukin-3 receptor α-chain)-PE (BD Pharmingen), anti-CD14-PECy7, anti-CD4-PECy7 (both from BD Biosciences), anti-CD1a-APC (BioLegend), anti-CD3-APC (Beckman Coulter), anti-CD7-APC (eBioscience, San Diego, CA, USA), anti-CD79a-APC (BioLegend), anti-CD303 (BDCA2)-APC (Miltenyi Biotec), and anti-CD8-APC-Cy7, CD11c-APC-Cy7, and CD34-APC-Cy7 (all from BioLegend). IgG_1-FITC, IgG_{2a}-FITC (both from BD Biosciences), IgG_1-PE, IgG_{2a}-PE, IgG_{2b}-PE, IgG1-PerCP-Cy5·5, IgG_1-APC, or IgG_1-APC-Cy7 (all from BD Pharmingen), IgG_1-PECy7 (BioLegend), served as isotype controls. Dead cells were excluded by staining with propidium iodide (BD Pharmingen) or 7-Aminoactinomycin D (BD Biosciences).

Cytoplasmic CD3 and CD79a staining was performed as previously described (Ohishi *et al*, 2002) with some modifications. Briefly, cells were incubated with various antibodies against surface antigens and streptavidin-conjugated tricolour (CALTAG, Burlingame, CA, USA) for 30 min at 4°C. After washing, the cells were permeabilized and fixed with Permea-Fix (Ortho, Raritan, NJ, USA) for 20 min at room temperature, washed again, and incubated with APC-conjugated antibodies against CD3 or CD79a for 30 min at 4°C. Dead cells were distinguished by positive staining with streptavidin-conjugated tricolour (Levelt & Eichmann, 1994; Ohishi *et al*, 2002).

Flow cytometric analysis was performed using a FACSCanto II flow cytometer (BD Biosciences) and the data were analysed using BD FACSDiva software (BD Biosciences).

Cocultures

Human telomerase reverse transcriptase (hTERT)-transduced telomerized stromal cells were obtained from the Riken BioResource Centre (Tsukuba, Japan). Before cocultures, the telomerized stromal cells were plated in a 25 cm^2 cell culture flask (Corning, NY, USA), or 12- or 96-well tissue culture plates (Nunc, Roskilde, Denmark) with Dexter-type long-term culture medium comprising minimum essential medium-α (αMEM; Gibco-Invitrogen, Grand Island, NY, USA), 12·5% horse serum (Invitrogen, Carlsbad, CA, USA), 12·5%

fetal calf serum (FCS; Invitrogen), and 1×10^{-6} mol/l hydrocortisone (Sigma-Aldrich, St Louis, MO, USA) as previously described (Kawano *et al*, 2003, 2006; Fujimi *et al*, 2008). Human primary bone marrow stromal cells derived from healthy adults were purchased from Lonza (Walkersville, MD, USA), and plated in a 25 cm^2 cell culture flask with long-term haematopoietic progenitor cell culture medium (MyeloCult; Stem Cell Technologies, Vancouver, BC, Canada). On the first day of coculture, the stromal cells were washed with αMEM. Five or ten thousand sorted haematopoietic progenitors were then seeded onto a pre-established monolayer of telomerized stromal cells along with 5–10 ml of αMEM supplemented with 20% FCS, 50 U/ml of penicillin, and 50 μg/ml of streptomycin in the presence or absence of cytokines. Half of the medium was exchanged for fresh medium containing the same concentrations of cytokines every 4–5 d. For single-cell cultures, individual cells were cultured in 96-well tissue culture plates (Nunc) containing a pre-established monolayer of telomerized stromal cells, in the presence or absence of flt3L. Cell culture inserts for 6-well plates with 0·4-μm pores (BD Biosciences) were used to separate haematopoietic progenitor cells from stromal cells in 6-well tissue culture plates (BD Biosciences). For serum-free cultures, α-MEM/20% FCS was replaced with serum-free medium (StemSpan; Stem Cell Technologies).

To induce T cell differentiation, cells were cocultured with a monolayer of OP9 stromal cells expressing the Notch ligand DLL1 (OP9-DL1; a gift from Dr. Juan Carlos Zúñiga-Pflücker, Department of Immunology, University of Toronto, Toronto, ON, Canada) pre-established in a 25 cm^2 cell culture flask for 28 d in the presence of 5 ng/ml of flt3L and 5 ng/ml of IL-7 (Schmitt & Zuniga-Pflucker, 2002; Awong *et al*, 2007). A stabilized form of vitamin C, phosphorylated ascorbate (100 ng/ml; Sigma-Aldrich), was added during the last 14 d of culture to enhance T cell differentiation (Manning *et al*, 2010). On days 3–4, the cocultures were disaggregated by vigorous pipetting, filtered through a 70 μm nylon filter (BD Biosciences) to reduce stromal cell aggregates and contamination with OP9-DL1 cells, and replated to new flasks containing fresh medium and the same concentrations of cytokines (Schmitt & Zuniga-Pflucker, 2002; Awong *et al*, 2007). Viable cells were counted using the trypan blue exclusion method.

Cytokine concentrations

Concentrations of flt3L, IL-7, and IL-15 in the culture media were analysed, using MILLIPLEX MAP Human Cytokine/Chemokine Panel I (Millipore, Billerica, MA, USA) with the manufacturer's recommended method.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted using an RNeasy Micro Kit (Qiagen, Valencia, CA, USA). Total RNA (1 μg) was reverse-transcribed in a total volume of 20 μl using a QuantiTect Reverse Transcription Kit (Qiagen). The PCR amplifications were performed for 35 cycles under the conditions shown in Table I, using either EX Taq (Takara, Shiga, Japan) or KOD FX (ToYoBo, Osaka, Japan) DNA polymerase. Primers for the following genes were prepared, as previously reported by others: *PAX5* (Reynaud *et al*, 2003); early B cell factor 1 (*EBF1*) (Gisler *et al*, 2000); *GATA3* (Garcia-Peydro *et al*, 2006); transcription factor 12 [*TCF12*; also termed HeLa E-box-binding factor (HEB)] (Schotte *et al*, 2010); inhibitor of DNA binding 2 (*ID2*) (Gudmundsson *et al*, 2007); pre-T cell receptor-α *PTCRA*) (Hao *et al*, 2001); and

Table I. RT-PCR primers and conditions.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Application condition			Size (bp)	*
PAX5	AGCAGGACAGGACATGGAGGA	ATCCTGTTGATGGAACTGACGC	98°C 10 s	65°C 30 s	72°C 30 s	377	(1)
EBF1	CAGGAAAGCATCCAACGGAGTGG	TGAGCAAGACTCGGCACATTTCTG	98°C 10 s	61°C 30 s	68°C 30 s	454	(2)
GATA3	GAAGGCATCCAGACCCGAAAC	ACCCATGGCGGTGACCATGC	95°C 30 s	62°C 30 s	72°C 20 s	255	(1)
TCF12	CCGTGGCAGTCATCCTTAGT	GCCGATACGGCAGAAACTT	98°C 10 s	57°C 30 s	68°C 10 s	109	(2)
ID2	CCCAGAACAAGAAGGTGAGC	AATTCAGAAGCCTGCAAGGA	95°C 10 s	68°C 60 s	–	200	(2)
PTCRA	TCCAGCCCTACCCACAGGTG	ATGAAGCCTCTCCTGACAGATGCAT	98°C 10 s	65°C 30 s	72°C 30 s	350	(1)
B2M	CCAGCAGAGAATGGAAAGTC	GATGCTGCTTACATGTCTCG	98°C 10 s	65°C 30 s	72°C 30 s	269	(1)

*(1) ExTaq or (2) KOD FX DNA polymerase was used.

β_2-microglobulin (*B2M*) (Cerdan *et al*, 2000). The sequences of the primers and the product lengths are also shown in Table I.

The PCR products were electrophoresed in a 2% agarose gel in conjunction with a molecular weight ladder, visualized by ethidium bromide staining, and viewed under ultraviolet illumination.

Quantitative RT-PCR analysis

Total cellular RNA was extracted from hTERT-transduced stromal cells, using an RNeasy Micro Kit (Qiagen), and total RNA was reverse-transcribed, using a QuantiTect Reverse Transcription Kit (Qiagen). Specific cDNA fragments were amplified for *DLL1* (Buchler *et al*, 2005) and *DLL4* (Nijjar *et al*, 2002) by using previously described primers. For *DLL1*, the primer set was 5′-CCTACTGCACAGAGCCGATCT-3′ and 5′-ACAGCCTGGATAGCGGATACAC-3′. For *DLL4*, the primers were 5′-TGACCACTTCGGCCACTATG-3′ and 5′-AGTTGGAGCCGGTGAAGTTG-3′. As a standard, *GAPDH* gene expression was amplified using the primers 5′-CCATC ACCATCTTCCAGGAGCGAG-3′ and 5′-CACAGTCTTCTG GGTGGCAGTGAT-3′. Equal amounts of cDNA were used for 45 cycles of amplification. Absolute Quantitative PCR was performed using a QIAGEN Quantitect SYBR Green PCR Kit (Qiagen) and the Mx3000p Real-Time QPCR System (Agilent Technologies, La Jolla, CA, USA), and then analysed with MXPRO software (Agilent Technologies). The reaction conditions were as follows: an initial denaturation step at 95°C for 15 min was followed by 45 cycles of denaturation at 94°C for 15 s, annealing at 60°C (*DLL1, GAPDH*) and at 57°C (*DLL4*) for 30 s and extension at 72°C for 30 s. Transcript quantification was performed in duplicate for each of the four samples. *DLL1* and *DLL4* values were reported as the normalized quotient, derived by dividing the *DLL1* or *DLL4* copy number by the *GAPDH* copy number.

Variable, diverse, and joining (VDJ) rearrangements in the immunoglobulin heavy chain (IGH@) gene

VDJ rearrangements of *IGH@* were assessed using Rearrangement and Translocation Assays (In VivoScribe Technologies, San Diego, CA, USA). The genomic DNA between the primers targeting the conserved framework 3 (FR3) of the immunoglobulin variable heavy chain (VH) and the joining region (JH) consensus regions was amplified by PCR and analysed by capillary electrophoresis on an ABI 310/3100 capillary electrophoresis instrument (Applied Biosystems, Foster City, CA, USA) (van Krieken *et al*, 2007). Human normal tonsil tissue was used as a positive control.

Statistical analysis

Statistical comparisons were made using Student's *t* test. Values of $P < 0{\cdot}05$ were considered significant.

Results

Telomerized stromal cells support the generation of early lymphoid precursors from haematopoietic progenitors

It has been reported that the $CD19^+$, $CD10^+$, or $CD7^+$ populations within $CD34^+$ cells in cord blood predominantly exhibit B or T lymphoid differentiation potential (Haddad *et al*, 2004; Blom & Spits, 2006), and that the expression of cytoplasmic CD79a (cyCD79a), a component of the B cell receptor complex, is detected from an early stage of B cell differentiation (Dworzak *et al*, 1998; Reynaud *et al*, 2003). To isolate human haematopoietic progenitors negative for these surface and cytoplasmic lymphoid antigens, we first examined the expression of CD19, CD10, or CD7, and the relationships of these lymphoid antigens with the differentiation marker CD38 on $CD34^+$ cells. The relationships between the surface expressions of these lymphoid antigens and cyCD79a were also assessed. The $CD34^+$ cell populations that were positive for CD19, CD10, or CD7 tended to express higher levels of CD38. Most $CD19^+$ or $CD10^+$ cells overlapped with each other, expressed cyCD79a, and were mutually exclusive of $CD7^+$ cells (Fig. S1). These findings suggest that $CD19^+$ or $CD10^+$ cells within the $CD34^+$ cells coexpress cyCD79a and high levels of CD38, and that isolation of the surface $CD38^{lo/-}CD19^-CD10^-CD7^-$ fraction from $CD34^+$ cells leads to the exclusion of $cyCD79a^+$ cells.

Next, we examined whether telomerized human bone marrow-derived stromal cells support the generation of B or T lineage cells from human haematopoietic progenitors. $CD34^+CD38^{lo/-}CD7^-CD19^-CD10^-$ cells were isolated (Fig. 1A) [the purity after sorting was $96{\cdot}1 \pm 1{\cdot}5\%$ ($n = 8$)], and incubated on the stromal cells (5×10^3 cells/well) in the absence of exogenous cytokines. After 3 weeks, the cultures produced only $6{\cdot}5 \pm 1{\cdot}8 \times 10^4$ ($n = 4$) cells but contained $CD19^+$, $CD7^+$ and $CD14^+$ cells (Fig. 1B). Some of the $CD19^+$ cells became negative for CD34 and positive for CD10, CD20 and cyCD79a, but all of the $CD19^+$ cells remained negative for VpreB, a component of the pre-B cell receptor complex. These phenotypes of the $CD19^+$ cells were consistent with those of early B cell precursors at the pro-B stage. In addition to $CD19^+cyCD79a^+$ cells, $CD19^-cyCD79a^+$ cells were observed, which represent B cell precursors in an early stage of B cell development. Some $CD7^+$ cells became negative for CD34 but coexpressed CD34 and CD45RA. The $CD7^+$ cells were partially positive for CD2, but low or negative for the NK marker CD56 and pre-T cell-related antigens including CD5, CD1a and cyCD3. These phenotypes of the $CD7^+$ cells corresponded to those of early T/NK cell precursors (Blom & Spits, 2006). These cocultures also contained a significant population of $CD14^+$ cells [$35{\cdot}2 \pm 20{\cdot}7\%$ (n = 5)], and a portion of the $CD14^+$ cells expressed CD1a (Fig. 1C). These $CD1a^+$ cells were positive for HLA-DR, CD11c and IL-3Rα, indicative of myeloid dendritic cells. We also observed the presence of $CD14^-$HLA-$DR^+CD11c^-CD1a^-IL\text{-}3R\alpha^{high}CD303^+$

cells, which are phenotypically considered to be plasmacytoid dendritic cells (Rossi & Young, 2005; Ueno *et al*, 2011) (Fig. 1C).

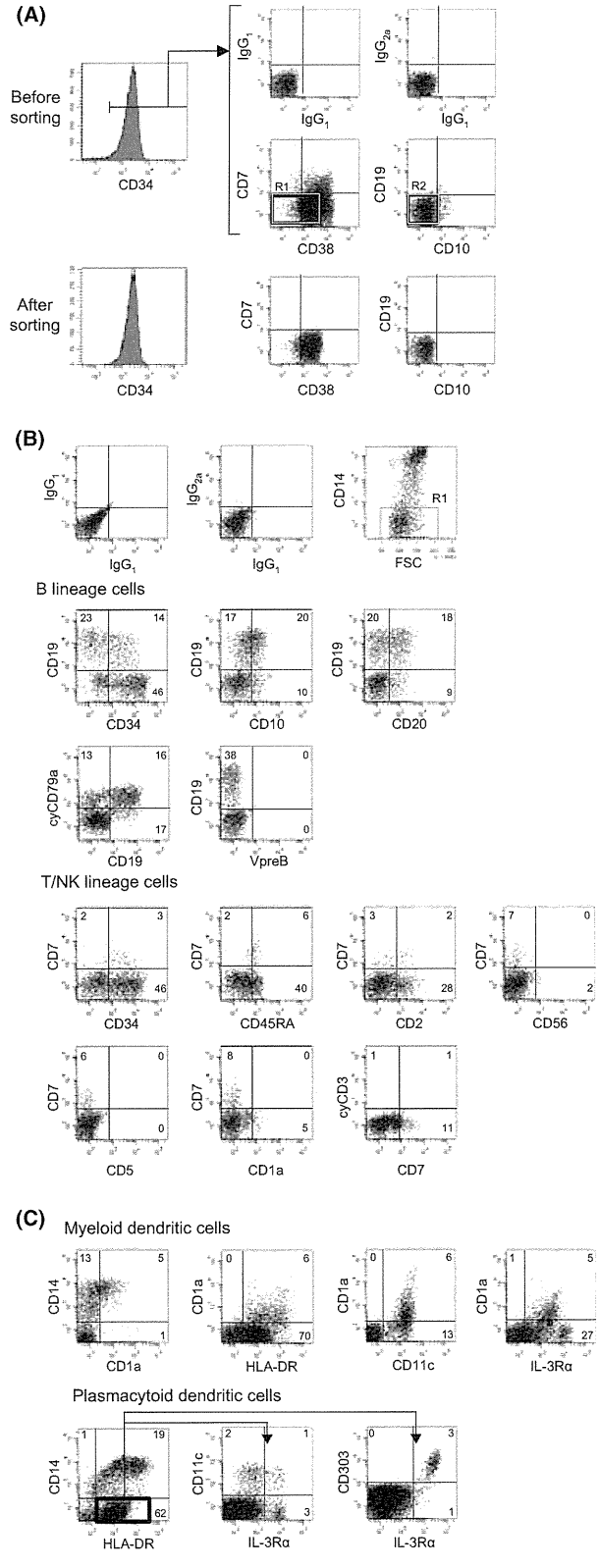

Fig 1. Expression of lymphoid-lineage antigens in cells cultured with telomerized stromal cells. (A) Phenotype of the $CD38^{low/-}CD19^-CD10^-CD7^-$ cell fraction before and after sorting of immunomagnetically-enriched $CD34^+$ cells. (B) $CD34^+CD38^{low/-}CD19^-CD10^-CD7^-$ cells (5×10^3 cells/well) were cocultured for 3 weeks with telomerized stromal cells without cytokines. The expression of B and T/NK lymphoid antigens after excluding $CD14^+$ cells (R1 gate) is shown. (C) The expression of CD14, HLA-DR, CD11c and IL-3Rα in $CD1a^+$ cells was analysed to assess the presence of myeloid dendritic cells (upper row). For plasmacytoid dendritic cells, $CD14^-$HLA-DR^+ cell fractions were gated, and analysed for the presence of IL-$3R\alpha^{high}CD11c^-CD303^+$ cells (lower row).

Flt3L enhances the generation of $CD19^+$cyCD79a$^+$, $CD19^-$cyCD79a$^+$ and $CD7^+$ cells from haematopoietic progenitors cocultured on telomerized stromal cells

Several mouse studies have demonstrated that flt3L plays an important role in early lymphopoiesis (Sitnicka *et al*, 2002; Buza-Vidas *et al*, 2007). To examine the role of flt3L in the generation of early B and T/NK cell precursors from human haematopoietic progenitors, $CD34^+CD38^{lo/-}CD19^-CD10^-CD7^-$ cells were cultured on the telomerized stromal cells for 3 weeks in the presence of various concentrations of flt3L. As shown in Fig. 2A, flt3L increased both the percentages and absolute numbers of cyCD79a$^+$, $CD19^+$cyCD79a$^+$ and $CD7^+$ cells in the cultures in dose-dependent manners. These effects reached a plateau at 10 ng/ml. Flt3L also promoted the generation of $CD1a^+$HLA-DR^+CD11c^+ myeloid (Fig. S2A) and $CD14^-$HLA-DR^+CD11c^-IL-$3R\alpha^{high}CD303^+$ plasmacytoid dendritic cells (Fig. S2B).

Next, we investigated the effects of flt3L (10 ng/ml) on the generation of $CD19^+$cyCD79a$^+$, $CD19^-$cyCD79a$^+$ and $CD7^+$ cells from more immature haematopoietic progenitors cultured on telomerized stromal cells. For this, $CD38^-$ cell subset from the $CD34^+CD19^-CD10^-CD7^-$ cell population was sorted (Fig. 2B) and cultured with or without flt3L at 10 ng/ml. The results showed that, even in the absence of flt3L, the stromal cells supported the generation of $CD19^+$cyCD79a$^+$, $CD19^-$cyCD79a$^+$ and $CD7^+$ lymphoid precursors from $CD34^+CD38^-CD19^-CD10^-CD7^-$ haematopoietic progenitor cells, which was considerably enhanced by flt3L (Fig. 2B).

To clarify the effects of flt3L on B- and T/NK-lineage precursors in more detail, similar experiments were performed using single-cell assays. To this end, single $CD34^+CD38^-CD19^-CD10^-CD7^-$ cells were individually cultured on telomerized stromal cells, either with or without 10 ng/ml of flt3L (288 wells per group), and the numbers of wells containing 50–99, 100–499, 500–999 and $\geq$1000 cells were counted. As shown in Fig. 2C, the numbers of wells showing significant cell proliferation were markedly higher in cultures containing flt3L. The generated cells in the wells that contained more than 50 cells (nine wells with flt3L and two wells without flt3L) were harvested and their phenotypes were analysed by flow cytometry. A significant portion of these wells contained either $CD19^+$ or $CD7^+$ cells in addition

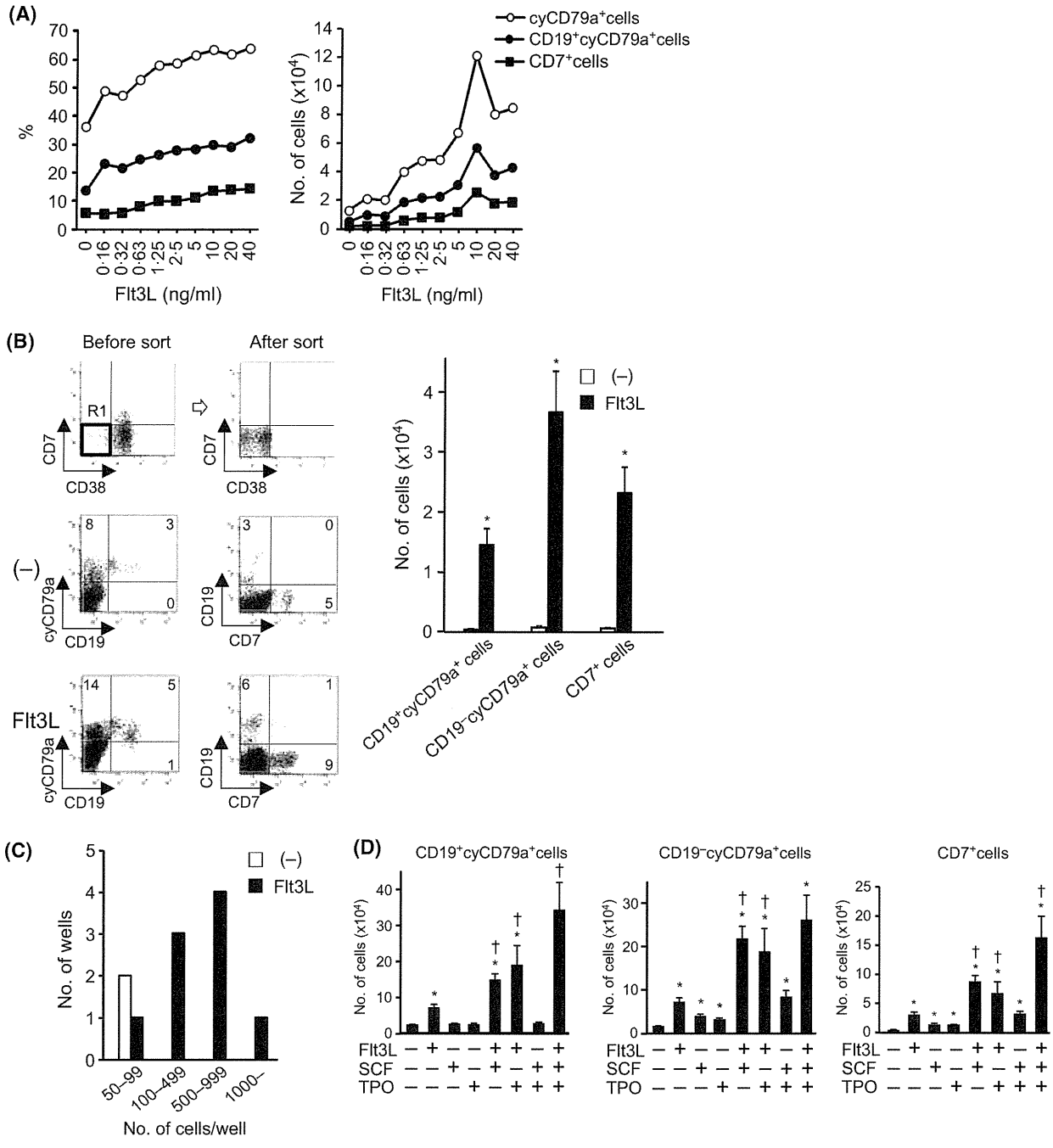

Fig 2. Effects of flt3L, SCF and TPO on lymphopoiesis. The effects of flt3L on $CD34^+CD38^{low/-}CD19^-CD10^-CD7^-$ (1×10^4 cells/well) (A) and $CD34^+CD38^-CD19^-CD10^-CD7^-$ (1×10^4 cells/well) (B) cultured on telomerized stromal cells. The percentage and number of $CD19^+cyCD79a^+$, $CD19^-CD79a^+$ and $CD7^+$ cells were assessed. The percentages after excluding $CD14^+$ cells are shown. Data represent means of duplicate (A) and means ± SD of triplicate (B) cultures. $^*P < 0{\cdot}05$ compared with control cultures. Data are representative of five independent experiments. (C) $CD34^+CD38^-CD19^-CD10^-CD7^-$ cells were individually cultured with or without 10 ng/ml of flt3L (288 wells for each group), and the number of wells containing 50–99, 100–499, 500–999, or ≥1000 cells was scored by observation on an inverted microscope. (D) $CD34^+CD38^{low/-}CD19^-CD10^-CD7^-$ cells (5×10^3 cells/well) were cocultured with telomerized stromal cells in the presence of flt3L (10 ng/ml), SCF (10 ng/ml), TPO (10 ng/ml) alone, or in various combinations. Data represent the mean ± SD of triplicate cultures. $^*P < 0{\cdot}05$ compared with control cultures (without cytokines); $^\dagger P < 0{\cdot}05$ compared with cultures containing flt3L. Data are representative of three independent experiments.

to $CD14^+$ cells, and in some wells both $CD19^+$ and $CD7^+$ cells were detected (Table SI). These findings indicate that flt3L enhances the growth of both $CD19^+$ and $CD7^+$ lymphoid precursors from primitive haematopoietic progenitors.

SCF and TPO are potent growth factors for human haematopoietic progenitors (Heike & Nakahata, 2002). To examine the roles of SCF, TPO and flt3L in the generation of lymphoid precursors from haematopoietic progenitors,

$CD34^+CD38^{lo/-}CD19^-CD10^-CD7^-$ cells were incubated with SCF, TPO, flt3L, or various combinations of these three cytokines, for 3 weeks. Unlike flt3L, TPO and SCF showed little or no effect when used alone. However, SCF or TPO, in combination with flt3L, significantly increased the generation of $CD19^+$cyCD79a$^+$, $CD19^-$cyCD79a$^+$ and $CD7^+$ cells (Fig. 2D). The percentages of CD19 and CD7 appeared relatively higher in the wells containing flt3L than those without flt3L (Fig. S3). Nevertheless, $CD19^+$ cells did not express VpreB and $CD7^+$ cells showed little or no expression of CD1a (data not shown). Thus, the differentiation stage of the generated cells did not appear to be different under the various culture conditions. To assess the generation of lymphoid cells from haematopoietic progenitors in the presence of SCF, flt3L and TPO more precisely, single $CD34^+CD38^{lo/-}CD19^-CD10^-CD7^-$ cells were individually cultured across 768 wells with SCF, flt3L and TPO. Wells that contained more than 300 cells (n = 43) were analysed (Table SIIA). Although cyCD79a$^+$ and $CD14^+$ cells were detected in the majority of wells (n = 24), all of cyCD79a$^+$, $CD7^+$ and $CD14^+$ cells were generated in some wells (n = 5) (Table SIIB). These data indicate that flt3L plays a central role in early B and NK/T cell generation from haematopoietic progenitors, and that SCF and TPO act synergistically with flt3L to promote the generation of both lineages of lymphoid precursors.

Besides flt3L, IL-7 and IL-15 are considered to be important cytokines for lymphopoiesis (Alpdogan & den Brink, 2005). Indeed, we observed the presence of low levels of flt3L (11·4 pg/ml), IL-7 (5·5 pg/ml), and IL-15 (4·5 pg/ml) in the culture medium of haematopoietic progenitor cells cocultured with telomerized stromal cells. We therefore examined the effect of IL-7 and IL-15 on the generation of early lymphoid precursors. Neither IL-7 alone nor IL-7+ flt3L affected the generation of $CD19^+$cyCD79a$^+$, $CD19^-$cyCD79a$^+$, or $CD7^+$ cells (Fig. S4). Similarly, IL-15 did not affect the differentiation of B-lineage cells (Fig. S5A). While IL-15 remarkably increased the population of $CD7^-CD56^+$ NK cells, $CD7^+$ cells remained negative for CD1a (Fig. S5B). Total cell numbers were not significantly different (Fig. S5C).

Characteristics of $CD19^+$ and $CD7^+$ cells generated by stromal cells in the presence of flt3L

We characterized the generated $CD19^+$ and $CD7^+$ cells, which were phenotypically equivalent to pro-B and T/NK precursors, respectively. RT-PCR analyses showed that cultured $CD19^+CD7^-$ cells expressed the genes for PAX5 and EBF1, which are critical transcription factors for B cell development (Blom & Spits, 2006; Rothenberg, 2010). However, little or no expression was seen in freshly isolated $CD34^+CD38^{lo/-}CD19^-CD10^-CD7^-$ cells (Fig. 3A). Moreover, we analysed whether VDJ rearrangements of *IGH@* occurred in the generated $CD19^+CD7^-$ cells using Rearrangement and Translocation Assays and primers for the VH-FR3 and JH consensus regions of *IGH@* (van Krieken *et al*, 2007). As expected, normal tonsil tissue, in which heterogeneous VDJ rearrangements of IgH occur, showed a bell-shaped curve for the PCR-amplified products (amplicons) within the valid size range. Similar observations were made for $CD19^+CD7^-$ cells (Fig. 3B), indicating polyclonal VDJ rearrangements of *IGH@*. These findings are consistent with the characteristics associated with the pro-B stage of B cell precursors (LeBien, 2000; Blom & Spits, 2006). These amplicons were not observed with $CD34^+CD38^{lo/-}CD19^-CD10^-CD7^-$ cells, suggesting that VDJ rearrangements of *IGH@* did not occur in these cells before cultures.

On the other hand, GATA3 (Blom & Spits, 2006; Hosoya *et al*, 2010), TCF12 (Blom & Spits, 2006; Braunstein & Anderson, 2011), and ID2 (Blom & Spits, 2006) were reported to be essential transcription factors for T or NK cell development. The gene expression of these transcription factors was maintained from $CD34^+CD38^{lo/-}CD19^-CD10^-CD7^-$ to $CD7^+CD56^-$ cells. The expression of *PTCRA*, which is involved in early T cell development (Yamasaki & Saito, 2007), was detected in $CD7^+CD56^-$cells, but not in $CD34^+CD38^{lo/-}CD19^-CD10^-CD7^-$ cells (Fig. 3C). Recombination of *TRG@*, *TRD@* or *TRB@* was not detected in $CD7^+CD56^-$ cells by the Rearrangement and Translocation Assays (data not shown).

Next, we examined the potential of $CD7^+CD56^-$ cells to differentiate toward T and NK lineage cells. Following coculture with OP9-DL1 cells in the presence of flt3L, IL-7 and phospho-ascorbate for 28 d, $CD7^+CD56^-$ cells gave rise to $CD7^+CD5^+$ or $CD5^+CD4^+$ cells, which correspond to early T cell precursors (Napolitano *et al*, 2003; Blom & Spits, 2006), and a portion of these cells differentiated into the $CD4^+CD8^-$ or $CD4^+CD8^+$ stage of T cells (Fig. 3D). $CD56^+CD3^-$ NK cells were generated after incubation of $CD7^+CD56^-$ cells with telomerized stromal cells in the presence of flt3L (10 ng/ml), IL-7 (10 ng/ml) and IL-15 (10 ng/ml) for 11 d (Fig. 3D).

Early B and T/NK lymphopoiesis occurs on primary bone marrow stromal cells

The characteristics of telomerized stromal cells were reported to be similar to those of primary human bone marrow stromal cells (Kawano *et al*, 2003; Kobune *et al*, 2005). Early B cell differentiation is observed in cocultures of human haematopoietic progenitors with primary bone marrow stromal cells (LeBien, 2000), but the generation of T lineage cells has not been described. Therefore, we investigated whether not only early B but also early T/NK cell generation occurred and whether the same effects of flt3L were observed, when $CD34^+CD38^{lo/-}CD19^-CD10^-CD7^-$ cells were cocultured with primary bone marrow stromal cells in the presence or absence of flt3L. As shown in Fig. 4A,B, $CD19^+$cyCD79a$^+$, $CD19^-$cyCD79a$^+$ and $CD7^+CD56^-$ cells were generated in cocultures with primary stromal cells even in the absence of

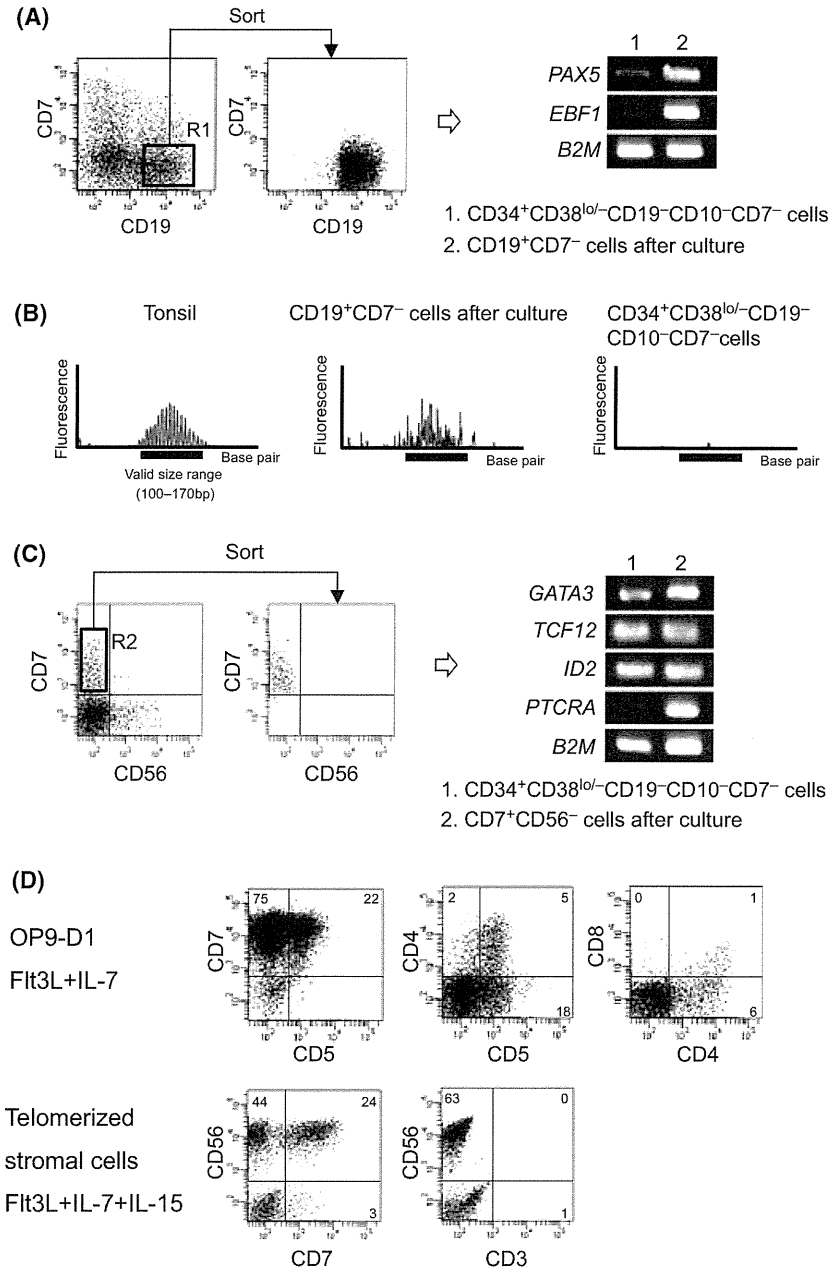

Fig 3. Characteristics of generated $CD19^+$ and $CD7^+CD56^-$ cells. (A) Gene expression of *PAX5*, *EBF1* and *B2M* in $CD34^+CD38^{low/-}CD19^-CD10^-CD7^-$ and $CD19^+CD7^-$ cells. (B) VDJ rearrangements of *IGH@* genes. (C) Gene expressions of *GATA3*, *TCF12*, *PTCRA* and *B2M* in $CD34^+CD38^{low/-}CD19^-CD10^-CD7^-$ and $CD7^+CD56^-$ cells. (D) To induce T cell differentiation, $CD7^+CD56^-$ cells were cocultured with OP9-DL1 cells in the presence of flt3L (5 ng/ml), IL-7 (5 ng/ml) and phospho-ascorbate for 28 d, and analysed for expression CD7, CD5, CD4 and CD8. To induce NK cell differentiation, $CD7^+CD56^-$ cells were cocultured with telomerized stromal cells in the presence of flt3L (10 ng/ml), IL-7 (10 ng/ml) and IL-15 (10 ng/ml) for 11 d and assessed for expression of CD7, CD56 and CD3.

flt3L, although the generation of these cells was lower compared with cocultures with telomerized stromal cells. The generation of these lymphoid precursors was promoted by ftl3L on primary stromal cells, as observed on telomerized stromal cells. These data indicate that the findings observed with telomerized stromal cells can be seen with primary stromal cells.

Early B and T/NK cell precursors develop without direct contact with stromal cells

To elucidate the mechanism by which the lymphopoiesis occurs on stromal cells, we examined whether direct contact between haematopoietic progenitors and stromal cells is required for the generation of early B and T/NK lineage pre-

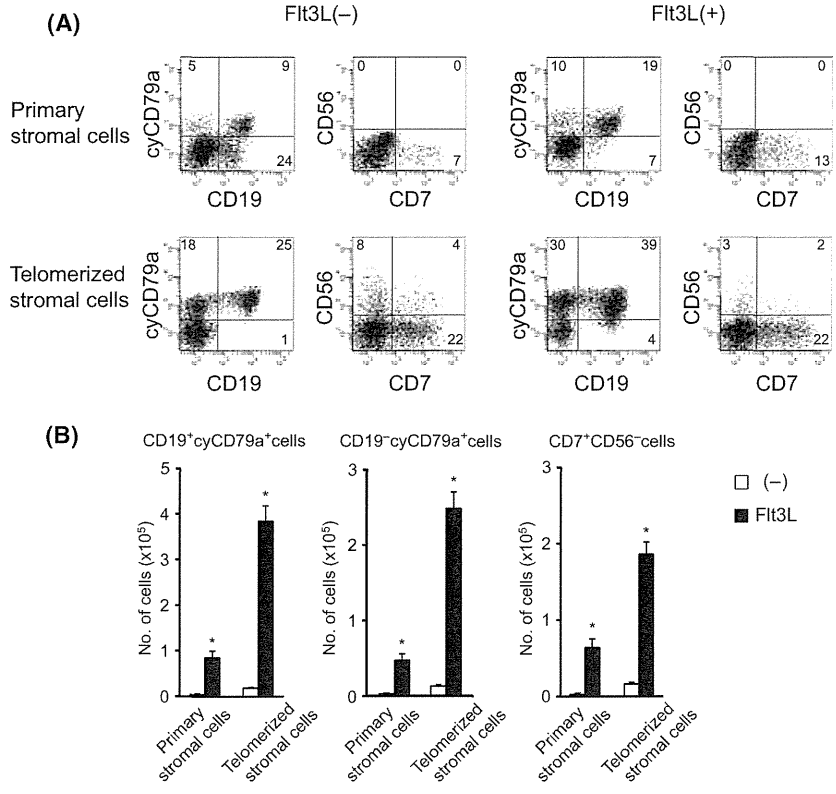

Fig 4. B and T/NK lymphopoiesis occurs on primary human stromal cells. $CD34^+CD38^{low/-}CD19^-CD10^-CD7^-$ cells (1×10^4 cells/well) were cocultured with primary or telomerized stromal cells, with or without flt3L, and analysed for the cell phenotypes (A) and numbers (B). The phenotypes after excluding $CD14^+$ cells are shown. Data represent means ± SD of triplicate cultures. $^*P < 0{\cdot}05$ compared with control cultures. Representative data of three independent experiments are shown.

cursors. To this end, $CD34^+CD38^{lo/-}CD19^-CD10^-CD7^-$ cells were incubated separately from the stromal cells using cell culture inserts of 0·4-μm pore size, or without inserts, in the presence or absence of flt3L. In the absence of flt3L, low cell numbers were detected after culture with or without inserts ($2{\cdot}5 \pm 1{\cdot}5 \times 10^4$ cells with inserts; $7{\cdot}8 \pm 7{\cdot}0 \times 10^4$ cells without inserts). Flt3L similarly increased the total cell numbers in the cultures with or without inserts ($6{\cdot}0 \pm 0{\cdot}5 \times 10^5$ cells with inserts; $2{\cdot}7 \pm 0{\cdot}5 \times 10^5$ cells without inserts). By phenotypical analysis, in the cultures with inserts but without flt3L, only a low number of $CD19^+cyCD79a^+$ and $CD19^-cyCD79a^+$ B and $CD7^+CD19^-$ T lineage cells were detected. However, the percentage and number of these lymphoid cells were significantly increased by flt3L (Fig. 5A,B). Similar results were obtained in the cultures without inserts (data not shown). These data imply that early B and T/NK cell development can be induced, at least in part, by soluble factors produced from the stromal cells, which is enhanced by flt3L.

Effect of flt3L in serum-free cultures

To investigate the role of flt3L during early B and T/NK cell generation more precisely, we analysed its effects in serum-free cultures. Without flt3L, low numbers of $CD7^+$ cells were observed, but no $CD19^+cyCD79a^+$ cells were seen. Even $CD19^-cyCD79a^+$ cells were rarely detected (Fig. 6A,B). These findings indicate that B cell differentiation is minimally supported in serum-free cultures. Nevertheless, $CD19^+cyCD79a^+$ as well as $CD19^-cyCD79a^+$ cells developed in the presence of flt3L. The number of $CD7^+$ cells was also increased by flt3L. These observations support the notion that flt3L plays a crucial role during early B and T/NK cell generation from haematopoietic progenitors, and indicate that flt3L is particularly important for early B cell differentiation. We also tested the effect of SCF and TPO under serum-free culture conditions. As in serum-containing cultures, SCF or TPO alone exerted little or no effect but, in combination with flt3L, enhanced the generation of $CD19^+cyCD79a^+$, $CD19^-cyCD79a^+$ and $CD7^+$ cells (Fig. S6). These data confirmed our notion that flt3L plays a central role in early B and T/NK cell generation from haematopoietic progenitors, which is promoted by SCF and TPO.

Discussion

The results of the present study show that human bone marrow stromal cells simultaneously support the *ex vivo* generation of early B and T/NK lineage precursors from human haematopoietic progenitors. These findings enabled us to

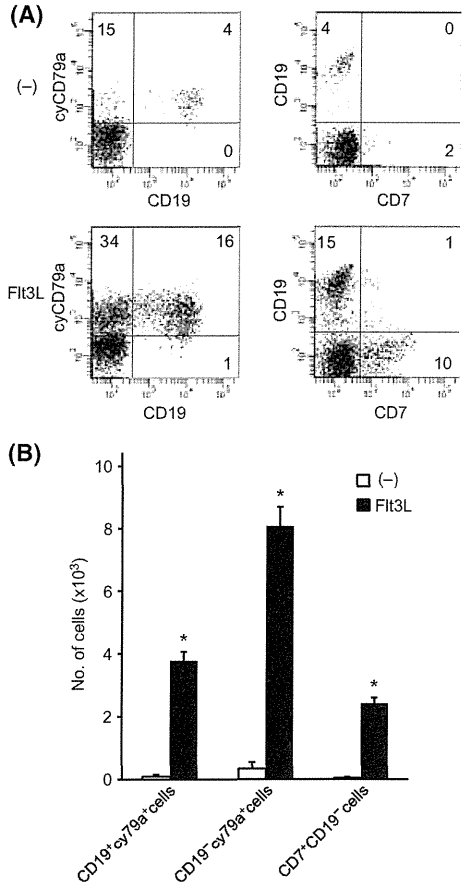

Fig 5. B and T/NK lymphopoiesis can occur separately from stromal cells. $CD34^+CD38^{lo/-}CD19^-CD10^-CD7^-$ cells (1×10^4 cells/well) were cultured separately from telomerized stromal cells using cell culture inserts in the presence or absence of flt3L. The phenotype (A) and number (B) of the cultured cells were analysed. The phenotypes after excluding $CD14^+$ cells are shown. $^*P < 0{\cdot}05$ compared with control cultures. Data are representative of three independent experiments.

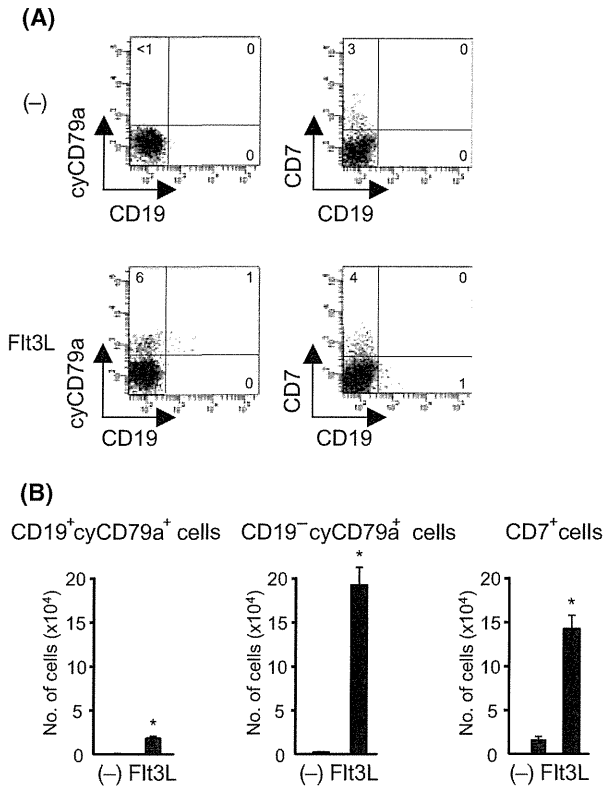

Fig 6. Effect of flt3L in serum-free cultures. $CD34^+CD38^{low/-}CD19^-CD10^-CD7^-$ cells (1×10^4 cells/well) were cultured with telomerized stromal cells in serum-free cultures with or without flt3L, and the phenotype (A) and the number (B) of $CD19^+cyCD79a^+$, $CD19^-cyCD79a^+$ and $CD7^+$ cells were assessed. The phenotypes after excluding $CD14^+$ cells are shown. Data represent means ± SD of triplicate cultures. $^*P < 0{\cdot}05$ compared with control cultures. Representative data of five independent experiments are shown.

demonstrate that flt3L plays a principal role in the generation of early B and T/NK lymphoid precursors from human haematopoietic progenitors.

Early B cell differentiation has been observed during cocultures with human or murine bone marrow stromal cells (LeBien, 2000). However, to the best of our knowledge, this is the first culture system in which B- and T/NK-lineage lymphoid precursors simultaneously developed from haematopoietic progenitors seeded on human bone marrow stromal cells. We postulate that the T/NK-lineage precursors were generated from primitive multipotent haematopoietic progenitors, and not merely from T-lineage committed precursors, cultured on the stromal cells because of the following reasons. Firstly, PTCRA, which is expressed in T cell precursors, was not observed in $CD34^+CD38^{lo/-}CD7^-CD19^-CD10^-$ cells, but was detected in the $CD7^+CD56^-$ cells after coculture with telomerized stromal cells. Secondly, $CD7^+CD56^-$ cells were generated not only from $CD34^+CD38^{lo/-}CD7^-CD10^-CD19^-$ but also from more primitive $CD34^+CD38^-CD7^-CD10^-CD19^-$ haematopoietic progenitors. Thirdly, both B- and T-lineage cells were generated from single haematopoietic progenitors.

Interestingly, we found that the early B and T/NK lineage cell development was able to take place without direct contact with the stromal cells. These findings suggest that early lymphopoiesis can be induced, at least in part, by soluble factors produced from human bone marrow stromal cells. Consistent with our study, it was recently reported that human mesenchymal stem cells support human B cell development without direct cell-to-cell contact (Ichii *et al*, 2010). In serum-free cultures, B cell development was severely reduced, indicating that other factors present in the serum were involved in the B cell development. These findings point to the importance of humoral factors in the regulation of human early lymphopoiesis. Besides flt3L, telomerized stromal cells produced low levels of IL-7 and IL-15. However, IL-7 showed little or no effect on the generation of $CD19^+$ B cells and $CD7^+CD56^-$ T/NK cell pre-

cursors, as previously reported (Prieyl & LeBien, 1996; Napolitano *et al*, 2003; Parrish *et al*, 2009). IL-15 mainly stimulated the $CD7^{-}CD56^{+}$ NK cell generation. Notch ligands DLL1 and DLL4, which are important for T cell development in mice (Koch *et al*, 2008; Koch & Radtke, 2011), were expressed in hTERT stromal cells (*DLL1/GAPDH*, $8{\cdot}62 \pm 0{\cdot}72 \times 10^{-4}$: *DLL4/GAPDH*, $6{\cdot}65 \pm 6{\cdot}60 \times 10^{-5}$). However, the soluble form of Notch ligand has been shown to be much less effective in activating Notch signalling than membranous or immobilized forms of Notch ligand (Varnum-Finney *et al*, 2000). Consistently, the expression level of one Notch target gene, *HES1*, in haematopoietic progenitors was not remarkably changed by coculture with the stromal cells (data not shown). Further studies are required to identify the critical factors required for early B and T/NK lymphopoiesis.

The coculture assays allowed us to demonstrate that flt3L simultaneously promotes the generation of not only early B and T/NK lymphoid precursors but also myeloid and plasmacytoid dendritic cells from haematopoietic progenitors on stromal cells. Flt3L has a positive effect on the generation of $CD19^{+}$ B cell precursors (Rawlings *et al*, 1997; Parrish *et al*, 2009), $CD34^{+}CD7^{-}CD122$ (IL-2/15 receptor $\beta)^{+}$ NK cell precursors (Yu *et al*, 1998), and myeloid or plasmacytoid dendritic cells (Chen *et al*, 2004; Rossi & Young, 2005; Ueno *et al*, 2011). The present study confirms these observations, and further demonstrates that flt3L also enhances the development of $CD7^{+}$ lymphoid precursors with T cell potential without significantly affecting their differentiation. The single-cell assays suggest that flt3L may directly stimulate the growth of lymphohaematopoietic progenitors. The plasma concentrations of flt3L are as low as approximately 20 pg/ml under steady-state conditions, but increase by more than 100 times during the haematopoietic recovery phase after chemotherapy or transplantation (Wodnar-Filipowicz *et al*, 1996). From these findings, we speculate that flt3L is a critical stimulator of B and T/NK lineage cell generation, at least when large numbers of B, T and NK cells need to be rapidly regenerated after myelosuppression. Notably, in the serum-free cultures, even $CD19^{-}cyCD79a^{+}$ early B cell precursors were rarely observed in the absence of cytokines, whereas $CD19^{+}cyCD79a^{+}$ pro-B cells, in addition to a significant number of $CD19^{-}cyCD79a^{+}$ cells, were generated in the presence of flt3L. These observations imply that flt3L is particularly important for human B cell development from haematopoietic progenitors.

SCF and TPO are involved in the survival and proliferation of primitive haematopoietic progenitors (Heike & Nakahata, 2002). Unlike flt3L, SCF or TPO alone had little or no effect on the generation of B and T/NK lymphoid precursors. However, SCF or TPO, in combination with flt3L, significantly augmented the generation of B and T/NK lymphoid precursors without considerably affecting their differentiation. These findings indicate that flt3L is a principal cytokine involved in early B and T/NK cell development, and that SCF and TPO act synergistically with flt3L to enhance lymphoid lineage development, presumably by stimulating the growth of primitive haematopoietic progenitors with lymphoid differentiation potential.

Not only telomerized but also primary bone marrow stromal cells supported the generation of early B and T/NK cell precursors from human haematopoietic progenitors, and similar stimulatory effects by flt3L were observed in cocultures with both types of stromal cells. These data are consistent with the notion that the telomerized human bone marrow stromal cells maintain the phenotype of primary human bone marrow stromal cells (Kawano *et al*, 2003; Kobune *et al*, 2005). Altogether, our findings indicate that the telomerized stromal cells provide an excellent culture system for assessing the lymphoid differentiation potential of primary or leukaemic haematopoietic progenitors. For example, in single cell assays with the telomerized stromal cells, we observed that B and/or T/NK lymphoid precursors were generated in accompaniment with $CD14^{+}$ cells. These findings are consistent with the observation that human multipotent lymphoid progenitors retain the potential to generate monocytic cells (Doulatov *et al*, 2010). These investigations will yield novel insights into the mechanisms involved in human normal and malignant haematopoiesis.

Acknowledgements

We thank Dr Juan Carlos Zúñiga-Pflücker (Department of Immunology, University of Toronto, Toronto, Ontario, Canada), for providing the OP9 stromal cells expressing DLL1. The authors are grateful to Yuki Orito (Department of Immunno-Gene Therapy, Mie University Graduate School of Medicine, Tsu, Mie, Japan) for technical assistance with the RT-PCR analyses and helpful suggestions. The assessment of the *IGH@* VDJ rearrangements using Rearrangement and Translocation Assays was performed by Mitsubishi Chemical Medience, Tokyo, Japan. This study was supported, in part, by a Japan Society for the Promotion of Science Grant-in-Aid for Scientific Research (21591240) and Grants-in-Aid for Scientific Research from Mie University Graduate School of Medicine.

Author's contributions

Y.N. and B.L. performed the research and analysed data; K. O. designed the research, analysed the data, and wrote the manuscript; K.S., K.I., T.M., M.M. analysed and interpreted the data; H.N. and H.S. analysed the data; H.H. contributed essential reagents or tools; N.K designed the research, analysed the data, and wrote the manuscript.

Conflict of interest

The authors declare no competing financial interests.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Expression of surface or cytoplasmic lymphoid antigens in freshly isolated $CD34^+$ cells.

Fig. S2. The effect of flt3L on the generation of dendritic lineage cells from haematopoietic progenitor cells cultured on telomerized stromal cells.

Fig. S3. The effect of flt3L, SCF, and TPO on the differentiation of lymphoid cells in serum-containing cultures.

Fig. S4. IL-7 has little to no effect on lymphopoiesis.

Fig. S5. The effect of IL-15 on lymphopoiesis.

Fig. S6. The effect of flt3L, SCF, and TPO on the generation of lymphoid cells in serum-free cultures.

Table SI. The generation of $CD19^+$, $CD7^+$, and/or $CD14^+$ cells in single-cell assays with or without flt3L.

Table SII. (A) Cell growth in single cell cultures with SCF + flt3L + TPO. (B) The generation $cyCD79a^+$, $CD7^+$ and/or $CD14^+$ cells in the single-cell assay.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

References

Akashi, K., Reya, T., Dalma-Weiszhausz, D. & Weissman, I.L. (2000) Lymphoid precursors. *Current Opinion in Immunology*, **12**, 144–150.

Alpdogan, O. & van den Brink, M.R. (2005) IL-7 and IL-15: therapeutic cytokines for immunodeficiency. *Trends in Immunology*, **26**, 56–64.

Awong, G., La Motte-Mohs, R.N. & Zuniga-Pflucker, J.C. (2007) Generation of pro-T cells *in vitro*: potential for immune reconstitution. *Seminars in Immunology*, **19**, 341–349.

Blom, B. & Spits, H. (2006) Development of human lymphoid cells. *Annual Review of Immunology*, **24**, 287–320.

Braunstein, M. & Anderson, M.K. (2011) HEB-deficient T-cell precursors lose T-cell potential and adopt an alternative pathway of differentiation. *Molecular and Cellular Biology*, **31**, 971–982.

Buchler, P., Gazdhar, A., Schubert, M., Giese, N., Reber, H.A., Hines, O.J., Giese, T., Ceyhan, G. O., Muller, M., Buchler, M.W. & Friess, H. (2005) The Notch signaling pathway is related to neurovascular progression of pancreatic cancer. *Annals of Surgery*, **242**, 791–801.

Buza-Vidas, N., Cheng, M., Duarte, S., Nozad, H., Jacobsen, S.E. & Sitnicka, E. (2007) Crucial role of FLT3 ligand in immune reconstitution after bone marrow transplantation and high-dose chemotherapy. *Blood*, **110**, 424–432.

Cerdan, C., Serfling, E. & Olive, D. (2000) The C-class chemokine, lymphotactin, impairs the induction of Th1-type lymphokines in human CD4(+) T cells. *Blood*, **96**, 420–428.

Chen, W., Antonenko, S., Sederstrom, J.M., Liang, X., Chan, A.S., Kanzler, H., Blom, B., Blazar, B. R. & Liu, Y.J. (2004) Thrombopoietin cooperates with FLT3-ligand in the generation of plasmacytoid dendritic cell precursors from human hematopoietic progenitors. *Blood*, **103**, 2547–2553.

Doulatov, S., Notta, F., Eppert, K., Nguyen, L.T., Ohashi, P.S. & Dick, J.E. (2010) Revised map of the human progenitor hierarchy shows the origin of macrophages and dendritic cells in early lymphoid development. *Nature Immunology*, **11**, 585–593.

Dworzak, M.N., Fritsch, G., Froschl, G., Printz, D. & Gadner, H. (1998) Four-color flow cytometric investigation of terminal deoxynucleotidyl transferase-positive lymphoid precursors in pediatric bone marrow: CD79a expression precedes CD19 in early B-cell ontogeny. *Blood*, **92**, 3203–3209.

von Freeden-Jeffry, U., Vieira, P., Lucian, L.A., McNeil, T., Burdach, S.E. & Murray, R. (1995) Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. *Journal of Experimental Medicine*, **181**, 1519–1526.

Fujimi, A., Matsunaga, T., Kobune, M., Kawano, Y., Nagaya, T., Tanaka, I., Iyama, S., Hayashi, T., Sato, T., Miyanishi, K., Sagawa, T., Sato, Y., Takimoto, R., Takayama, T., Kato, J., Gasa, S., Sakai, H., Tsuchida, E., Ikebuchi, K., Hamada, H. & Niitsu, Y. (2008) Ex vivo large-scale generation of human red blood cells from cord blood CD34 + cells by co-culturing with macrophages. *International Journal of Hematology*, **87**, 339–350.

Garcia-Peydro, M., de Yebenes, V.G. & Toribio, M.L. (2006) Notch1 and IL-7 receptor interplay maintains proliferation of human thymic progenitors while suppressing non-T cell fates. *Journal of Immunology*, **177**, 3711–3720.

Gisler, R., Jacobsen, S.E. & Sigvardsson, M. (2000) Cloning of human early B-cell factor and identification of target genes suggest a conserved role in B-cell development in man and mouse. *Blood*, **96**, 1457–1464.

Gudmundsson, K.O., Thorsteinsson, L., Sigurjonsson, O.E., Keller, J.R., Olafsson, K., Egeland, T., Gudmundsson, S. & Rafnar, T. (2007) Gene expression analysis of hematopoietic progenitor cells identifies Dlg7 as a potential stem cell gene. *Stem Cells*, **25**, 1498–1506.

Haddad, R., Guardiola, P., Izac, B., Thibault, C., Radich, J., Delezoide, A.L., Baillou, C., Lemoine, F.M., Gluckman, J.C., Pflumio, F. & Canque, B. (2004) Molecular characterization of early human T/NK and B-lymphoid progenitor cells in umbilical cord blood. *Blood*, **104**, 3918–3926.

Hao, Q.L., Zhu, J., Price, M.A., Payne, K.J., Barsky, L.W. & Crooks, G.M. (2001) Identification of a novel, human multilymphoid progenitor in cord blood. *Blood*, **97**, 3683–3690.

Heike, T. & Nakahata, T. (2002) Ex vivo expansion of hematopoietic stem cells by cytokines. *Biochimica et Biophysica Acta*, **1592**, 313–321.

Hosoya, T., Maillard, I. & Engel, J.D. (2010) From the cradle to the grave: activities of GATA-3 throughout T-cell development and differentiation. *Immunological Reviews*, **238**, 110–125.

Ichii, M., Oritani, K., Yokota, T., Schultz, D.C., Holter, J.L., Kanakura, Y. & Kincade, P.W. (2010) Stromal cell-free conditions favorable for human B lymphopoiesis in culture. *Journal of Immunological Methods*, **359**, 47–55.

Jaleco, A.C., Neves, H., Hooijberg, E., Gameiro, P., Clode, N., Haury, M., Henrique, D. & Parreira, L. (2001) Differential effects of Notch ligands Delta-1 and Jagged-1 in human lymphoid differentiation. *Journal of Experimental Medicine*, **194**, 991–1002.

Kang, J. & Der, S.D. (2004) Cytokine functions in the formative stages of a lymphocyte's life. *Current Opinion in Immunology*, **16**, 180–190.

Kawano, Y., Kobune, M., Yamaguchi, M., Nakamura, K., Ito, Y., Sasaki, K., Takahashi, S., Nakamura, T., Chiba, H., Sato, T., Matsunaga, T., Azuma, H., Ikebuchi, K., Ikeda, H., Kato, J., Niitsu, Y. & Hamada, H. (2003) Ex vivo expansion of human umbilical cord hematopoietic progenitor cells using a coculture system with human telomerase catalytic subunit (hTERT)-transfected human stromal cells. *Blood*, **101**, 532–540.

Kawano, Y., Kobune, M., Chiba, H., Nakamura, K., Takimoto, R., Takada, K., Ito, Y., Kato, J., Hamada, H. & Niitsu, Y. (2006) Ex vivo expansion of G-CSF-mobilized peripheral blood CD133 + progenitor cells on coculture with human stromal cells. *Experimental Hematology*, **34**, 150–158.

Kobune, M., Kato, J., Chiba, H., Kawano, Y., Tanaka, M., Takimoto, R., Hamada, H. & Niitsu, Y. (2005) Telomerized human bone marrow-derived cell clones maintain the phenotype of hematopoietic-supporting osteoblastic and myofibroblastic stromal cells after long-term culture. *Experimental Hematology*, **33**, 1544–1553.

Koch, U. & Radtke, F. (2011) Mechanisms of T cell development and transformation. *Annual Review of Cell and Developmental Biology*, **27**, 539–562.

Koch, U., Fiorini, E., Benedito, R., Besseyrias, V., Schuster-Gossler, K., Pierres, M., Manley, N.R., Duarte, A., Macdonald, H.R. & Radtke, F. (2008) Delta-like 4 is the essential, nonredundant ligand for Notch1 during thymic T cell lineage commitment. *Journal of Experimental Medicine*, **205**, 2515–2523.

van Krieken, J.H., Langerak, A.W., Macintyre, E. A., Kneba, M., Hodges, E., Sanz, R.G., Morgan, G.J., Parreira, A., Molina, T.J., Cabecadas, J., Gaulard, P., Jasani, B., Garcia, J.F., Ott, M., Hannsmann, M.L., Berger, F., Hummel, M., Davi, F., Bruggemann, M., Lavender, F.L., Schuuring, E., Evans, P.A., White, H., Salles, G., Groenen, P.J., Gameiro, P., Pott, C. & Dongen, J.J. (2007) Improved reliability of lymphoma diagnostics via PCR-based clonality testing: report of the BIOMED-2 Concerted Action BHM4-CT98-3936. *Leukemia*, **21**, 201–206.

LeBien, T.W. (2000) Fates of human B-cell precursors. *Blood*, **96**, 9–23.

Levelt, C.N. & Eichmann, K. (1994) Streptavidin-tricolor is a reliable marker for nonviable cells subjected to permeabilization or fixation. *Cytometry*, **15**, 84–86.

Liu, B., Ohishi, K., Yamamura, K., Suzuki, K., Monma, F., Ino, K., Nishii, K., Masuya, M., Sekine, T., Heike, Y., Takaue, Y. & Katayama, N. (2010) A potential activity of valproic acid in the stimulation of interleukin-3-mediated megakaryopoiesis and erythropoiesis. *Experimental Hematology*, **38**, 685–695.

Manning, J., Mitchell, B., Appadurai, D., Ornelas, L. & Spangrude, G. (2010) Ascorbic acid promotes increased mRNA expression of rearranged TCR-alpha and TCR-beta genes during *in vitro* T Cell development. *Blood*(ASH Annual Meeting Abstracts), **116**, 1083. Abstract 2623.

Matsunaga, T., Tanaka, I., Kobune, M., Kawano, Y., Tanaka, M., Kuribayashi, K., Iyama, S., Sato, T., Sato, Y., Takimoto, R., Takayama, T., Kato, J., Ninomiya, T., Hamada, H. & Niitsu, Y. (2006) Ex vivo large-scale generation of human platelets from cord blood CD34 + cells. *Stem Cells*, **24**, 2877–2887.

Meek, B., Cloosen, S., Borsotti, C., Van Elssen, C. H., Vanderlocht, J., Schnijderberg, M.C., van der Poel, M.W., Leewis, B., Hesselink, R., Manz, M. G., Katsura, Y., Kawamoto, H., Germeraad, W. T. & Bos, G.M. (2010) *In vitro*-differentiated T/natural killer-cell progenitors derived from human CD34+ cells mature in the thymus. *Blood*, **115**, 261–264.

Napolitano, L.A., Stoddart, C.A., Hanley, M.B., Wieder, E. & McCune, J.M. (2003) Effects of IL-7 on early human thymocyte progenitor cells *in vitro* and in SCID-hu Thy/Liv mice. *Journal of Immunology*, **171**, 645–654.

Nijjar, S.S., Wallace, L., Crosby, H.A., Hubscher, S. G. & Strain, A.J. (2002) Altered Notch ligand expression in human liver disease: further evidence for a role of the Notch signaling pathway in hepatic neovascularization and biliary ductular defects. *American Journal of Pathology*, **160**, 1695–1703.

Ohishi, K., Varnum-Finney, B. & Bernstein, I.D. (2002) Delta-1 enhances marrow and thymus repopulating ability of human CD34(+)CD38 (−) cord blood cells. *The Journal of Clinical Investigation*, **110**, 1165–1174.

Parrish, Y.K., Baez, I., Milford, T.A., Benitez, A., Galloway, N., Rogerio, J.W., Sahakian, E., Kagoda, M., Huang, G., Hao, Q.L., Sevilla, Y., Barsky, L.W., Zielinska, E., Price, M.A., Wall, N.R., Dovat, S. & Payne, K.J. (2009) IL-7 Dependence in human B lymphopoiesis increases during progression of ontogeny from cord blood to bone marrow. *Journal of Immunology*, **182**, 4255–4266.

Peschon, J.J., Morrissey, P.J., Grabstein, K.H., Ramsdell, F.J., Maraskovsky, E., Gliniak, B.C., Park, L.S., Ziegler, S.F., Williams, D.E., Ware, C. B., Meyer, J.D. & Davison, B.L. (1994) Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. *Journal of Experimental Medicine*, **180**, 1955–1960.

Prieyl, J.A. & LeBien, T.W. (1996) Interleukin 7 independent development of human B cells. *Proceedings of the National Academy of Sciences of the USA*, **93**, 10348–10353.

Rawlings, D.J., Quan, S., Hao, Q.L., Thiemann, F. T., Smogorzewska, M., Witte, O.N. & Crooks, G.M. (1997) Differentiation of human CD34+ CD38- cord blood stem cells into B cell progenitors *in vitro*. *Experimental Hematology*, **25**, 66–72.

Reynaud, D., Lefort, N., Manie, E., Coulombel, L. & Levy, Y. (2003) *In vitro* identification of human pro-B cells that give rise to macrophages, natural killer cells, and T cells. *Blood*, **101**, 4313–4321.

Rossi, M. & Young, J.W. (2005) Human dendritic cells: potent antigen-presenting cells at the crossroads of innate and adaptive immunity. *Journal of Immunology*, **175**, 1373–1381.

Rothenberg, E.V. (2010) Lineage determination in the immune system. *Immunological Reviews*, **238**, 5–11.

Schmitt, T.M. & Zuniga-Pflucker, J.C. (2002) Induction of T cell development from hematopoietic progenitor cells by delta-like-1 *in vitro*. *Immunity*, **17**, 749–756.

Schotte, R., Dontje, W., Nagasawa, M., Yasuda, Y., Bakker, A.Q., Spits, H. & Blom, B. (2010) Synergy between IL-15 and Id2 promotes the expansion of human NK progenitor cells, which can be counteracted by the E protein HEB required to drive T cell development. *Journal of Immunology*, **184**, 6670–6679.

Sitnicka, E., Bryder, D., Theilgaard-Monch, K., Buza-Vidas, N., Adolfsson, J. & Jacobsen, S.E. (2002) Key role of flt3 ligand in regulation of the common lymphoid progenitor but not in maintenance of the hematopoietic stem cell pool. *Immunity*, **17**, 463–472.

Sitnicka, E., Buza-Vidas, N., Larsson, S., Nygren, J.M., Liuba, K. & Jacobsen, S.E. (2003) Human CD34 + hematopoietic stem cells capable of multilineage engrafting NOD/SCID mice express flt3: distinct flt3 and c-kit expression and response patterns on mouse and candidate human hematopoietic stem cells. *Blood*, **102**, 881–886.

Tokoyoda, K., Egawa, T., Sugiyama, T., Choi, B.I. & Nagasawa, T. (2004) Cellular niches controlling B lymphocyte behavior within bone marrow during development. *Immunity*, **20**, 707–718.

Ueno, H., Klechevsky, E., Schmitt, N., Ni, L., Flamar, A.L., Zurawski, S., Zurawski, G., Palucka, K., Banchereau, J. & Oh, S. (2011) Targeting human dendritic cell subsets for improved vaccines. *Seminars in Immunology*, **23**, 21–27.

Varnum-Finney, B., Wu, L., Yu, M., Brashem-Stein, C., Staats, S., Flowers, D., Griffin, J.D. & Bernstein, I.D. (2000) Immobilization of Notch ligand, Delta-1, is required for induction of notch signaling. *Journal of Cell Science*, **113**, 4313–4318.

Wodnar-Filipowicz, A., Lyman, S.D., Gratwohl, A., Tichelli, A., Speck, B. & Nissen, C. (1996) Flt3 ligand level reflects hematopoietic progenitor cell function in aplastic anemia and chemotherapy-induced bone marrow aplasia. *Blood*, **88**, 4493–4499.

Wu, J.Y., Scadden, D.T. & Kronenberg, H.M. (2009) Role of the osteoblast lineage in the bone marrow hematopoietic niches. *Journal of Bone and Mineral Research*, **24**, 759–764.

Yamasaki, S. & Saito, T. (2007) Molecular basis for pre-TCR-mediated autonomous signaling. *Trends in Immunology*, **28**, 39–43.

Yu, H., Fehniger, T.A., Fuchshuber, P., Thiel, K.S., Vivier, E., Carson, W.E. & Caligiuri, M.A. (1998) Flt3 ligand promotes the generation of a distinct CD34(+) human natural killer cell progenitor that responds to interleukin-15. *Blood*, **92**, 3647–3657.

Zhu, J., Garrett, R., Jung, Y., Zhang, Y., Kim, N., Wang, J., Joe, G.J., Hexner, E., Choi, Y., Taichman, R.S. & Emerson, S.G. (2007) Osteoblasts support B-lymphocyte commitment and differentiation from hematopoietic stem cells. *Blood*, **109**, 3706–3712.

Zlotoff, D.A., Schwarz, B.A. & Bhandoola, A. (2008) The long road to the thymus: the generation, mobilization, and circulation of T-cell progenitors in mouse and man. *Seminars in Immunopathology*, **30**, 371–382.

Cancer Science
The official journal of the Japanese Cancer Association JCA

T-cell receptor gene therapy targeting melanoma-associated antigen-A4 inhibits human tumor growth in non-obese diabetic/SCID/γc^{null} mice

Yoshitaka Shirakura,[1,2,7] Yukari Mizuno,[1,2,7] Linan Wang,[2] Naoko Imai,[2] Chisaki Amaike,[2] Eiichi Sato,[3] Mamoru Ito,[4] Ikuei Nukaya,[5] Junichi Mineno,[5] Kazutoh Takesako,[5] Hiroaki Ikeda[2,6] and Hiroshi Shiku[1,2,6]

Departments of [1]Cancer Vaccine, [2]Immuno-Gene Therapy, Mie University Graduate School of Medicine, Tsu; [3]Department of Pathology, Tokyo Medical University, Tokyo; [4]Central Institute for Experimental Animals, Kawasaki; [5]Center for Cell and Gene Therapy, Takara Bio Inc., Otsu, Japan

(Received June 20, 2011/Revised September 14, 2011/Accepted September 17, 2011/Accepted manuscript online September 23, 2011/Article first published online November 9, 2011)

Adoptive cell therapy with lymphocytes that have been genetically engineered to express tumor-reactive T-cell receptors (TCR) is a promising approach for cancer immunotherapy. We have been exploring the development of TCR gene therapy targeting cancer/testis antigens, including melanoma-associated antigen (MAGE) family antigens, that are ideal targets for adoptive T-cell therapy. The efficacy of TCR gene therapy targeting MAGE family antigens, however, has not yet been evaluated *in vivo*. Here, we demonstrate the *in vivo* antitumor activity in immunodeficient non-obese diabetic/SCID/γc^{null} (NOG) mice of human lymphocytes genetically engineered to express TCR specific for the MAGE-A4 antigen. Polyclonal T cells derived from human peripheral blood mononuclear cells were transduced with the $\alpha\beta$ TCR genes specific for MAGE-A4, then adoptively transferred into NOG mice inoculated with MAGE-A4 expressing human tumor cell lines. The transferred T cells maintained their effector function *in vivo*, infiltrated into tumors, and inhibited tumor growth in an antigen-specific manner. The combination of adoptive cell therapy with antigen peptide vaccination enhanced antitumor activity, with improved multifunctionality of the transferred cells. These data suggest that TCR gene therapy with MAGE-A4-specific TCR is a promising strategy to treat patients with MAGE-A4-expressing tumors; in addition, the acquisition of multifunctionality *in vivo* is an important factor to predict the quality of the T-cell response during adoptive therapy with human lymphocytes. (*Cancer Sci* 2012; 103: 17–25)

T-cell receptor (TCR) gene transfer using retroviral vectors has been shown to be an attractive strategy to redirect the antigen specificity of polyclonal T cells to create tumor- or pathogen-specific lymphocytes.[1–6] This approach is a promising method for the treatment of patients with malignancies that might overcome the limitations of current adoptive T-cell therapies that have been hampered by difficulties in the isolation and expansion of pre-existing, antigen-specific lymphocytes in patients.[7–10] For the treatment of metastatic melanoma, clinical trials using autologous lymphocytes that have been retrovirally transduced with melanoma/melanocyte antigen-specific TCR have reported objective cancer regression.[11,12] These reports suggest that adoptive cell therapy using TCR gene-modified lymphocytes is a promising approach to immunotherapy in cancer patients; such reports have encouraged the development of novel TCR gene therapy-based approaches.

On-target adverse events, however, have been reported for TCR gene therapies targeting melanocyte differentiation antigens, such as melanoma antigen recognized by T-cells (MART)-1 or gp100. Normal tissues in which melanocytic cells exist, such as the skin, eyes, and inner ears, exhibited severe histological destruction, especially when high-avidity TCR were used.[12] Gene-modified T cells targeting carcinoembryonic antigen also induced a severe transient inflammatory colitis that served as a dose-limiting toxicity for all three patients enrolled.[13] Case reports exploring the severe adverse events seen in patients receiving T cells transduced with chimeric antigen receptors bearing the variable regions of human epidermal growth factor receptor type 2 (HER2)/neu- or CD19-reactive antibodies have suggested that these adverse events might be related to the release of cytokines from transferred cells.[14,15] These observations highlight the potential risk in the usage of receptor genes that render T cells reactive to both tumor cells and a subset of normal cells.

Cancer/testis antigens are particularly attractive targets for immunotherapy, because of their unique expression profiles. While these antigens are highly expressed on adult male germ cells or placenta, they are typically completely absent from other normal adult tissues, and demonstrate aberrant expression in a variety of malignant neoplasms.[16,17] As adult male germ cells do not express MHC class I, $CD8^+$ effector cells theoretically ignore these cells.[18] *MAGE-A*, *-B*, and *-C* genes exhibit such an expression pattern, and their immunogenicity as targets for cancer immunotherapy has been well studied.[19–21] MAGE-A4 expression was reported in 56.6% of serous carcinoma of the ovary, 61.4% of melanoma, 28.4% of non-small cell lung carcinoma, 20% of hepatocellular carcinoma, 22.3% of colorectal carcinoma, 90.2% of esophageal squamous cell carcinoma, and 6.7% of esophageal adenocarcinoma.[22–28] These results suggest that TCR gene therapy targeting the MAGE family of antigens, including MAGE-A4, represents a promising treatment for malignancies that minimizes the risk of severe on-target toxicity. The feasibility of TCR gene therapy targeting MAGE family antigens *in vivo*, however, has not previously been evaluated.

In the present study, we isolated rearranged *TCR$\alpha\beta$* genes from a human $CD8^+$ T-cell clone that recognizes a MAGE-A4-derived peptide, MAGE-$A4_{143-151}$, in the context of HLA-A*2402.[29] Polyclonal human lymphocytes that were retrovirally transduced with these TCR genes demonstrated stable transgene expression and specific cytotoxicity against MAGE-A4-expressing tumor cells *in vitro*.[30,31] These results prompted us to confirm the efficacy of the TCR gene-modified T cells *in vivo* prior to clinical evaluation.

In this study, we investigated if human lymphocytes genetically engineered to express this MAGE-A4-specific TCR could inhibit the growth of MAGE-A4-expressing tumors when adoptively transferred into immunodeficient non-obese diabetic/SCID/γc^{null} (NOG) mice. We evaluated the *in vivo* function of the transferred cells, as well as their migration to the tumor

[6]To whom correspondence should be addressed.
E-mail: shiku@clin.medic.mie-u.ac.jp; hikeda@clin.medic.mie-u.ac.jp
[7]These authors contributed equally to this work.

doi: 10.1111/j.1349-7006.2011.02111.x

site, and the resultant antitumor effect. We addressed if the combination of adoptive cell therapy and vaccination with peptide antigen could influence the antitumor activity of transferred cells.

Materials and Methods

Peripheral blood mononuclear cells. Peripheral blood mononuclear cells (PBMC) were isolated from healthy donors who provided informed consent. Peripheral blood mononuclear cells were cultured in GT-T503 media (Takara Bio, Otsu, Japan) supplemented with 1% autologous plasma, 0.2% human serum albumin (HSA; Sigma-Aldrich, St. Louis, MO, USA), 2.5 mg/mL fungizone (Bristol-Myers Squibb, New York, NY, USA), and 600 IU/mL interleukin-2. This study was approved by the ethics review committees of Mie University Graduate School of Medicine (Tsu, Japan) and Takara Bio.

Mice. Studies were conducted using 8-week-old female NOG mice (Central Institute for Experimental Animals, Kawasaki, Japan) that had been established as described previously.[32] Mice were maintained at the Animal Center of Mie University Graduate School of Medicine. All experimental protocols were approved by the Ethics Review Committee for Animal Experimentation (of Mie University Graduate School of Medicine).

Cell lines. The KE4 (MAGE-A4$^+$HLA-A*2402$^+$ human esophageal carcinoma), QG56 (MAGE-A4$^+$HLA-A*2402$^-$ human lung carcinoma), and T2-A*2402 (human T, B hybridoma transfected with *HLA-A*2402* cDNA)[29] cell lines were maintained in RPMI-1640 media (Sigma-Aldrich) supplemented with 10% FCS, penicillin (100 U/mL), and streptomycin (100 mg/mL).

Retroviral transduction. A retroviral vector encoding MAGE-A4-specific *TCRα* (*TRAV8-1*) and *TCRβ* (*TRBV7-9*) genes (MS-bPa retroviral vector) was described previously.[30] Peripheral blood mononuclear cells were stimulated with 30 ng/mL OKT-3 (Janssen Pharmaceutical, Titusville, NJ, USA) and 600 IU/mL interleukin-2 prior to transduction with MS-bPa particles. Briefly, retroviral solutions were preloaded onto RetroNectin-coated plates and centrifuged at 2000*g* for 2 h, then rinsed with PBS, according to the RetroNectin (Takara Bio)-bound virus infection method. Cells were then applied onto preloaded plates; PBMC transduced with the MS-bPa retroviral vector were designated as gene-modified cells. Control PBMC were treated similarly, except that MS-bPa was omitted from the cultures; these specimens were designated as unmodified cells.

Tumor challenge. KE4 tumor cells (2.5×10^6 in 0.2 mL PBS) were subcutaneously inoculated into the right flanks of mice. In the indicated experiments, QG56 tumor cells (2.5×10^6 in 0.2 mL PBS) were subcutaneously inoculated in a similar manner. Tumor size was determined by the product of perpendicular diameters measured with calipers. The mice were killed before the mean diameter of the tumor reached 20 mm, according to institutional guidelines. The statistical significance of the difference between groups in tumor growth was evaluated at the last time point.

Adoptive cell transfer. After two washes in saline containing 1% human serum albumin (HSA), gene-modified or unmodified cells (1×10^8) were suspended in 0.3 mL saline and intravenously injected into a lateral tail vein of the NOG mice. Prior to injection, gene-modified cells were analyzed for staining with MAGE-A4$_{143-151}$/HLA-A*2402 tetramer and antihuman CD8 mAb to calculate the proportion of tetramer$^+$CD8$^+$ T cells infused. When indicated, HLA-A*2402-positive PBMC were pulsed with 1 μM MAGE-A4$_{141-153}$ peptide and co-administered intravenously as a peptide vaccination.

***In vitro* stimulation and staining of cells.** Cells were incubated for 2 h at 37°C with irradiated (45 Gy) stimulator T2-A*2402 cells, which had been pulsed with 1 μM MAGE-A4$_{141-153}$ or HER2$_{63-71}$ (an irrelevant peptide with HLA-A*2402 binding activity) peptide, at an effector/stimulator ratio of four in the presence of 0.1 mg/mL phycoerythrin (PE)-conjugated anti-CD107a (BD Bioscience, San Diego, CA, USA). We then incubated samples for an additional 6 h in 1 mL/mL GolgiStop (BD Bioscience). The cells were then stained with FITC-conjugated anti-CD8 (BD Bioscience) mAb. After permeabilization and fixation using a Cytofix/Cytoperm kit (BD Bioscience) according to the manufacturer's instructions, the cells were stained intracellularly with allophycocyanin (APC)-conjugated anti-γ-interferon (IFN-γ) (BD Bioscience) and PE–Cy7-conjugated antitumor necrosis factor (TNF) (BD Bioscience) mAb.

Flow cytometric analysis. PE-conjugated MAGE-A4$_{143-151}$/HLA-A*2402 tetramer (provided by the Ludwig Institute for Cancer Research, New York, NY, USA) and FITC-conjugated antihuman CD4 (BD Bioscience), human CD8 (BD Bioscience), and PerCP–Cy5.5-conjugated antihuman CD3 (BD Bioscience) mAb were used to detect transduced TCR in specific cell populations. Polychromatic analyses were performed as previously described.[33] Cell staining data were acquired using a FACS CantoI flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA), and analyzed using FACSDiva (Becton Dickinson) and FlowJ (Tree Star, Ashland, OR, USA) software.

Immunohistochemical analysis. Formalin-fixed and paraffin-embedded specimens were used. After deparaffinization, tissue sections were pretreated with antigen retrieval solution (DAKO high pH solution, DAKO, Glostrum, Denmark) at 95°C for 20 min. As a primary antibody, antihuman CD8 (clone C8/144B; DAKO) was used. Dextran polymer method with EnVision plus (DAKO) was adopted for secondary detection. 3,3′-Diaminobenzidine was used as chromogen, and hematoxylin counterstain was performed. Infiltrated CD8-positive tumor infiltrating lymphocytes (TIL) were counted in the selected 10 independent areas with most abundant TIL infiltration. Tumor-infiltrated, CD8-positive cells per high power field (0.0625 mm^2) were counted using an ocular grid at ×400 magnification. Three independent counts were performed by a board-certified pathologist (E.S) with no knowledge of the earlier results. The average TIL counts of 10 fields was used for the statistical analyses.

Statistical analyses. Data were expressed as mean ± SD. Differences between groups were examined for statistical significance using the Student's *t*-test. A *P*-value less than 0.01 denoted a statistically-significant difference.

Results

Adoptive transfer of MAGE-A4-specific, TCR-transduced lymphocytes inhibits tumor progression in a dose-dependent and antigen-specific manner. We previously reported the successful retroviral transduction of *TCRαβ* genes recognizing the MAGE-A4$_{143-151}$ peptide in an HLA-A*2402-restricted manner into polyclonally-activated human CD8$^+$ T cells. The TCRαβ-transduced CD8$^+$ T cells exhibited IFN-γ production and cytotoxic activity against both peptide-loaded T2-A*2402 cells and human tumor cell lines, such as KE4, that express both MAGE-A4 and HLA-A*2402.[30] To confirm the efficacy of these gene-modified T cells *in vivo* prior to clinical evaluation, we examined the antitumor efficacy of adoptive cell therapy with MAGE-A4-specific *TCR* gene-modified lymphocytes into NOG mice. We anticipated that a clinical trial to evaluate this therapy would involve the transduction of polyclonally-activated PBMC with *TCR* genes, followed by the transfer of these cells into patients without purification of the CD8$^+$ T-cell subset. To mimic these conditions, the NOG mice received *TCR* gene-modified lymphocytes without further purification. The *TCR* gene-modified and unmodified cells used for the transfer experiments were stained with anti-CD8 mAb and a MAGE-A4$_{143-151}$/HLA-A*2402 tetramer that specifically detected the transduced TCR (Fig. 1A). As we reported previously, this TCR bound the tetramer in a

doi: 10.1111/j.1349-7006.2011.02111.x

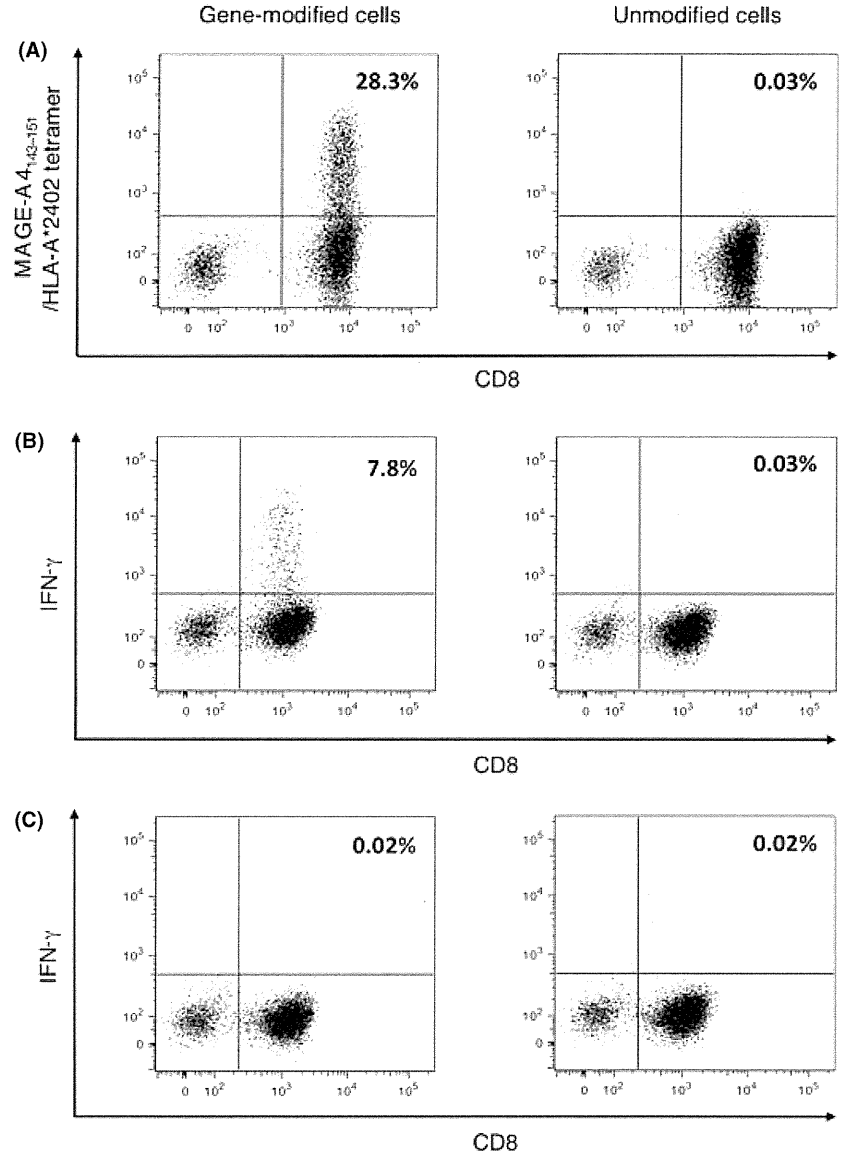

Fig. 1. Transduction of melanoma-associated antigen (MAGE)-A4-specific T-cell receptor (TCR) in human lymphocytes. Peripheral blood mononuclear cells from healthy donors were stimulated with anti-CD3 mAb and interleukin-2. Cells were cultured with or without retroviral vector encoding MAGE-A4-specific TCR, designated gene-modified or unmodified cells, respectively. (A) Representative staining for gene-modified and unmodified cells with MAGE-$A4_{143-151}$/HLA-A*2402 tetramer and antihuman CD8 mAb are shown. (B,C) Gene-modified and unmodified cells were stimulated with T2-A*2402 cells pulsed with the MAGE-$A4_{143-151}$ peptide (B) or HLA-A*2402-binding irrelevant control peptide (C). Representative specific intracellular interferon (IFN)-γ staining is displayed. Numerical value indicates the percentage of the tetramer$^+$ cells or IFN-g$^+$ cells among CD8$^+$ cells.

CD8 molecule-dependent manner.[34] These T cells were tested for specific reactivity against antigen peptide presented on HLA-A*2402 (Fig. 1B,C).

Before transfer, we stained the cells with the MAGE-$A4_{143-151}$/HLA-A*2402 tetramer to calculate the number of tetramer$^+$CD8$^+$ cells. The growth of implanted MAGE-A4$^+$HLA-A*2402$^+$ KE4 tumor cells was significantly inhibited when 9×10^6 of tetramer$^+$CD8$^+$ cells were intravenously injected into NOG mice on day 0 (Fig. 2A). The inhibition of KE4 growth required specific recognition of the MAGE-$A4_{141-153}$/HLA-A*2402 complex by the TCR, because unmodified cells derived from the same donor did not alter KE4 growth. In this experiment, 1×10^8 gene-modified or unmodified lymphocytes derived from the same donor were administered to mice. Although the CD4/CD8 ratio of the *in vitro* expanded lymphocytes depends on the donor, gene-modified and unmodified cells derived from the same donor demonstrated similar phenotypes, determined by the expression of cell surface markers, including CD3, CD4, CD8, CD45RA, CD45RO, CD62L, CCR7, CD152, CD25, CD27, and CD28 (data not shown). The growth of the QG56 tumors, which expressed MAGE-A4, but lacked HLA-A*2402, was indistinguishable in mice receiving either gene-modified or unmodified cells (Fig. 2D). Only a modest inhibition of KE4 growth was seen when mice received only 3×10^6 of tetramer$^+$CD8$^+$ cells (Fig. 2B), while no effect was seen upon administration of 1×10^6 of tetramer$^+$CD8$^+$ cells (Fig. 2C).

We addressed the effect of the adoptive transfer of the gene-modified cells into the mice with established tumors. We adoptively transferred TCR-engineered T cells into NOG mice that were inoculated with KE4 tumor cells 3 days earlier. On the day of adoptive T-cell transfer, we observed the establishment of a KE4 tumor mass in the mice. As shown in Figure 2(E), the administration of gene-modified cells significantly inhibited the growth of KE4 tumors, although the effect was limited and appeared later compared to the treatment on day 0. Taken together, the adoptive transfer of MAGE-A4-specific TCR gene-modified lymphocytes inhibited human tumor growth in NOG mice in a dose-dependent and TCR-specific manner.

Adoptively-transferred human lymphocytes persist in NOG mice. We monitored the persistence of transferred human lymphocytes in peripheral blood by staining Ficoll-purified PBMC from NOG mice with mAb specific for human CD8 and CD4.

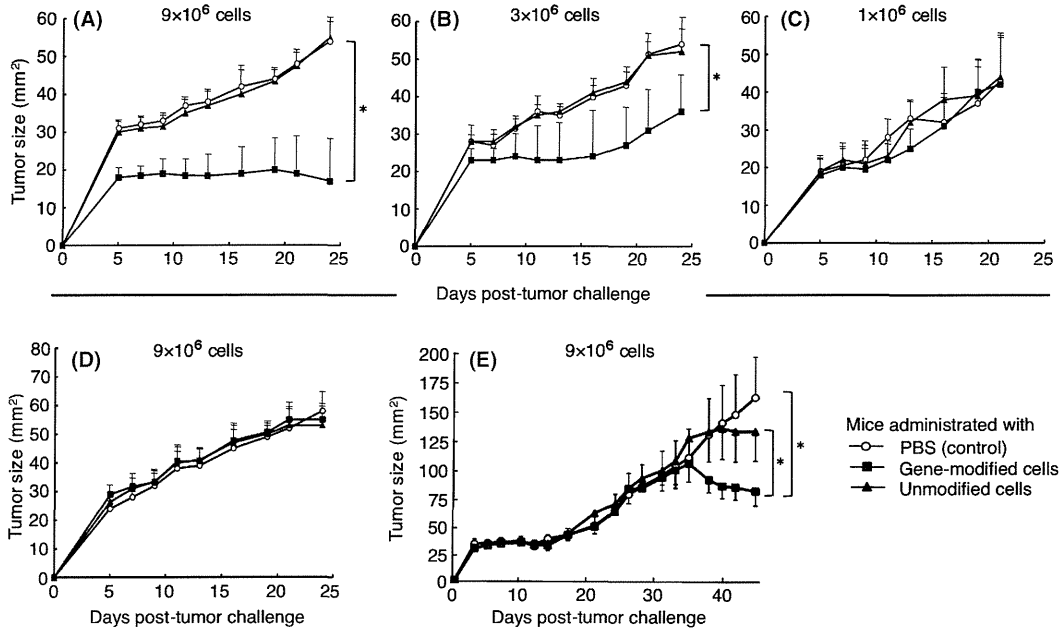

Fig. 2. Adoptive transfer of lymphocytes genetically engineered to express MAGE-A4-specific T-cell receptor inhibits human tumor progression in non-obese diabetic/SCID/γc^{null} mice. Non-obese diabetic/SCID/γc^{null} mice (n = 4 per group) were subcutaneously inoculated with 2.5×10^6 KE4 (A–C) or QG56 (D) tumor cells, and intravenously administered $\sim 1 \times 10^8$ gene-modified (■) or unmodified (▲) cells or PBS alone (control, ○) on day 0. Total of 9×10^6 (A,D), 3×10^6 (B), or 1×10^6 (C) tetramer$^+$CD8$^+$ cells were confirmed to be adoptively transferred; we subsequently monitored tumor growth over time. (E) Non-obese, diabetic/SCID/γc^{null} mice (n = 4 per group) received the treatment 3 days after the subcutaneous inoculation of 2.5×10^6 KE4. Total of 9×10^6 tetramer$^+$CD8$^+$ cells were transferred. Mean tumor size for each group is represented as the average + SD of four mice. Results are representative of three independent experiments. Differences between groups were examined for statistical significance using the Student's t-test. *$P < 0.01$. Numerical value indicates the number of tetramer$^+$CD8$^+$ cells administrated.

Human CD8$^+$ T cells persisted in NOG mice for more than 40 days after transfer (Fig. 3A). The transferred human CD8$^+$ cells comprised between 10% and 30% of the total peripheral mononuclear cells in NOG mice at almost all time points following transfer of 1×10^8 human lymphocytes. In these experiments, approximately 9×10^6 of the transferred 1×10^8 gene-modified cells were tetramer$^+$CD8$^+$. The percentage of specifically staining cells in the total peripheral mononuclear cell population was significantly less when mice received 5×10^7 human lymphocytes (Fig. 3B). There was no significant difference in transferred cell survival or percentages between mice receiving gene-modified and unmodified cells (Fig. 3A,B). Human CD4$^+$ cells comprised less than 10% of all lymphocytes for the first 2 weeks following transfer, but a rapid increase in this population was evident after day 21(Fig. 3C,D). This observation was consistent with reports suggesting that CD4$^+$ T cells play a dominant role in the induction of graft-versus-host (GVH) reactions in hosts receiving transfusions.[35,36] The NOG mice receiving human lymphocyte transfers demonstrated significant weight loss after day 21, a sign of GVH reactions (Fig. 3E).

Transferred TCR gene-modified T cells retain their ability to recognize specific antigens in NOG mice. Lymphocytes harvested from the peripheral blood of NOG mice administered TCR gene-modified lymphocytes were tested for their antigen-specific reactivity by intracellular cytokine staining with anti-IFN-γ mAb after incubation with peptide-loaded T2-A*2402 cells. Antigen-specific IFN-γ secretion was detectable by peripheral blood CD8$^+$ cells isolated from mice throughout the 40-day period after adoptive transfer with either 1×10^8 (Fig. 4A) or 5×10^7 (Fig. 4B) gene-modified cells. No reactivity of these lymphocytes was seen against T2-A*2402 cells without loaded peptide (data not shown). Cells from mice that received unmodified lymphocytes did not demonstrate a specific response (Fig. 4A,B). These results indicate that transferred TCR gene-modified cells remained functional *in vivo*, recognizing the MAGE-A4$_{141-153}$ peptide in the context of HLA-A*2402. When 5×10^7 cells were transferred, these cells expanded more rapidly in the early phase compared to the group with 1×10^8 cells transferred. We speculate that the adoptive transfer of a lower number of antigen-specific T cells might induce these cells to expand more rapidly *in vivo* in the early expansion phase. At the later time points, more antigen-specific cells persisted in mice receiving 1×10^8 cells.

Intratumor infiltration of transferred human CD8$^+$ T cells. To confirm the infiltration of transferred cells into tumor tissue, we examined implanted KE4 and QG56 tumors by immunohistochemical analysis. As antibodies specifically recognizing the transferred TCR (TCRα V8-1 or TCRβ V7-9) are not available, we stained tumor specimens with a mAb against human CD8. Significant infiltration of human CD8$^+$ cells was detectable in KE4 tumors harvested from mice as early as 2 weeks after the transfer of gene-modified cells (Fig. 5A,B). CD8$^+$ cell infiltration in KE4 tumor specimens in the mice that received gene-modified cells was slightly better than in the mice that received unmodified lymphocytes. However, the difference was not statistically significant (Fig. 5A,B). A similar degree of infiltration was also observed in QG56 tumors. These data were consistent with previous reports analyzing the migration of tumor-specific T cells by two-photon laser microscopy that indicated tumor-specific T cells accumulate in both antigen-positive and -negative tumor tissues to comparable extents, but at different migratory velocities, according to tumor antigen expression.[37] The KE4 tumors in mice that did not receive human lymphocytes lacked any positive staining (Fig. 5B).

Combination of TCR gene therapy and peptide vaccine enhances antitumor efficacy. In animal models of adoptive cell therapy examining the effects against murine tumors with tumor-specific CD8$^+$ T cells, *in vivo* vaccinations using agents, such as antigen-peptide or antigen-encoding viruses,

doi: 10.1111/j.1349-7006.2011.02111.x

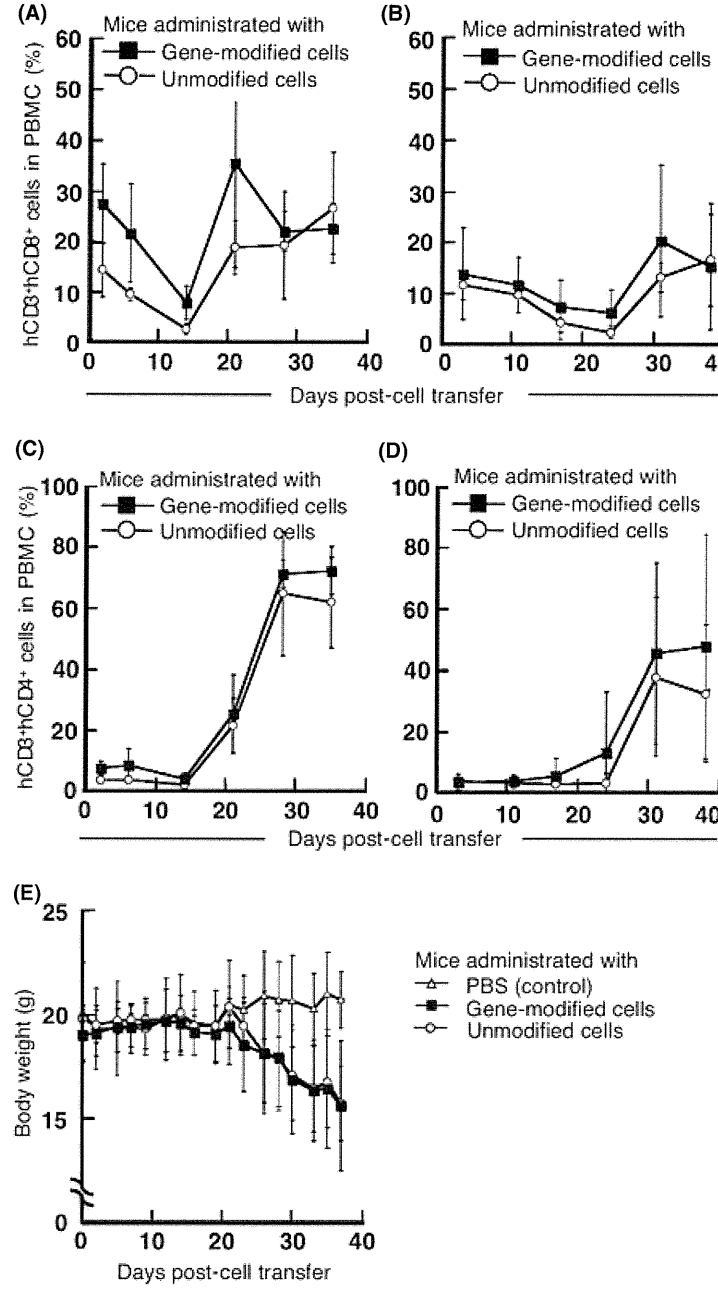

Fig. 3. Persistence of adoptively transferred human lymphocytes in non-obese, diabetic/SCID/γc^{null} (NOG) mice. Non-obese, diabetic/SCID/γc^{null} mice ($n = 4$ per group) were subcutaneously inoculated with 2.5×10^6 KE4 tumor cells, then intravenously administered 1×10^8 (A,C) or 5×10^7 (B,D) gene-modified (■) or unmodified (○) cells on day 0. Mononuclear cells were purified from peripheral blood collected from mice on the indicated days. We evaluated the proportion of human $CD3^+CD8^+$ (A,B) or $CD3^+CD4^+$ (C,D) cells among the mononuclear cell population. (E) We also monitored the body weight of NOG mice administered 1×10^8 gene-modified (■) or unmodified (○) cells or PBS (control, △) over time. Results are representative of three independent experiments. PBMC, peripheral blood mononuclear cells.

can increase the antitumor efficacy of adoptive cell therapy.[9,38] Therefore, we explored if a peptide vaccination in conjunction with TCR gene-modified cell transfer could increase the inhibition of tumor growth seen in this model. As the administration of 1×10^6 $tetramer^+CD8^+$ cells alone was incapable of inducing tumor growth inhibition in this model (Fig. 2C), we examined if the combination of an *in vivo* peptide vaccination with cell transfer under these conditions could enhance tumor inhibition. As NOG mice do not possess endogenous antigen-presenting cells capable of presenting peptide in an HLA-A*2402-restricted manner, we used HLA-A*2402-positive human PBMC pulsed with the MAGE-$A4_{143-151}$ peptide. Tumor-inoculated NOG mice receiving gene-modified cells were also administered peptide-loaded HLA-A*2402-positive PBMC derived from the same donor on days 2 and 8 of the tumor challenge. KE4 tumor growth was significantly inhibited in the mice receiving a combination of cell therapy and peptide vaccination in comparison to mice treated by cell therapy alone (Fig. 6A). The peptide vaccination did not alter KE4 growth when combined with the transfer of unmodified cells. The growth of the HLA-A*2402-negative QG56 tumor was identical in both groups (Fig. 6B).

Increased multifunctionality in adoptively-transferred cells when inoculated with peptide vaccine. We previously reported that the multifunctionality of effector cytotoxic T cells (CTL) is a critical determinant of the quality of the T-cell response and the resultant immunological control of tumor.[33,39] We therefore compared the multifunctionality of transferred cells from NOG mice treated with TCR gene-modified cells and peptide vaccination with that from mice treated by TCR gene cell therapy alone. We assessed IFN-γ and TNF-α production and CD107a mobilization by $CD8^+$ T cells at the single-cell level in specimens harvested from mice on days 2, 7, and 14 after transfer. We

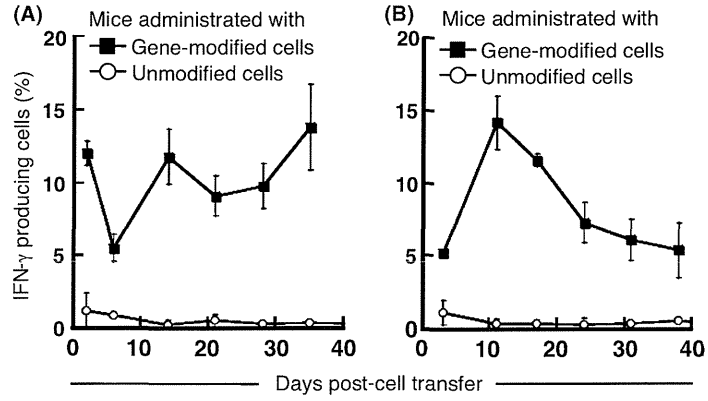

Fig. 4. Lymphocytes genetically engineered to express MAGE-A4-specific T-cell receptor-maintained specific reactivity after *in vivo* passage. Non-obese, diabetic/SCID/γc^{null} mice ($n = 4$ per group) were subcutaneously inoculated with 2.5×10^6 KE4 tumor cells, then intravenously administered 1×10^8 (A) or 5×10^7 (B) gene-modified (■) or unmodified (○) cells on day 0. Mononuclear cells were purified from peripheral blood collected from mice on the indicated days. Intracellular γ-interferon (IFN-γ) production by these cells was assessed after being stimulated with 1 μM MAGE-$A4_{141-153}$ peptide for 6 h. Data are shown as the percentage of IFN-γ-producing cells within the total human $CD8^+$ cell population. Results are representative of three independent experiments.

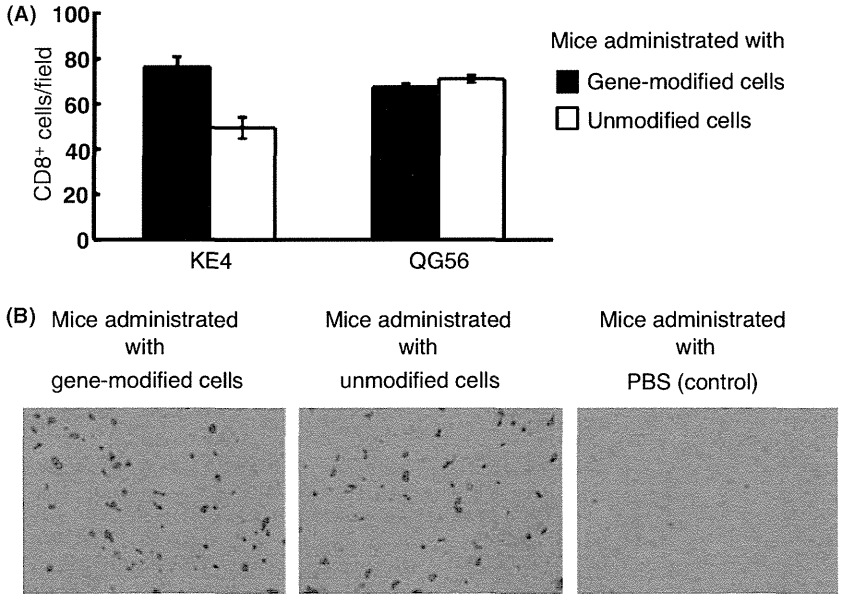

Fig. 5. Adoptively-transferred human $CD8^+$ T cells infiltrate into tumor tissues. Tumor specimens were harvested from non-obese, diabetic/SCID/γc^{null} mice 14 days after subcutaneous inoculation with 2.5×10^6 KE4 or QG56 tumor cells, and intravenous administration of 1×10^8 gene-modified or unmodified cells or PBS (control). We stained formalin-embedded tumor specimens with an antihuman CD8 monoclonal antibody, clone C8/144B. Average $CD8^+$ TIL counts ± SD in KE4 or QG56 (A) and the representative images from KE4 tissue sections (B) are shown.

selected these functional measures because multifunctionality assessed by these factors defines a sensitive correlate of the immunological control of tumors.[33,39]

The mice received human lymphocytes with or without peptide vaccination; isolated peripheral blood specimens were tested for their antigen-specific reactivity of component $CD8^+$ T cells at the indicated time points. On day 2 or 7 after adoptive transfer, we were barely able to detect cells with two or three functions in mice receiving gene-modified cells without peptide vaccination (Fig. 7); cells with three functions comprised 3.7% of all $CD8^+$ T cells, while bifunctional cells comprised 2.4% on day 14. In contrast, mice receiving combination therapy with gene-modified cells and peptide vaccination exhibited a population of cells with three and two functions of 1.4% and 2% of the total $CD8^+$ cells, respectively, as early as day 2. Therefore, multifunctional effector $CD8^+$ T cells appear earlier in mice receiving combination therapy in comparison to those receiving cell therapy alone. On day 7, trifunctional and bifunctional cells in mice receiving combination therapy comprised 1.7% and 4.8% of all cells, respectively. The cells with three or two functions were retained as part of the peripheral mononuclear cell population in these animals on day 14.

Discussion

Successful clinical responses using adoptive cell therapy with tumor-reactive T cells in patients with advanced melanoma have encouraged the development of genetic engineering approaches

doi: 10.1111/j.1349-7006.2011.02111.x

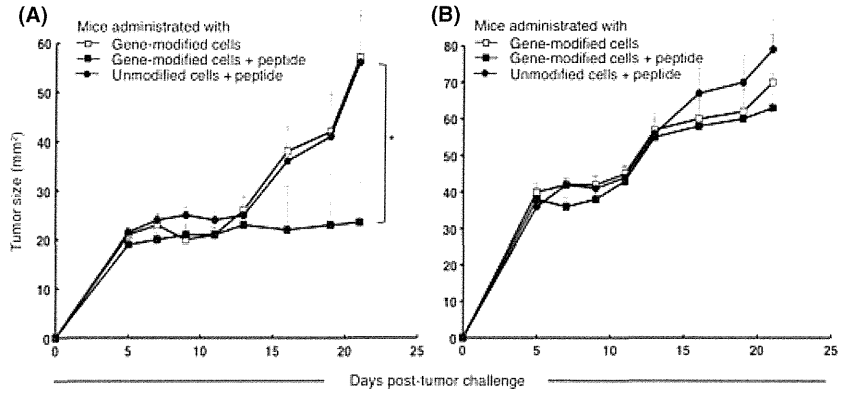

Fig. 6. Peptide vaccination enhanced the antitumor efficacy of adoptive therapy using T-cell receptor, gene-modified cells. Non-obese, diabetic/SCID/γc^{null} mice (n = 4 per group) were subcutaneously inoculated with 2.5×10^6 KE4 (A) or QG56 (B) tumor cells, and intravenously administered 1×10^8 gene-modified (□) or unmodified (●) cells on day 0. Gene-modified population included 1×10^6 tetramer$^+$CD8$^+$ cells. We pulsed 4×10^7 peripheral blood mononuclear cells derived from the same donor (HLA-A*2402 positive) with 1 μM MAGE-A4$_{141-153}$ peptide, and intravenously administered these cells into the animals on days 1 and 8 (■ and ●). Results are representative of three independent experiments.

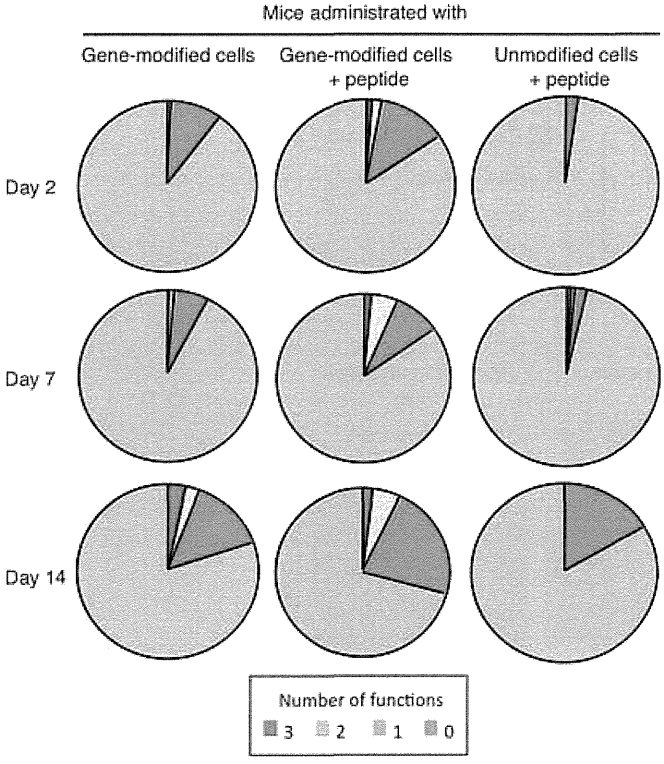

Fig. 7. Peptide vaccination increased the multifunctionality of transferred gene-modified cells. Mice were subcutaneously inoculated with 2.5×10^6 KE4 tumor cells, then intravenously administered 1×10^8 gene-modified or unmodified cells with or without peptide vaccination. Two, 7, and 14 days after transfer, we collected peripheral blood from mice. After purifying the mononuclear cells in these samples, we evaluated their multifunctionality by measuring γ-interferon (IFN-γ) and tumor necrosis factor-α (TNF-α) production and CD107a mobilization. Data are summarized in the pie chart, where each wedge represents the frequency of human CD8$^+$ cells expressing all three functions (3), any two functions (2), a single function (1), or no function (0). Results are representative of three independent experiments.

using patient lymphocytes; these studies aim to extend the range of tumor types that can be treated with this technique and to improve the quality of the lymphocytes employed.[40–42] In a recent clinical trial for metastatic synovial cell sarcoma and melanoma, patients were administered autologous lymphocytes genetically engineered to express a high-avidity TCR against NY-ESO-1; objective clinical responses were observed in four (60%) of six patients with synovial cell sarcoma, and five (45%) of 11 patients with melanoma.[43] In this trial, the transferred TCR contained two amino-acid substitutions in the third complementary determining region of the native TCRα chain that conferred CD8$^+$ T cells with an enhanced avidity. No on-target toxicities were seen in this trial, in contrast to previous observations of vigorous on-target toxicity in patients receiving lymphocytes engineered to express melanocyte differentiation antigen-specific TCR. Genetic engineering also offers the means to endow T cells with enhanced function, as well as resistance to tumor-mediated immunosuppression through the addition of genes encoding homeostatic or pro-inflammatory cytokines,[44,45] chemokine receptors,[46] anti-apoptotic molecules,[47] and costimulatory molecules,[48.49] as well as the silencing of co-inhibitory molecules,[50] although these modifications await clinical evaluation. As increased effector function and/or *in vivo* persistence of cells bearing these modifications might increase on-target toxicity during therapy, the selection of appropriate target antigens is critical to induce favorable antitumor effects and avoid severe adverse events.

The establishment of an animal model suitable for evaluating the *in vivo* efficacy and safety of human adoptive cell therapy is an important challenge to facilitate the development of these therapies and prevent toxicity. Non-obese diabetic/SCID/γc^{null}-immunodeficient mice that lack T, B, and natural killer cells, and demonstrate impaired dendritic cell activity, are a helpful animal model to evaluate the *in vivo* activity of human hematopoietic cells.[32] The NOG mouse model, however, still has limitations, including a homeostatic expansion effect on infused T cells, an allo-reactive response between infused effector cells and transplanted target cells, and potential GVH reactions. In this study, mice receiving human lymphocytes exhibited severe weight loss, consistent with GVH reaction, which worsened after day 21. Therefore, antitumor efficacy in this model is best evaluated before day 21. Future studies will need to evaluate if the homeostatic proliferation of infused cells and/or a suboptimal allo-reactivity influenced the treatment effect seen in this model. The lack of an effect by unmodified cells (Fig. 2) and the increased efficacy upon co-administration of an antigen-peptide vaccine (Fig. 6), however, strongly suggest that the observed antitumor effect was achieved in a MAGE-A4-specific, TCR-mediated manner. The future devel-