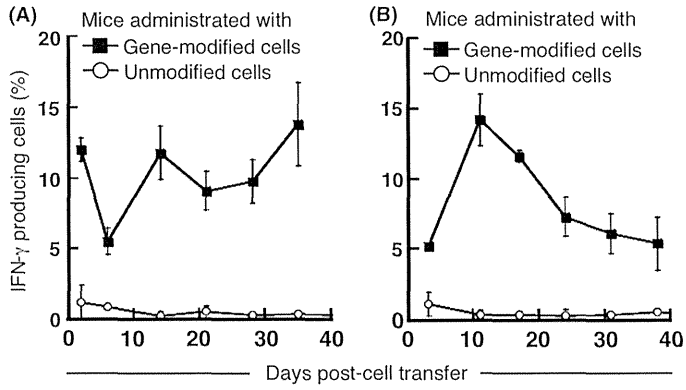

Fig. 4. Lymphocytes genetically engineered to express MAGE-A4-specific T-cell receptor-maintained specific reactivity after *in vivo* passage. Non-obese, diabetic/SCID/γc^{null} mice ($n = 4$ per group) were subcutaneously inoculated with 2.5×10^6 KE4 tumor cells, then intravenously administered 1×10^8 (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.

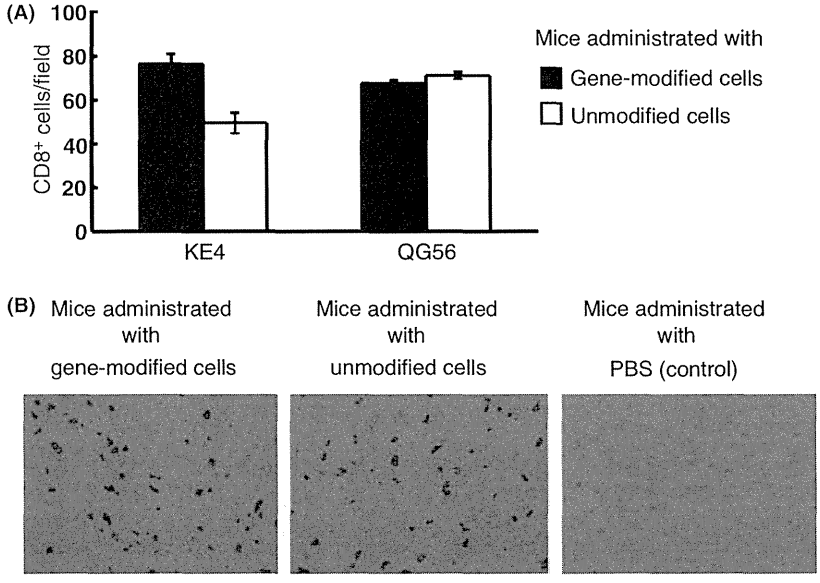

Fig. 5. Adoptively-transferred human $CD8^+$ T cells infiltrate into tumor tissues. Tumor specimens were harvested from non-obese, diabetic/SCID/γc^{null} mice 14 days after subcutaneous inoculation with 2.5×10^6 KE4 or QG56 tumor cells, and intravenous administration of 1×10^8 gene-modified or unmodified 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

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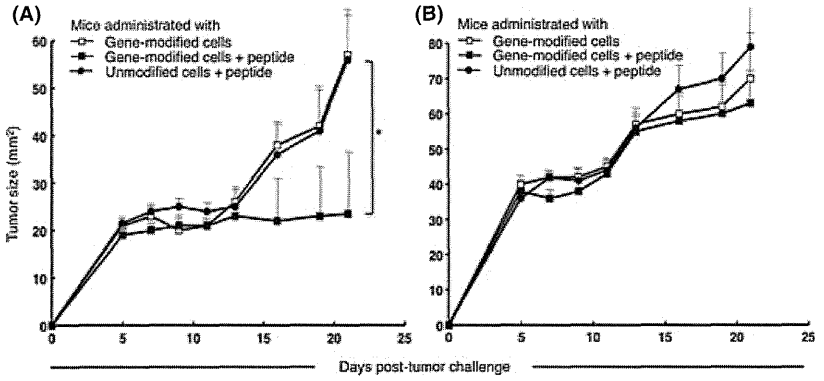

Fig. 6. Peptide vaccination enhanced the antitumor efficacy of adoptive therapy using T-cell receptor, gene-modified cells. Non-obese, diabetic/SCID/γc^{null} mice (n = 4 per group) were subcutaneously inoculated with 2.5×10^6 KE4 (A) or QG56 (B) tumor cells, and intravenously administered 1×10^8 gene-modified (□) 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.

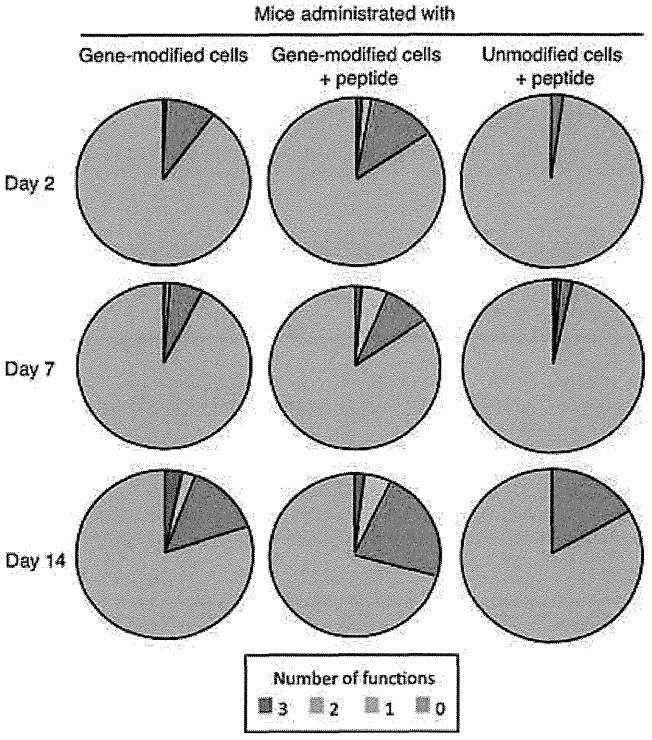

Fig. 7. Peptide vaccination increased the multifunctionality of transferred gene-modified cells. Mice were subcutaneously inoculated with 2.5×10^6 KE4 tumor cells, then intravenously administered 1×10^8 gene-modified or unmodified cells with or without peptide vaccination. Two, 7, and 14 days after transfer, 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-

opment of improved humanized mice will help to better evaluate the optimization of human immunotherapy.

Multifunctionality is the ability of T cells to exhibit multiple functions, including the simultaneous secretion of multiple cytokines, chemokines, or cytotoxic granules at the single-cell level.[51] The importance of T-cell multifunctionality has been reported in multiple animal infection models[52,53] and in humans infected with HIV, cytomegalovirus, hepatitis B virus, or tuberculosis.[53–60] We reported the importance of effector T-cell multifunctionality in antitumor immune response. Specifically, the appearance of multifunctional $CD8^+$ effector cytotoxic T cells *in vivo* is a critical determinant of effective immunological control of tumors. Regulatory T cells were found to play a role in the inhibition of transferred tumor antigen-specific T-cell multifunctionality.[33,39] In the present study, effector T-cell multifunctionality appeared to correlate with the quality of T-cell responses in adoptive T-cell therapy utilizing genetically-engineered human lymphocytes (Figs 6,7). The peptide vaccination did not significantly change the percentage of human $CD3^+CD8^+$ cells in the PBMC of NOG mice (data not shown). The TCR-transduction efficiency in this study was not very high in general. We found that the combination of vaccination with the adoptive transfer of antigen-specific T cells increased effector T-cell multifunctionality and made the antitumor effect visible, even with a low number of specific TCR-transduced T cells transferred. The unmodified cells with background reactivity were the IFN-γ single producers. We speculate that these cells are positive for IFN-γ because of their non-specific activation due to GVH reaction.

To our knowledge, this study represents the first demonstration *in vivo* of an antitumor effect following the adoptive transfer of human lymphocytes genetically engineered to express a TCR specific for MAGE family antigen. The retroviral vector used in this report is currently under evaluation in a phase I clinical trial designed to treat patients with MAGE-A4-expressing esophageal cancer.

In summary, our data suggest that adoptive cell therapy with human lymphocytes engineered to express MAGE-A4-specific TCR through retroviral transduction is a promising strategy to treat patients with MAGE-A4-expressing tumors. Combination therapy with gene-modified cell-adoptive transfer and *in vivo* vaccination might improve antitumor efficacy, even with low numbers of transferred tumor-reactive T cells. These data support the rationale to explore clinical trials utilizing gene-modified lymphocytes prepared using the vector described in this report.

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Disclosure Statement

No potential conflicts of interest were disclosed.

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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

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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).

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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.

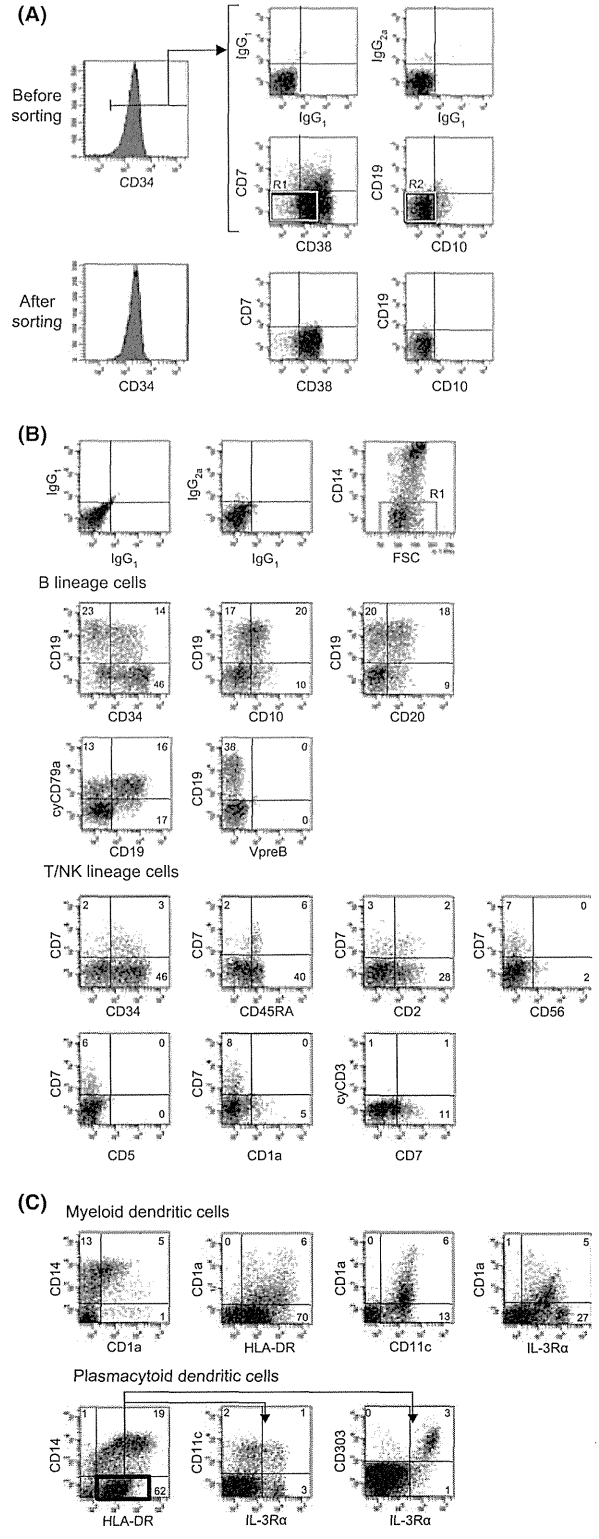
Results

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-$3R\alpha^{high}CD303^+$

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



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-3Rα^{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 ≥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 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-3Rα^{high}CD11c$^-$CD303$^+$ cells (lower row).

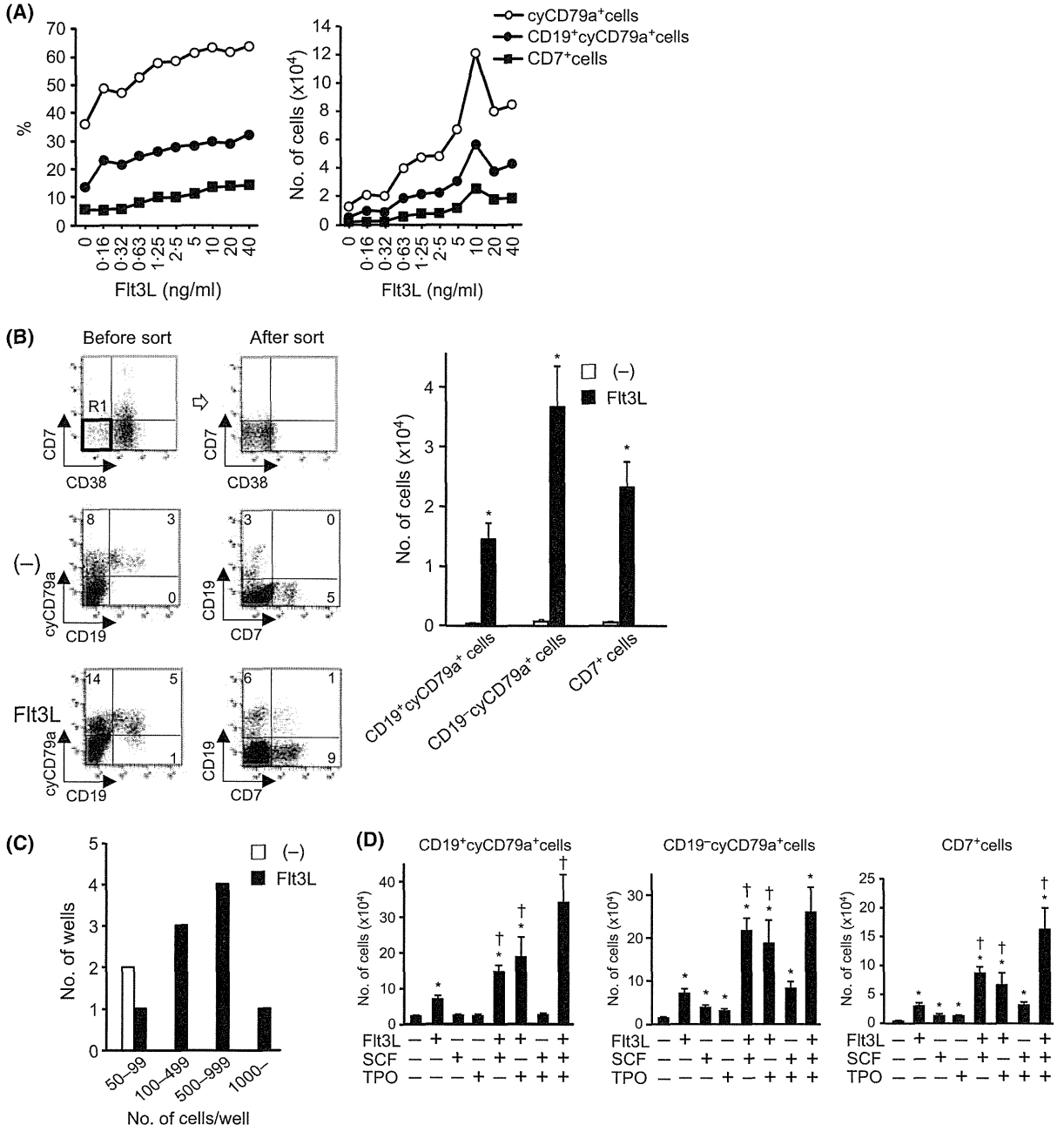


Fig 2. Effects of flt3L, SCF and TPO on lymphopoiesis. The effects of flt3L on $CD34^+CD38^{low/-}CD19^-CD10^-CD7^-$ (1×10^4 cells/well) (A) and $CD34^+CD38^-CD19^-CD10^-CD7^-$ (1×10^4 cells/well) (B) cultured on telomerized stromal cells. The percentage and number of $CD19^+cyCD79a^+$, $CD19^-CD79a^+$ 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 $\geq$ 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

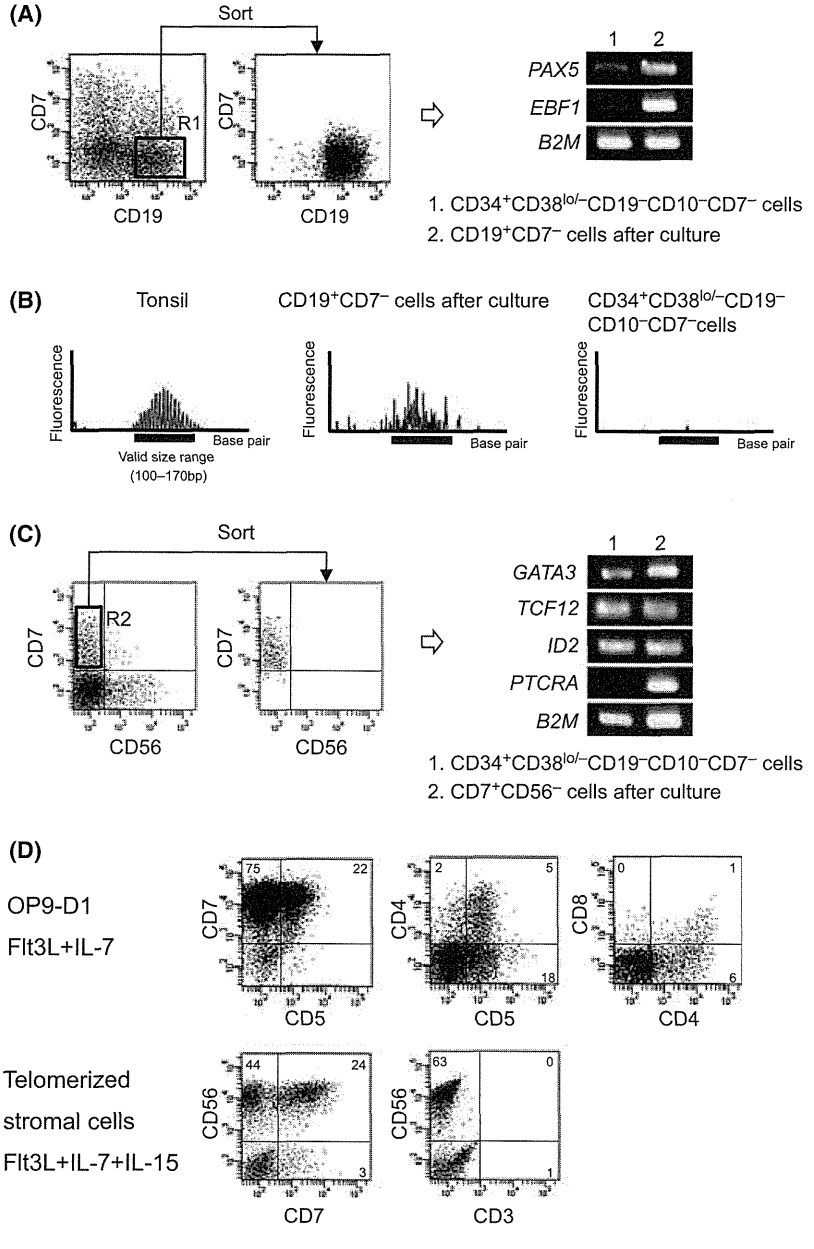


Fig 3. Characteristics of generated $CD19^+$ and $CD7^+CD56^-$ cells. (A) Gene expression of *PAX5*, *EBF1* and *B2M* in $CD34^+CD38^{low/-}CD19^-CD10^-CD7^-$ and $CD19^+CD7^-$ cells. (B) VDJ rearrangements of *IGH@* genes. (C) Gene expressions of *GATA3*, *TCF12*, *PTCRA* and *B2M* in $CD34^+CD38^{low/-}CD19^-CD10^-CD7^-$ 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-

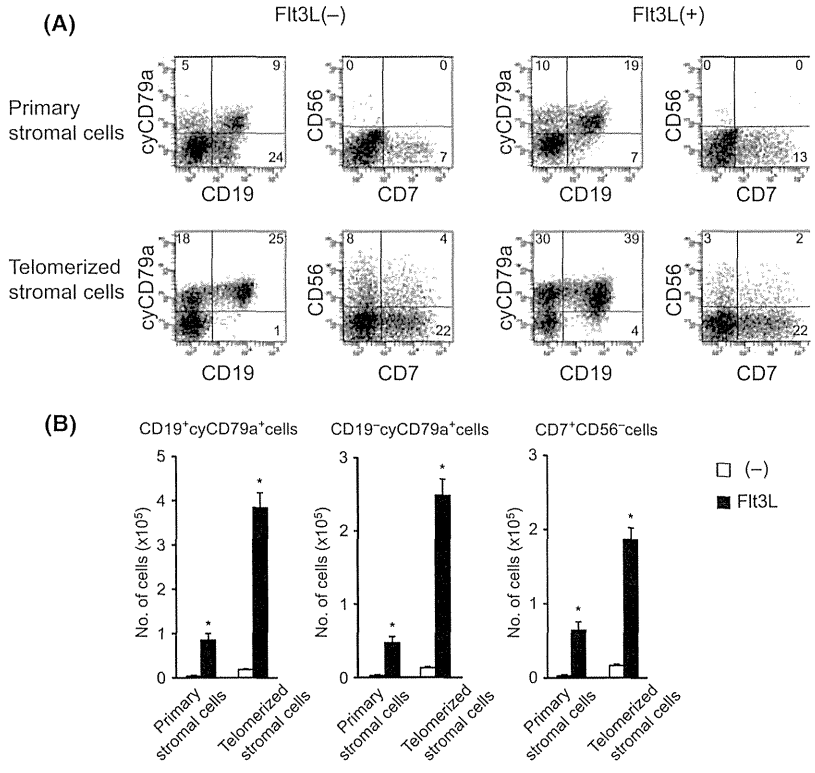


Fig 4. B and T/NK lymphopoiesis occurs on primary human stromal cells. $CD34^+CD38^{low/-}CD19^-CD10^-CD7^-$ cells (1×10^4 cells/well) were cocultured with primary or telomerized stromal cells, with or without flt3L, and analysed for the cell phenotypes (A) and numbers (B). The phenotypes after excluding $CD14^+$ 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

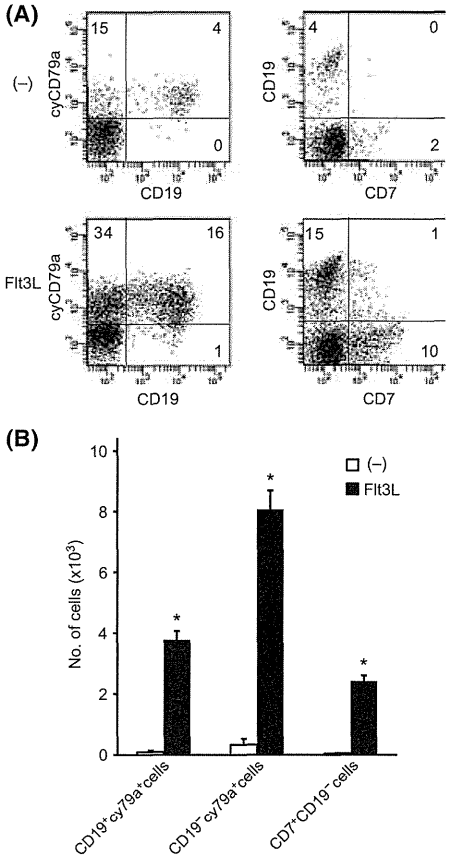


Fig 5. B and T/NK lymphopoiesis can occur separately from stromal cells. $CD34^+CD38^{lo/-}CD19^-CD10^-CD7^-$ cells (1×10^4 cells/well) were cultured separately from telomerized stromal cells using cell culture inserts in the presence or absence of flt3L. The phenotype (A) and number (B) of the cultured 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.

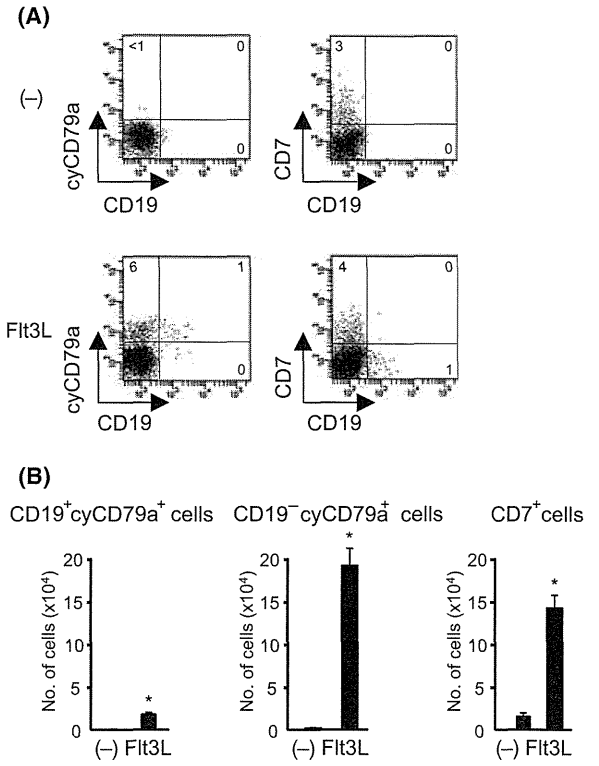


Fig 6. Effect of flt3L in serum-free cultures. $CD34^+CD38^{low/-}CD19^-CD10^-CD7^-$ cells (1×10^4 cells/well) were cultured with telomerized stromal cells in serum-free cultures with or without flt3L, and the phenotype (A) and the number (B) of $CD19^+cyCD79a^+$, $CD19^-cyCD79a^+$ and $CD7^+$ cells 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.

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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.

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Microenvironment and Immunology

Cancer Research

Intracellular Tumor-Associated Antigens Represent Effective Targets for Passive Immunotherapy

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Abstract

Monoclonal antibody (mAb) therapy against tumor antigens expressed on the tumor surface is associated with clinical benefit. However, many tumor antigens are intracellular molecules that generally would not be considered suitable targets for mAb therapy. In this study, we provide evidence challenging this view through an investigation of the efficacy of mAb directed against NY-ESO-1, a widely expressed immunogen in human tumors that is expressed intracellularly rather than on the surface of cells. On their own, NY-ESO-1 mAb could neither augment antigen-specific $CD8^+$ T-cell induction nor cause tumor eradication. To facilitate mAb access to intracellular target molecules, we combined anti-NY-ESO-1 mAb with anticancer drugs to accentuate the release of intracellular NY-ESO-1 from dying tumor cells. Strikingly, combination therapy induced a strong antitumor effect that was accompanied by the development of NY-ESO-1–specific effector/memory $CD8^+$ T cells that were not elicited by single treatments alone. The combinatorial effect was also associated with upregulation of maturation markers on dendritic cells, consistent with the organization of an effective antitumor T-cell response. Administration of Fc-depleted F(ab) mAb or combination treatment in Fcγ receptor–deficient host mice abolished the therapeutic effect. Together, our findings show that intracellular tumor antigens can be captured by mAbs and engaged in an efficient induction of $CD8^+$ T-cell responses, greatly expanding the possible use of mAb for passive cancer immunotherapy. *Cancer Res; 72(7); 1672–82. ©2012 AACR.*

Introduction

With the molecular identification of tumor antigens recognized by the human immune system, antigen-specific immunotherapy for cancers has been developed and is explored in the clinic (1–3). Particularly, monoclonal antibodies (mAb) that recognize surface antigens, such as trastuzumab (anti-Her2/neu) and rituximab (anti-CD20), as a single agent or in combination with chemotherapy, are used in the clinic for frontline or salvage therapy and have resulted in objective and durable clinical responses (3–5). One of the major therapeutic mechanisms of mAb is considered to be the selective interruption of vital signaling pathways in which the targeted antigens are critically involved (3, 5). In addition, there is accumulating evidence that mAb therapy also works through antibody-dependent cellular cytotoxicity (ADCC) by natural killer (NK) cells or through the activation of complement, both of which depend on the Fc portion of the mAbs (6–9). Furthermore, Fc receptor–mediated uptake of immune complexes results in activation of antigen-presenting cells (APC) and facilitates cross-presentation of those antigens to tumor-specific $CD8^+$ T cells and inhibition of tumor growth, as was shown recently in HER2/*neu* and melanoma differentiation antigen tyrosinase–related protein-1 (Trp1; gp75) models (10–13).

However, many well-characterized tumor-associated antigens, including cancer/testis (CT) antigens, are intracellular antigens and thus not accessible for antibodies (14–16). An exception is mAb TA99, which targets gp75 and was shown to induce NK and $CD4^+$ T-cell–dependent antitumor responses *in vivo* (17). However, the fact that gp75 is expressed both on the cell surface and intracellularly makes it difficult to define the precise targets for the antitumor responses induced by mAb TA99 (12, 17).

NY-ESO-1, a CT antigen discovered by SEREX (serologic identification of antigens by recombinant expression cloning) using the serum of a patient with esophageal cancer, is frequently expressed in cancer cells of various tissue origins

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but not in normal somatic cells except for germ cells in the testis (2, 18). Spontaneous cellular and humoral immune responses against NY-ESO-1 are found in patients with cancer, which underscores its immunogenicity (2, 18). It has an intracellular location and lacks cell surface expression (2, 18), thus curtails it from being a candidate of mAb therapy. Interestingly, NY-ESO-1 protein/IgG antibody complexes (immune complexes, IC) are efficiently cross-presented to the MHC class I pathway (19, 20) and there is a close correlation between antibody and $CD8^+$ T-cell responses (2, 21), suggesting that NY-ESO-1–specific $CD8^+$ T-cell induction by cross-priming *in vivo* is associated with the induction of specific antibodies. These data prompted us to analyze the possibility whether mAb therapy could be applied to an intracellular molecule NY-ESO-1 and inhibit tumor growth by enhancing $CD8^+$ T-cell induction.

We have established syngeneic tumor models in BALB/c mice using CT26 colon carcinoma cells and CMS5a sarcoma cells that are stably transfected with NY-ESO-1 (22, 23). Using these models, we addressed whether NY-ESO-1 mAb combined with chemotherapy augmented NY-ESO-1–specific $CD8^+$ T-cell induction and inhibited tumor growth.

Materials and Methods

Mice

Female BALB/c mice and BALB/$c^{nu/nu}$ mice were obtained from SLC Japan or Jackson laboratory and used at 7 to 10 weeks of age. BALB/c mice deficient in the γ-chain subunit of Fc receptors were obtained from Taconic and used at 7 to 10 weeks of age. Mice were maintained in accordance with the NIH and American Association of Laboratory Animal Care Regulations. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Mie University Graduate School of Medicine (Mie, Japan) and by the Memorial Sloan-Kettering Cancer Center Institutional Animal Care and Use Committee (New York, NY).

Tumors

CT26 is a colon epithelial tumor derived by intrarectal injections of *N*-nitroso-*N*-methylurethane in BALB/c mice (24). CT26 expressing NY-ESO-1 (CT26-NY-ESO-1) was established as described previously (23). CMS5a is a subcloned cell line obtained from CMS5 (25). CMS5a-NY-ESO-1 was established as described previously (22).

Antibodies and reagents

Anti-NY-ESO-1 mAbs [E978 (mouse IgG1) recognizing NY-ESO-1_{71-90}, ES121 (mouse IgG1) recognizing NY-ESO-1_{91-110}, 219–510 (mouse IgG1) recognizing NY-ESO-1_{21-40} (Supplementary Fig. S3; ref. 26)]; anti-CD4 (GK1.5, rat IgG2b), anti-CD8 (19/178, mouse IgG2a), and anti-MAGE-A4 (MCV1, mouse IgG1) were purified from hybridoma supernatant by protein G affinity chromatography. The F(ab) fragment of E978 was generated using the ImmunoPure Fab Preparation Kit (Thermo Fisher Scientific). Anti-CD8 (53–6.7), anti-CD45RB (16A), anti-CD80 (16-10A1), anti-CD86 (GL-1), anti-CD40 (3/23), anti-IFN-γ (XMG1.2), anti-CD62L (MEL-14), anti-CD11c (HL3), anti-TNF-α (MP6-XT22), and antimouse IgG1 (A85-1) mAbs were purchased from BD Biosciences, Biolegend, or eBioscience. Phycoerythrin (PE)-labeled NY-ESO-1_{81-88}-D^d tetramers were provided by Drs. P. Guillaume and I. Luescher (Ludwig Institute Core Facility, Lausanne, Switzerland). An anti-NY-ESO-1 human IgG1 mAb (12D7) was obtained from CT Atlantic. p63 (T) peptide TYLPTNASL (27), AH-$1_{138-147}$ peptide SPSYVHQF (28), and NY-ESO-1_{81-88} peptide RGPESRLL (23) were purchased from Operon Biotechnologies and BioSynthesis and Sigma.

Chemotherapeutic agents

5-Fluorouracil (5-FU; Kyowa Hakko Kirin,), doxorubicin (Kyowa Hakko Kirin), CPT-11 (Yakult), and paclitaxel (Bristol-Myers Squibb) were injected intraperitoneally as indicated.

Tumor challenge

Mice were inoculated with 0.5×10^6 to 1×10^6 CT26-NY-ESO-1 cells, 1×10^6 CMS5a-NY-ESO-1, or 1×10^6 CT26-MAGE-A4 cells in the right hind flank subcutaneously. Mice were monitored 3 times a week and were sacrificed when tumors reached greater than 20 mm.

Staining and flow cytometry

To collect tumor-infiltrating T cells, tumors were minced and treated with 1 mg/mL of collagenase IA (Sigma) in Hanks' balanced salt solution (HBSS) for 90 minutes at room temperature.

Cells harvested from draining lymph node (dLN) and tumors were stained for surface markers in PBS with 0.5% FBS for 15 minutes at 4°C. For intracellular cytokine staining, 1×10^6 to 3×10^6 cells from tumors or dLNs were cultured with peptide for 5 hours at 37°C, and GolgiPlug was added for the last 4 hours of culture. These cells were stained for surface markers and intracellularly with allophycocyanin-conjugated anti-IFN-γ and PE-conjugated anti-TNF-α mAbs after permeabilization and fixation using Cytofix/Cytoperm Kit (BD Bioscience). Dead cells were excluded by LIVE/DEAD Fixable Dead Cell Stain Kits (Invitrogen). Cells were analyzed on FACSCanto or FACSCalibur (BD Bioscience) and FlowJo software (Tree Star).

Fluorescent immunohistochemistry

Three micrometers of tissue sections prepared from fresh-frozen tumor specimens were fixed with ice-chilled acetone for 15 minutes. Alexa 488–labeled antihuman IgG antibody (Invitrogen) was applied and incubated at room temperature for 2 hours. For double immunolabeling, sections were fixed with 3% paraformaldehyde for 15 minutes, incubated with anti-cleaved caspase-3 (Cell Signaling Technology) at room temperature for 2 hours and then incubated with Alexa 488–labeled anti-human IgG antibody and Alexa 568–labeled antirabbit IgG Ab (Invitrogen) at room temperature for 2 hours. Sections were rinsed with PBS, counterstained with 4′,6-diamidino-2-phenylindole (DAPI), and mounted. Images were captured using ×40 magnification objective by Zeiss Axiocam system (Carl Zeiss).

Statistical analysis

Tumor curves were assessed by one-way ANOVA with a Bonferroni multiple comparisons posttest. Single measurement

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comparison between 2 groups was evaluated by 2-sided Student *t* test. *P* values <0.05 were considered statistically significant.

Results

Establishment of CT26-NY-ESO-1

We established a syngeneic colon carcinoma model (CT26-NY-ESO-1) with stable NY-ESO-1 expression (2, 22, 23). NY-ESO-1 expression in CT26-NY-ESO-1 cells was exclusively intracellular, and no NY-ESO-1 protein was detected on the cell surface (Supplementary Fig. S1A), consistent with the expression of NY-ESO-1 protein in human cancer cells (2). These CT26-NY-ESO-1 cells maintained the same tumor growth capacity as their parental CT26 cells in both wild-type Balb/c and C.B-17 SCID (severe combined immunodeficient) mice, indicating that there was no alteration of tumorigenicity caused by the NY-ESO-1 transfection (Supplementary Fig. S1B). When BALB/c mice were inoculated with CT26-NY-ESO-1 cells, spontaneous antibody and $CD8^+$ T-cell responses were detected after 7 days and increased thereafter (Supplementary Fig. S1C and S1D). These spontaneous immune responses closely paralleled spontaneous NY-ESO-1–specific immune responses found in humans (2).

We used this tumor model to explore the antitumor effects of mAbs against NY-ESO-1 alone and in combination with an anticancer drug. To select anticancer drugs suitable for this model, we examined the antitumor capacity of several anticancer drugs (5-FU, CPT-11, paclitaxel, and doxorubicin) against CT26-NY-ESO-1. Of the 4 drugs, 5-FU exhibited a significant antitumor effect (Supplementary Fig. S2A). When CT26-NY-ESO-1 cells were cultured with 5-FU, NY-ESO-1 protein was released from CT26-NY-ESO-1 cells into the culture supernatant but not from parental CT26 cells (Supplementary Fig. S2B). On the basis of these data, we chose 5-FU for our further experiments.

Combination treatment with anti-NY-ESO-1 mAb and 5-FU results in augmented tumor growth inhibition

BALB/c mice were inoculated with CT26-NY-ESO-1 and were injected with 5-FU (75 mg/kg) and anti-NY-ESO-1 mAb (clone; E978, 100 μg, 2 days after 5-FU injection) when the tumor was palpable (around 25 mm^2). Treatment was repeated after 1 week. The combination treatment with anti-NY-ESO-1 mAb and 5-FU exhibited a significantly augmented antitumor effect and longer survival compared with control mice or mice that had received either 5-FU or anti-NY-ESO-1 mAb alone (Fig. 1A and B). This augmented antitumor effect was also observed when another anti-NY-ESO-1 mAb (clone; ES121, 100 μg) was used, but not with a control mAb, against another immunogenic CT antigen MAGE-A4, which is not expressed in the CT26-NY-ESO-1 cells (Fig. 1C and D). In contrast, combination treatment with anti-MAGE-A4 mAb (clone; MCV1, 100 μg), but not control antibody and 5-FU, exhibited an augmented antitumor effect against CT26-MAGE-A4 (Fig. 1E). To show that the effect of this combination treatment is not limited to the CT26, we examined the antitumor effect using CMS5a fibrosarcoma cells. BALB/c mice were inoculated with CMS5a-NY-ESO-1 and were injected with doxorubicin (50 μL intratumoral injection, 0.25 mmol/L) and anti-NY-ESO-1 mAb. As systemic administration of doxorubicin did not induce effective killing of CMS5a-NY-ESO-1, we used an intratumoral injection method. This combination treatment with anti-NY-ESO-1 mAb (but not an isotype control antibody) and doxorubicin exhibited a significantly augmented antitumor effect as well (Fig. 1F). These data suggest that the augmented antitumor effect is an antigen-specific phenomenon and that this combination treatment could be applicable to a broader range of intracellular antigens and tumors.

We next investigated whether a cocktail of 2 different anti-NY-ESO-1 mAbs (E978 50 μg and ES121 50 μg) that recognize 2 different nonoverlapping epitopes on the NY-ESO-1 protein (Supplementary Fig. S3) further augmented antitumor effects. We observed no additive antitumor effects when mice were treated with the combination of 2 different anti-NY-ESO-1 mAbs and 5-FU compared with mice treated with a single anti-NY-ESO-1 mAb and 5-FU (Fig. 1G).

Augmented tumor growth inhibition by combination treatment with anti-NY-ESO-1 mAb and 5-FU is dependent on $CD8^+$ T cells

To gain insight into the cellular components involved in the augmented antitumor effects by the combination treatment, we initially examined the role of T cells using BALB/$c^{nu/nu}$ mice. BALB/$c^{nu/nu}$ mice were inoculated with CT26-NY-ESO-1 and combination treatment with 5-FU and anti-NY-ESO-1 mAb was initiated when the tumor was palpable. The augmented antitumor effect by the combination treatment in wild-type BALB/c mice was abrogated in BALB/$c^{nu/nu}$ mice (Fig. 2A).

Given the critical role of T cells in this augmentation of antitumor effects, we next explored the outcome of $CD4^+$/$CD8^+$ T-cell depletion on the augmented antitumor effect. BALB/c mice bearing CT26-NY-ESO-1 tumors were injected with 5-FU and anti-NY-ESO-1 mAb and received anti-CD4 (days 7, 14, and 21) or anti-CD8 mAb (days 7 and 21). The depletion of $CD8^+$ T cells totally abolished the augmented antitumor effects (Fig. 2B). In contrast, $CD4^+$ T-cell depletion did not affect the augmented antitumor effects (Fig. 2B).

Combination treatment with anti-NY-ESO-1 mAb and 5-FU enhances NY-ESO-1–specific $CD8^+$ T-cell induction

Considering a critical role of $CD8^+$ T cells, we examined NY-ESO-1–specific T cells in dLNs. BALB/c mice were inoculated with CT26-NY-ESO-1 and received the combination treatment. dLNs and tumors were harvested on days 14 to 16, and cells were incubated with NY-ESO-1_{81-88} (23) or control peptide, and cytokine secretion was analyzed. Combination treatment with anti-NY-ESO-1 mAb and 5-FU elicited significantly higher numbers of NY-ESO-1–specific $CD8^+$ T cells producing IFN-γ and/or TNF-α than 5-FU alone (Fig. 3A). Furthermore, there was a trend of higher numbers of NY-ESO-1–specific $CD8^+$ T cells in tumors treated with the combination treatment than those treated with 5-FU alone (Fig. 3C).

To explore further differences in NY-ESO-1_{81-88}–specific $CD8^+$ T cells, the effector/memory status was analyzed. The