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Aurora kinase A-specific T-cell receptor gene transfer redirects T lymphocytes to display effective antileukemia reactivity

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Aurora kinase A (AURKA) is overexpressed in leukemias. Previously, we demonstrated that AURKA-specific CD8$^+$ T cells specifically and selectively lysed leukemia cells, indicating that AURKA is an excellent target for immunotherapy. In this study, we examined the feasibility of adoptive therapy using redirected T cells expressing an HLA-A*0201–restricted AURKA$_{207-215}$-specific T-cell receptor (TCR). Retrovirally transduced T cells recognized relevant peptide-pulsed but not control target cells. Furthermore, TCR-redirected CD8$^+$ T cells lysed AURKA-overexpressing human leukemic cells in an HLA-A*0201–restricted manner, but did not kill HLA-A*0201$^+$ normal cells, including hematopoietic progenitors. In addition, AURKA$_{207-215}$-specific *TCR*-transduced CD4$^+$ T cells displayed target-responsive Th1 cytokine production. Finally, AURKA$_{207-215}$-specific *TCR*-transduced CD8$^+$ T cells displayed antileukemia efficacy in a xenograft mouse model. Collectively, these data demonstrate the feasibility of redirected T cell–based AURKA-specific immunotherapy for the treatment of human leukemia. (*Blood*. 2012;119(2):368-376)

Introduction

Aurora kinase A (AURKA) is a member of the serine-threonine kinase family that regulates mitotic cell division from G_2 through to M phase of the cell cycle.[1] The *AURKA* gene maps to chromosome region 20q13.2. AURKA is expressed at low levels in normal cells, including dividing cells, and overexpression of AURKA has clear oncogenic potential.[2,3] Indeed, the *AURKA* gene is overexpressed in various types of cancer,[4] including leukemias.[5,6] Furthermore, correlations between the genetic dysregulation of *AURKA* and susceptibility to cancer, disease status, and prognosis have been described.[4] In particular, *AURKA* gene overexpression correlates with genetic instability and poor differentiation of cancer cells.[7,8] As AURKA expression is tightly regulated in normal tissues and overexpression correlates with malignant transformation, small molecular inhibitors have been developed that selectively target this protein in various tumors. A number of such molecules are currently in early phase clinical trials and preliminary data are encouraging.[9-12]

The overexpression of AURKA in cancer cells, but not in normal tissues, makes it an attractive target for tumor immunotherapy. We have previously shown that testis is the only tissue that expresses detectable levels of AURKA, which suggests that this antigen behaves like cancer/testis antigens.[13] Based on these findings, we previously studied the immunotherapeutic potential of AURKA and identified an HLA-A*0201-restricted antigenic nonamer epitope derived from the kinase domain (residues 207-215). The AURKA$_{207-215}$ epitope (YLILEYAPL) was recognized by CD8$^+$ cytotoxic T lymphocytes (CTLs) generated in vitro.[6] Furthermore, leukemic cells endogenously expressing AURKA were killed by these CTLs, indicating that the cognate epitope is naturally processed and presented in the context of HLA-A*0201 at levels sufficient for immunotherapeutic applications. In addition, Kobayashi and colleagues have identified HLA-class II–restricted AURKA-derived pentadecamer epitopes to which they could generate CD4$^+$ helper T cells that expressed antitumor reactivity.[14]

Immunotherapeutic interventions based on tumor antigen-specific *T-cell receptor* (*TCR*) gene transfer to redirect the specificity of other T cells has shown clinical success in patients with advanced melanoma.[15] However, this approach is complicated by several potential problems: (1) on-target adverse events directed against normal tissues, especially when affinity-enhanced TCRs are used[16]; (2) issues related to chain mispairing between the introduced and endogenous *TCR* α/β genes; and (3) off-target adverse events because of inherent cross-reactivity of the introduced TCR.[17] Although various solutions have been explored to minimize TCR chain mispairing, all current approaches have intrinsic limitations. To this end, we have recently developed a unique vector system that simultaneously delivers *siRNA*s, which specifically down-regulate endogenous *TCR* expression, and a *siRNA*-resistant relevant *TCR* construct (si-*TCR* vector).[18] Furthermore, the likelihood of adverse events related to expression of the introduced TCR may be minimized by the selection of tumor-specific antigens or cancer/testis antigens, rather than tumor-associated antigens. Indeed, a recent clinical study reported that redirected T-cell therapy using NY-ESO-1–specific *TCR* gene

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transfer displayed antitumor efficacy against metastatic melanoma and metastatic synovial cell sarcoma without obvious toxicities mediated by the transferred T cells.[19]

In this study, we examined the antileukemic efficacy and safety of redirected T cells using HLA-A*0201–restricted $AURKA_{207-215}$–specific *TCR* gene transfer both in vitro and in vivo. The data demonstrate the feasibility of this approach for the treatment of human leukemias.

Methods

Cells and cell lines

Approval for this study was obtained from the Institutional Review Board of Ehime University Hospital (Protocol 0909001 and 0909002). Written informed consent was obtained from all patients, healthy volunteers, and parents of cord blood donors in accordance with the Declaration of Helsinki. B-lymphoblastoid cell lines (B-LCLs) were established by transformation of peripheral blood B-lymphocytes with Epstein-Barr virus. GANMO-1 (HLA-$A2^+$), MEG01 (HLA-$A2^-$), MEG01-A2 (*HLA-A*0201* gene-transduced MEG01), OUN-1 (HLA-$A2^-$), and KAZZ (HLA-$A2^-$) leukemia cell lines were cultured in RPMI 1640 with 10% FCS, antibiotics, and L-glutamine. The artificial antigen-presenting cell line C1R-A2 (HLA-$A*0201^+$) was a kind gift from Dr A. John Barrett (National Heart, Lung, and Blood Institute, Bethesda, MD). The Jurkat/MA cell line (kindly provided by Prof Erik Hooijberg, Vrije Universiteit Medisch Centrum, Amsterdam, The Netherlands) is a Jurkat cell subclone that lacks endogenous TCR expression and stably expresses both the human *CD8α* gene (*hCD8α*) and an *NFAT-luciferase* gene construct for the detection of signaling via newly introduced TCRs.[20] PBMCs and bone marrow mononuclear cells (BMMCs) from leukemia patients and healthy volunteers, and cord blood mononuclear cells (CBMCs) from healthy donors, were isolated by density gradient centrifugation and stored in liquid nitrogen until use. $CD34^+$ cells from CBMCs were isolated using $CD34^+$ cell-isolating immunomagnetic beads (Miltenyi Biotec).

Synthetic peptides and HLA-A*0201/peptide tetrameric complexes

The HLA-A*0201–restricted $AURKA_{207-215}$ nonameric peptide (YLILEYAPL) was purchased from Thermo Electron (Greiner Bio-One). Biotin-tagged soluble HLA-A*0201/AURKA monomers were produced as previously described.[21] Fluorochrome-labeled tetrameric complexes were generated by conjugation to streptavidin-PE (Prozyme) at a molar ratio of 4:1.[22] HLA-A*0201 tetramers were also produced with the HIV-1 p17 Gag-derived peptide epitope SL9 (SLYNTVATL, residues 77-85) for control purposes.

Generation of an $AURKA_{207-215}$-specific CTL clone

A novel $AURKA_{207-215}$-specific CTL clone designated AUR-2 was generated as previously described.[23] Briefly, monocyte-derived dendritic cells (Mo-DCs) were generated from $CD14^+$ PBMCs using 10 ng/mL recombinant human IL-4 and 75 ng/mL recombinant human GM-CSF (R&D systems), then matured with 100 U/mL recombinant human TNF-α (Dainippon Pharmaceutical). $CD8^+$ T cells (1×10^5) were stimulated with 10^4 autologous mature $AURKA_{207-215}$ peptide-loaded (10μM) Mo-DCs in a 96-well round-bottomed plate. One week later, the $CD8^+$ T cells were restimulated similarly, and 10 U/mL recombinant human IL-2 (Roche) was added after a further 4 days. Thereafter, $CD8^+$ T cells were restimulated weekly with 10^5 autologous $AURKA_{207-215}$ peptide-pulsed (10μM) PBMCs treated with mitomycin-C (MMC; Kyowa Hakko). Epitope-dependent target cell cytotoxicity was examined using standard ^{51}chromium (^{51}Cr)–release assays.

ELISPOT

ELISPOT assays were conducted as previously described.[22] Briefly, 96-well flat-bottomed MultiScreen-HA plates with a nitrocellulose base (Millipore) were coated overnight at 4°C with 10 μg/mL anti-IFN-γ monoclonal antibody (mAb; R&D Systems). After washing with PBS, cultured $CD8^+$ T cells were stimulated with 5×10^4 $AURKA_{207-215}$ peptide-pulsed (1μM) or unpulsed C1R-A2 cells/well for 20 hours at 37°C in a 5% CO_2 atmosphere. Subsequently, the wells were vigorously washed with PBS/0.05% tween 20 and incubated with polyclonal rabbit anti–IFN-γ Ab (Endogen) for 90 minutes at room temperature. The wells were then washed again and incubated for 90 minutes with peroxidase-conjugated goat anti–rabbit IgG Ab (Zymed). Spots were visualized by the addition of 100 μL substrate, comprising 0.1M sodium acetate buffer (pH 5.0) containing 3-amino-9-ethylcarbazole (Sigma-Aldrich) and 0.015% H_2O_2, for 40 minutes at room temperature and counted under a light microscope.

Cloning of full-length *TCR* α and β chain genes from the AUR-2 CTL clone and retroviral vector construction

Total RNA was extracted from the HLA-A*0201–restricted $AURKA_{207-215}$-specific CTL clone AUR-2 using the FastPure RNA Kit (Takara Bio) according to the manufacturer's instructions. Full-length *TCR* α and β genes were cloned as previously described.[24] Briefly, cDNA was amplified using a 5′-RACE primer and 3′-constant region primers as follows: (1) 5′-TCAGCTGGACCACAGCCGCAGCGT-3′ for TCR Cα; (2) 5′-TCAGAAATCCTTTCTCTTGAC-3′ for TCR Cβ1; and (3) 5′-CTAGCCTCTGGAATCCTTTCTCTT-3′ for TCR Cβ2. The conditions for PCR were: one cycle at 94°C for 3 minutes, followed by 30 cycles at 94°C for 40 seconds, 58°C for 40 seconds and 72°C for 1 minute, with a final extension phase at 72°C for 5 minutes. Each *TCR* α and β chain amplicon was cloned into the pMD20 TA cloning vector (Takara Bio), and sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and an ABI 3730xl sequencer (Applied Biosystems). Full-length *TCR* α and β genes were then cloned into the bicistronic pMS3 retroviral vector, which is a pME1-5 derivative that contains the murine stem cell virus (MSCV) LTR (Takara Bio) in place of the 3′LTR (pMS3-*AURKA-TCR*, Figure 1). Ecotropic retrovirus particles were obtained by transient transfection of HEK293 T cells with the TCR construct and helper plasmids (pGP vector and pE-eco vector; Takara Bio). GaLV-pseudotyped retrovirus particles were obtained by consecutive transfection into PG13 cells. The pMS3-*AURKA-TCR* GaLV-pseudotyped retroviruses were used for AURKA-specific *TCR* α and β gene transduction.

Transduction of $AURKA_{207-215}$-specific *TCR genes*

Jurkat/MA cells and healthy donor T cells were genetically modified to express the AURKA-specific TCR using RetroNectin (Takara Bio) as previously described.[18] Briefly, 1×10^6 healthy donor T cells per well in GT-T503 (Takara Bio) with 5% human serum, 0.2% human albumin, 50 U/mL recombinant human IL-2 (R&D Systems), 5 ng/mL recombinant human IL-7 (R&D Systems), 10 ng/mL recombinant human IL-15 (PeproTech Inc), and 100 ng/mL recombinant human IL-21 (Shenandoah Biotechnology Inc) were added on day 1 to a 24-well culture plate pretreated with antihuman CD3 mAb (BioLegend). Jurkat/MA cells were cultured in IMDM with 8% FCS and 50 μg/mL hygromycin B (Invitrogen). On day 3, cultured T cells or Jurkat/MA cells were transferred on to a retrovirus-preloaded RetroNectin-coated 24-well plate, centrifuged at 2000g for 2 hours and rinsed with PBS. Cells were then applied to the retrovirus-preloaded RetroNectin-coated 24-well plate again for the second transduction. AURKA-specific *TCR*-transduced T cells were stimulated weekly with MMC-treated C1R-A2 cells loaded with $AURKA_{207-215}$ peptide (1μM) for further functional experiments.

Flow cytometric analysis

The AUR-2 CTL clone expresses the *TRBV10-3* gene, denoted in IMGT nomenclature.[25] This corresponds to TCR Vβ12 in the Arden nomenclature.[26] Accordingly, anti-TCR Vβ12 mAb was used to detect $AURKA_{207-215}$-specific *TCR*-transduced cells. After 4 to 6 days, transduced cells were analyzed by flow cytometry using anti-TCR Vβ12-FITC (Beckman Coulter), anti-CD8–FITC (BD Biosciences) or anti-CD8–PE (BioLegend), and HLA-A*0201/$AURKA_{207-215}$ tetramer-PE (only with anti-CD8–FITC).

Intracellular expression of Foxp3 and $AURKA_{207-215}$–responsive IFN-γ production by $AURKA_{207-215}$–specific *TCR*-transduced $CD4^+$ T cells were analyzed using anti–Foxp3-PE (e-Bioscience) and anti–IFN-γ–FITC (BD Biosciences). Data were acquired using a FACS Calibur flow cytometer and analyzed with either Cell Quest (BD Biosciences) or FlowJo Version 7.2.2 software (TreeStar Inc).

CFSE dilution assay

To measure epitope-responsive proliferation of $AURKA_{207-215}$–specific *TCR*-transduced $CD8^+$ T cells in the presence or absence of similarly redirected $CD4^+$ T cells, $CD8^+$ T cells were labeled with CFSE (Molecular Probe Inc) as described previously.[27] After 3 days, CFSE dilution within the $CD8^+$ T-cell population was assessed by flow cytometry.

Epitope-responsive luciferase production by $AURKA_{207-215}$–specific *TCR*-transduced Jurkat/MA cells

To verify the functionality of the cloned $AURKA_{207-215}$–specific *TCR* α and β chains, we used the TCR^- Jurkat/MA cell line, which stably expresses *hCD8α* and an *NFAT-luciferase* reporter gene (Jurkat/MA/CD8α/luc), as follows. pMS3-*AURKA-TCR* was retrovirally transduced into Jurkat/MA/CD8α/luc cells. Cells expressing TCR Vβ12 were isolated for functional analysis. Briefly, HLA-A*0201$^+$ B-LCL cells were loaded with titrated doses of $AURKA_{207-215}$ peptide or the irrelevant SL9 peptide (10μM; HIV-1 p17 Gag, residues 77-85) and used to stimulate 8×10^5 *TCR* gene-modified Jurkat/MA/CD8α/luc cells (effector:target ratio 2:1) for 12 hours. The cells were then lysed and subjected to luciferase assay using the PicaGene-Dual-SeaPansy Kit (TOYOinki) according to manufacturer's instructions. Luciferase activity was measured using a Lumicounter700 (MicrotecNition).

IFN-γ secretion assay

$AURKA_{207-215}$–specific *TCR*-transduced $CD4^+$ or $CD8^+$ T cells (5×10^5) were incubated with 10^5 $AURKA_{207-215}$ peptide-pulsed (1μM) or unpulsed C1R-A2 cells for 24 hours. For the inhibition assay, cells were cultured in the presence of either an anti-HLA class I framework mAb (w6/32; ATCC) or a control anti–HLA-DR mAb (L243; ATCC). Cytokine production patterns were assessed using a bead-based immunoassay kit (Becton Dickinson). IFN-γ in the culture supernatant was measured using an ELISA kit (Pierce) according to the manufacturer's instructions. Streptavidin-HRP was used for color development, and luminointensity was measured using IMMUNO-MINI (NJ-2300; Microtec).

Cytotoxicity assay

Standard ^{51}Cr release assays were performed as previously described.[28] Briefly, 10^4 unpulsed or peptide-pulsed target cells were labeled with ^{51}Cr ($Na_2{}^{51}CrO_4$; MP Bio Japan) and incubated at various ratios with effector cells in 200 μL of culture medium in 96-well round-bottomed plates. To assess HLA class I restriction, target cells were incubated with 10 μg/mL w6/32 mAb or the control L243 mAb for 1 hour, then incubated with effector cells for 5 hours. After incubation, 100 μL supernatant was collected from each well to measure ^{51}Cr release. The percentage specific lysis was calculated as: (experimental release cpm − spontaneous release cpm)/(maximal release cpm − spontaneous release cpm) × 100 (%).

Quantitative analysis of *AURKA* mRNA expression

Quantitative real-time PCR (qRT-PCR) for *AURKA* mRNA was performed as described previously.[6] Briefly, total RNA was extracted using an RNeasy Mini Kit (QIAGEN) and cDNA was synthesized. qRT-PCRs for *AURKA* mRNA (Hs00269212_m1) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA (4326317E) as an internal control were performed using the TaqMan Gene Expression assay (Applied Biosystems) in accordance with the manufacturer's instructions and an ABI Prism 7700 Sequence Detection System (Applied Biosystems). The expression level of *AURKA* mRNA was corrected by reference to that of *GAPDH* mRNA, and the relative amount of *AURKA* mRNA in each sample was calculated by the comparative ΔCt method.

AURKA protein expression analysis by Western blotting

For the analysis of protein expression, Western blotting was performed as described previously.[6] Briefly, cell lysates were subjected to 10% SDS-PAGE (e-PAGEL, ATTO) and blotted onto PVDF membranes (Bio-Rad Laboratories). The blots were incubated first with anti-AURKA mouse mAb (Abcam), then with HRP-conjugated anti–mouse IgG (GE Healthcare). The probed proteins were visualized using an enhanced chemiluminescence system (GE Healthcare). Subsequently, the blotted membranes were stripped and reprobed with anti–β-actin mouse mAb (Sigma-Aldrich) to confirm equivalent protein loading between samples.

Antileukemia effect of $AURKA_{207-215}$–specific *TCR*-transduced T cells in xenograft mouse models

All in vivo experiments were approved by the Ehime University animal care committee. For the Winn assay, 5×10^6 GANMO-1 cells and 2.5×10^7 $AURKA_{207-215}$–specific *TCR* gene-transduced or non–gene-modified $CD8^+$ T cells were inoculated per mouse (n = 4 per group). The cells were suspended in 300 μL PBS and injected subcutaneously into the left flank of NOG mice (Non-Obese Diabetic/Severe Combined Immuno-Deficiency/IL-2 receptor γ-chain allelic mutation; *NOD*/Shi-*scid*/IL-2R γ^{null})[29] aged 5-6 weeks (Central Institute for Experimental Animals). Mice were subsequently injected intravenously with either 5×10^6 $AURKA_{207-215}$–specific *TCR* gene-modified cells, AUR-2 cells or unmodified $CD8^+$ T cells, as per the initial inoculation, on a weekly basis for a total of 5 infusions. Tumor size was measured every 5 days until the mice died or were euthanized because of tumor progression.

For adoptive transfer experiments, NOG mice aged 9 weeks were similarly inoculated with 5×10^6 of GANMO-1 cells. Intravenous administration of either 5×10^6 $AURKA_{207-215}$–specific *TCR* gene-transduced or non–gene-modified $CD8^+$ T cells commenced on the same day (day 0), and was continued on a weekly basis thereafter until the mice died or were euthanized because of tumor progression.

Statistical analysis

The paired *t* test was used to assess differences between groups; a P value $< .05$ was considered significant.

Results

Generation of a novel HLA-A*0201–restricted $AURKA_{207-215}$–specific CTL clone (AUR-2) and retroviral expression of the full-length *TCR* α and β genes

Characteristics of the newly established HLA-A*0201-restricted $AURKA_{207-215}$–specific CTL clone (AUR-2) are shown in Figure 1. AUR-2 was stained uniformly with the HLA-A*0201/$AURKA_{207-215}$ tetramer, but not with the irrelevant HLA-A*0201/Gag_{77-85} tetramer (Figure 1A). In cytotoxicity assays, AUR-2 displayed moderate levels of functional sensitivity in response to cognate peptide (Figure 1B). Epitope-dependent production of IFN-γ was confirmed in ELISPOT assays (Figure 1C). Peptide specificity and HLA restriction were further demonstrated in cytotoxicity assays with different target cells (Figure 1D). In addition, AUR-2 lysed the HLA-A*0201$^+$ leukemia cell line GANMO-1, which overexpresses *AURKA* mRNA, but not the HLA-A*0201$^-$ negative cell lines MEG01 and K562, both of which also express *AURKA* mRNA at high levels (Figure 1E). The rearranged *TCR* α and β genes of AUR-2 were sequenced and found to comprise the germ line gene segments *TRAV3/TRAJ20/TRAC* and *TRBV10-3/TRBJ1-1/TRBC1*, respectively; both full-length genes were cloned into a novel bicistronic retroviral vector (Figure 1F).

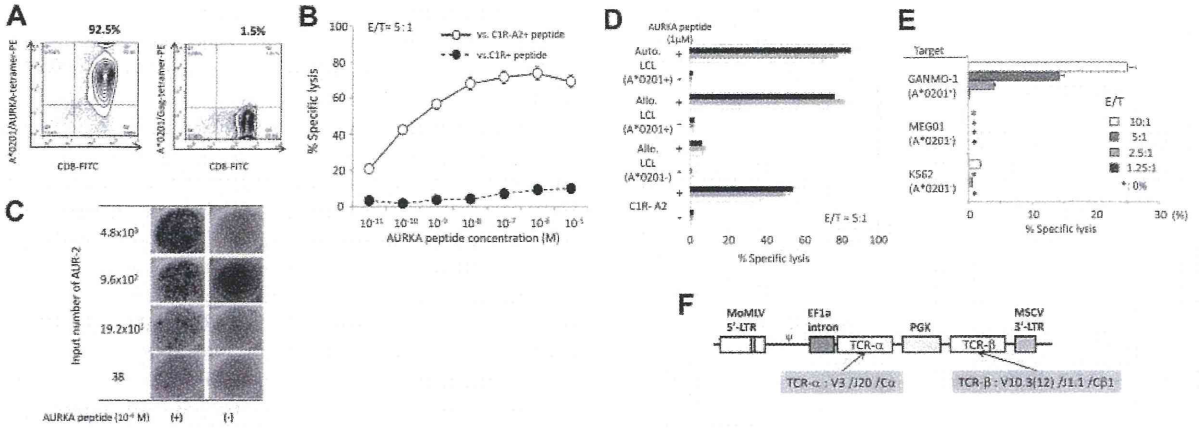

Figure 1. Characteristics of the AURKA$_{207-215}$–specific CTL clone AUR-2. (A) Representative flow cytometry plots showing staining of AUR-2 with the HLA-A*0201/AURKA$_{207-215}$ tetramer (left) and the irrelevant HLA-A*0201/Gag$_{77-85}$ tetramer (negative control; right). (B) The cytotoxic activity of AUR-2 was measured in ^{51}Cr-release assays against C1R-A2 or C1R (negative control) cells loaded with a range of AURKA$_{207-215}$ peptide concentrations as indicated. E/T indicates effector:target ratio. (C) IFN-γ ELISPOT assays were conducted using C1R-A2 target cells loaded with 1 μM AURKA$_{207-215}$ peptide and AUR-2 CTL at different input numbers as shown. (D) ^{51}Cr-release assays were conducted using AUR-2 CTL with unpulsed or AURKA$_{207-215}$ peptide-pulsed (1 μM) HLA-A*0201$^+$ autologous or allogeneic B-LCLs, C1R-A2 cells or HLA-A*0201$^-$ allogeneic B-LCLs as indicated. E/T indicates effector:target ratio. (E) The cytotoxic activity of AUR-2 CTL against the indicated leukemia cell lines was measured in ^{51}Cr-release assays. GANMO-1, HLA-A*0201$^+$; MEG01 and K562, HLA-A*0201$^-$. Expression of *AURKA* mRNA and AURKA protein in these leukemia cell lines is shown in supplemental Figure 2. E/T indicates effector:target ratio. (F) Construction of a novel retroviral vector encoding full-length AURKA–specific *TCR* α and β genes derived from AUR-2. MoMLV indicates Moloney murine leukemia virus; LTR, long terminal repeat; EF1a, elongation factor 1a; PGK, phosphoglycerate kinase promoter; and MSCV, murine stem cell virus. Error bars represent SDs.

Functional reconstitution of the AURKA$_{207-215}$–specific TCR heterodimer in Jurkat/MA cells

To validate the functionality of the cloned *TCR* genes, both chains were expressed in the TCR$^-$ cell line Jurkat/MA/CD8α/luc, which contains a luciferase reporter gene to monitor TCR signaling (Figure 2A). AUR-2*TCR*–transduced, Vβ12-selected Jurkat/MA/CD8α/luc cells (Figure 2B) were incubated with C1R-A2 cells pulsed with a range of AURKA$_{207-215}$ peptide concentrations, then assayed for luciferase activity. The *TCR* gene-modified Jurkat/MA/CD8α/luc cells produced luciferase in response to stimulation with AURKA$_{207-215}$ peptide-loaded C1R-A2 cells in a dose-dependent manner (Figure 2C). Compared with the parental AUR-2 CTL clone (Figure 1B), the *TCR*-transduced Jurkat/MA cells displayed low levels of peptide sensitivity. To address this functional discrepancy, we assessed cell-surface expression of TCR α/β, CD3, CD8α, CD11a, and CD28 (supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). The *TCR*-transduced Jurkat/MA cells expressed lower surface levels of TCR α/β, CD3 and CD8α compared with both similarly activated normal CD8$^+$ T cells and the parental AUR-2 CTL clone. Furthermore, CD11a and CD28 were almost absent from the transfectant cells. These findings may explain the observed differences in functional sensitivity between AUR-2 *TCR*-transduced Jurkat/MA cells and the parental CTL clone.

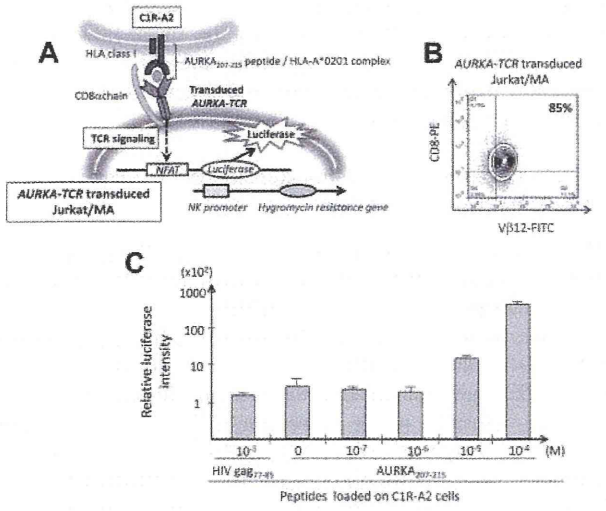

Figure 2. Functional retroviral expression of the AURKA$_{207-215}$–specific TCR. (A) Schematic representation of the luciferase assay using AURKA$_{207-215}$–specific *TCR*-transduced Jurkat/MA cells. NFAT indicates nuclear factor activated T cells; and NK, natural killer. (B) AURKA$_{207-215}$–specific *TCR*-transduced Jurkat/MA cells express Vβ12 but label poorly with cognate tetramer (data not shown), probably because of the low levels of surface CD8α expression. (C) AURKA$_{207-215}$–specific *TCR*-transduced Jurkat/MA cells were stimulated with peptide-pulsed C1R-A2 cells as shown and subjected to luciferase assay. Error bars represent SDs.

AURKA$_{207-215}$–specific *TCR* gene-transduced CD8$^+$ T cells exert antileukemia reactivity in vitro

Next, the AURKA$_{207-215}$–specific *TCR* was retrovirally introduced into normal CD8$^+$ T cells. Transduction efficiency determined by Vβ12 staining of *TCR* gene-modified T cells was 50%-70% (data not shown), and 20%-25% of the Vβ12$^-$ cells stained with the HLA-A*0201/AURKA$_{207-215}$ tetramer (Figure 3A). Isolated Vβ12$^+$ AURKA$_{207-215}$–specific *TCR* gene-transduced CD8$^+$ T cells displayed similar antigen sensitivity to the parental AUR-2 CTL clone (Figure 3B-C). Notably, however, the AURKA$_{207-215}$–specific *TCR* transductants produced higher quantities of IFN-γ in response to the same peptide-pulsed C1R-A2 targets (Figure 3C). On the basis of these observations, further experiments were carried out using these AURKA$_{207-215}$–specific *TCR* gene transfectants.

AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells displayed HLA class I–restricted, peptide-dependent IFN-γ production (Figure 3D), and target epitope–specific cytotoxic activity (Figure 3E). Furthermore, these redirected CD8$^+$ T cells selectively lysed the HLA-A*0201$^+$ leukemia cell line GANMO-1, which overexpresses AURKA, but not the HLA-A*0201$^-$ leukemia cell lines, MEG01, KAZZ, and OUN-1, which also overexpress AURKA (Figure 4A, supplemental Figure 2). In contrast,

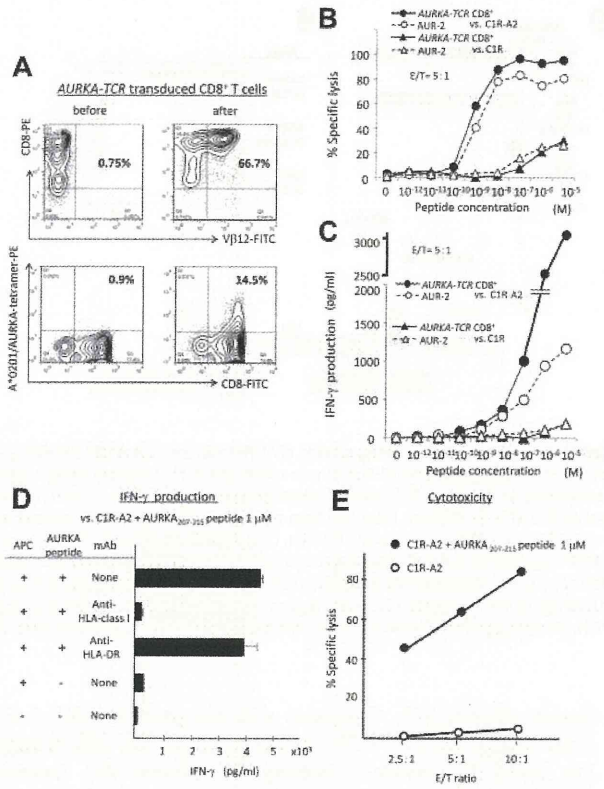

Figure 3. AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells display epitope-specific functionality. (A) Representative flow cytometry plots showing staining of AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells with anti-TCR Vβ12 mAb and HLA-A*0201/AURKA$_{207-215}$ tetramer. (B) The same AURKA$_{207-215}$ *TCR*-transduced CD8$^+$ T cells shown in panel A were tested in ^{51}Cr-release assays against C1R (negative control) and C1R-A2 cells pulsed with the indicated concentrations of AURKA$_{207-215}$ peptide. The parental AUR-2 CTL clone was tested in parallel. E/T indicates effector:target ratio. (C) IFN-γ production by AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells was measured in a similar format to that described for panel B. (D) Effects of HLA class I and class II blockade on the production of IFN-γ by AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells stimulated with cognate peptide-loaded (1μM) C1R-A2 cells. (E) Cytotoxic activity of AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells against unpulsed or cognate peptide-loaded (1μM) C1R-A2 cells as a function of effector:target (E/T) ratio. Error bars represent SDs.

HLA-A*0201$^+$ PBMCs (n = 3), PHA-stimulated lymphoblasts representing highly mitotic normal cells (n = 3), and normal cord blood CD34$^+$ cells (CB-CD34$^+$cells; n = 2) were not lysed by these AURKA$_{207-215}$–specific *TCR* transductants (Figure 4B). *AURKA* mRNA expression relative to K562 for each group (mean ± SD) was 0.02 ± 0.008 for PBMCs, 0.25 ± 0.005 for PHA-lymphoblasts and 0.21 ± 0.09 for CB-CD34$^+$ cells, which indicated relatively low expression levels of *AURKA* mRNA among these cells compared with K562. The cytotoxic activity of AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells against GANMO-1 and cognate peptide-loaded B-LCLs was significantly diminished by an anti-HLA class-1 mAb but not by an anti–HLA-DR mAb (Figure 4C-D). To confirm recognition of the endogenously processed AURKA$_{207-215}$ epitope in the context of HLA-A*0201 expressed by leukemia cells, we retrovirally transduced the *HLA-A*0201* gene into MEG01 cells (MEG01-A2; Figure 4E). Parental MEG01 cells do not express HLA-A*0201, but abundantly overexpress both *AURKA* mRNA and AURKA protein (supplemental Figure 2). Compared with MEG01, MEG01-A2 were susceptible to the cytotoxic effects of AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells (Figure 4F). Collectively, these data indicate that the antileukemia reactivity mediated by AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells occurs through the recognition of endogenously processed and presented AURKA$_{207-215}$ peptide in the context of HLA-A*0201 on the surface of leukemia cells.

Next, the antileukemia reactivity mediated by these redirected AURKA$_{207-215}$–specific CD8$^+$ T cells was tested against freshly isolated leukemia cells in vitro (Figure 5). *AURKA* mRNA was overexpressed in all 6 leukemia samples as determined by qRT-PCR. Leukemia cells isolated from HLA-A*0201$^+$ patients (1-3), but not HLA-A*0201$^-$ patients (4-6) were lysed by the AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells. Thus, our data show that the AUR-2 TCR confers AURKA$_{207-215}$ specificity to donor CD8$^+$ T cells transduced with both TCR chains, and that normal cells, including actively cycling cells and hematopoietic progenitor cells, are not lysed by these redirected T cells.

AURKA$_{207-215}$–specific *TCR*-transduced CD4$^+$ T cells respond to cognate antigen

Next, we examined antigen reactivity in isolated populations of Vβ12$^+$ AURKA$_{207-215}$–specific *TCR*-transduced CD4$^+$ T cells (Figure 6A). AURKA$_{207-215}$–specific *TCR*-transduced CD4$^+$ T cells successfully produced IFN-γ in response to stimulation with AURKA$_{207-215}$ peptide-loaded C1R-A2 cells; this response was substantially reduced by HLA class I blockade, and a partial response reduction was also observed with HLA class II blockade (Figure 6B). These redirected CD4$^+$ T cells did not express Foxp3, which is a key molecular signature of regulatory T cells (supplemental Figure 3A), and the cognate antigen-specific proliferative response of AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells was actually enhanced in the presence of redirected CD4$^+$ T cells but not in the presence of non–gene-modified CD4$^+$ T cells (supplemental Figure 3B). Furthermore, AURKA$_{207-215}$–specific *TCR*-transduced CD4$^+$ T cells produced significant amounts of IL-2, TNF-α, and IFN-γ, but not IL-4 or IL-10 (supplemental Figure 4).

These observations suggest that AURKA$_{207-215}$–specific *TCR*-transduced CD4$^+$ T cells might be able to function as epitope-specific Th1 helper T cells, and that the interaction between T cell–expressed CD4 and target cell-expressed HLA class II molecules facilitates HLA class I–restricted AURKA$_{207-215}$–specific IFN-γ production.

AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells exhibit antileukemia reactivity in vivo

The in vivo antileukemia reactivity of AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells was assessed using the Winn assay and a therapeutic adoptive transfer model.

In the Winn assay, NOG mice were initially coinjected with GANMO-1 cells (5×10^6) and either 2.5×10^7 AURKA$_{207-215}$-specific *TCR* gene-modified or non–gene-modified CD8$^+$ T cells; 5 weekly infusions of the respective CD8$^+$ T-cell populations (5×10^6 cells per infusion) were subsequently administered. Treatment with AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells completely prohibited the engraftment and growth of inoculated leukemia cells for more than 2 months (Figure 7A), and significantly prolonged survival (Figure 7B). Similar results were obtained with AUR-2 cells in a parallel regimen (supplemental Figure 5). In contrast, non–gene-modified CD8$^+$ T cells did not prohibit leukemia growth. In a therapeutic adoptive transfer model, intravenously injected AURKA$_{207-215}$–specific *TCR*-transduced

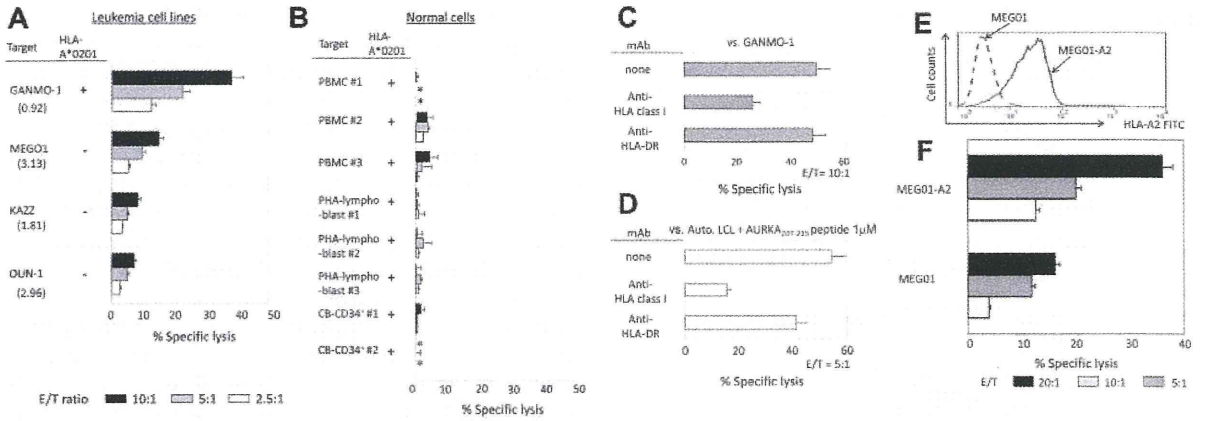

Figure 4. AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells can distinguish leukemia cells from normal cells on the basis of AURKA expression levels. (A) AURKA$_{207-215}$–specific *TCR*-transduced CD8$^-$ T cells exhibit antileukemia reactivity in an HLA-A*0201-dependent fashion. The HLA-A*0201$^+$ leukemia cell line GANMO-1 was lysed by AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells as a function of effector:target (E/T) ratio; no significant lysis was observed with the HLA-A*0201$^-$ leukemia cells lines MEG01, KAZZ and OUN-1. All of the tested leukemia cell lines overexpress *AURKA* mRNA; numbers in parentheses indicate *AURKA* mRNA expression relative to K562, and correlations with AURKA protein expression are shown in supplemental Figure 2. (B) The same AURKA$_{207-215}$–specific *TCR*-transduced CD8$^-$ T cells used in panel A at the same E/T ratios were tested in ^{51}Cr-release assays for potentially damaging effects against normal cells. No significant lysis was observed with HLA-A*0201$^+$ PBMCs (n = 3), PHA-lymphoblasts representing normal mitotic cells (n = 3) or normal cord blood–derived CD34$^+$ cells (CB-CD34$^+$) encompassing normal hematopoietic progenitor cells (n = 2). *AURKA* mRNA expression relative to K562 was 0.02 ± 0.008 for PBMCs, 0.25 ± 0.005 for PHA-lymphoblasts and 0.21 ± 0.09 for CB-CD34$^+$ cells (* indicates less than detectable). (C) Effects of HLA class I and class II blockade on the cytotoxic activity of AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells against GANMO-1 leukemia cells. E/T, effector:target ratio. (D) As for panel C, showing the effects of HLA class I and class II blockade on the lysis of autologous B-LCLs loaded with AURKA$_{207-215}$ peptide (1μM). (E) Flow cytometric confirmation of HLA-A*0201 expression by MEG01-A2 cells. (F) Enhanced lysis of MEG01-A2 cells relative to parental MEG01 cells by AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells confirms recognition of endogenously processed AURKA$_{207-215}$ peptide presented in the context of HLA-A*0201. E/T indicates effector:target ratio. Error bars represent SDs.

CD8$^+$ T cells, but not non–gene-modified CD8$^+$ T cells, significantly suppressed the growth of inoculated leukemia cells in vivo ($P < .02$; Figure 7C). Statistically significant tumor suppression was achieved on day 65, after 10 adoptive infusions. Thereafter, all mice (n = 4) treated with non–gene-modified CD8$^+$ T cells died by day 85. 2 mice treated with AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells died from other causes (1 on day 45 and 1 on day 70); the other 2 mice in this group survived longer than 90 days and were finally euthanized because of disease progression. Collectively, these observations indicate that AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells exhibit antileukemia reactivity in vivo.

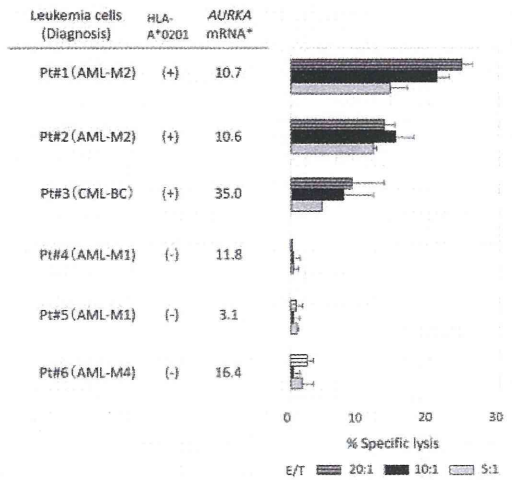

Figure 5. AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells kill freshly isolated leukemia cells in vitro. Freshly isolated HLA-A*0201$^-$ (n = 3) or HLA-A*0201$^-$ (n = 3) acute or chronic myeloid leukemia cells overexpressing *AURKA* mRNA were used as targets in ^{51}Cr-release assays with AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells at the indicated effector:target (E/T) ratios. AML, acute myeloid leukemia; BC, blast crisis; CML, chronic myeloid leukemia. M1, M2, and M4 refer to French-American-British classification subtypes (* indicates the expression of *AURKA* mRNA relative to the mean expression levels across 5 PBMC samples from healthy donors was determined by qRT-PCR and calculated using the comparative ΔCt method). Error bars represent SDs.

Discussion

In the setting of hematologic malignancies, *TCR* gene therapy targeting WT1 in leukemia,[30] and *chimeric antigen receptor* (*CAR*) gene therapy targeting CD33 in myeloid leukemias[31] and CD19, CD20, CD22, CD30, and the receptor tyrosine kinase-like orphan receptor 1 (ROR1) in B-cell malignancies,[32-38] are currently being investigated in preclinical studies or in early phase clinical trials.

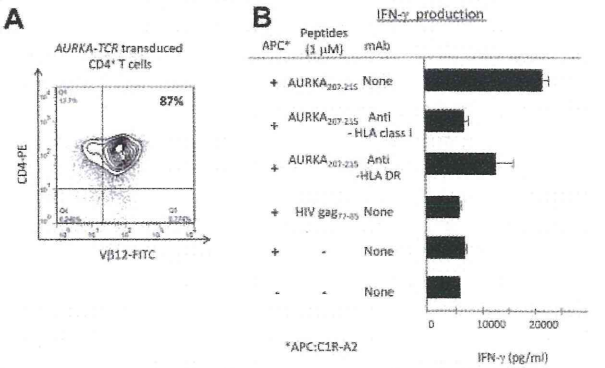

Figure 6. AURKA$_{207-215}$–*TCR* transduced CD4$^+$ T cells display antigen-specific Th1 cytokine production. (A) A representative flow cytometry plot showing surface Vβ12 expression by AURKA$_{207-215}$–specific *TCR*-transduced CD4$^+$ T cells. (B) *AURKA$_{207-215}$–TCR* transduced CD4$^+$ T cells produce IFN-γ in response to cognate peptide-loaded (1μM) C1R-A2 cells; unpulsed or irrelevant (HIV p17 Gag SL9) peptide-pulsed C1R-A2 cells were used as negative controls. Cognate antigen-specific IFN-γ production was reduced to background levels in the presence of anti-HLA class I blocking mAb and inhibited in the presence of anti-HLA class II blocking mAb. APC, antigen-presenting cell. Error bars represent SDs.

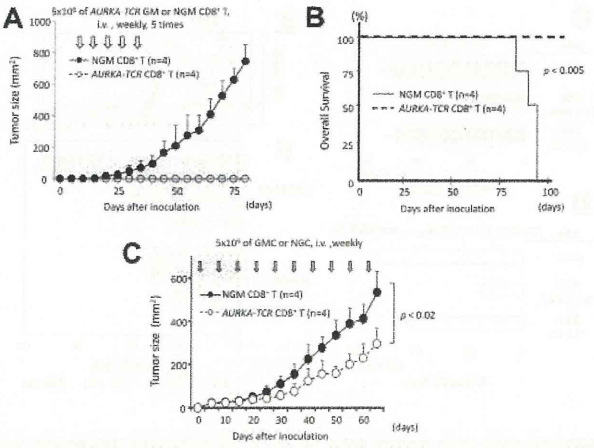

Figure 7. AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells mediate antileukemia reactivity in vivo. (A) Winn assay: tumor suppression curve. NOG mice were coinjected with GANMO-1 cells (5×10^6) and either 2.5×10^7 AURKA$_{207-215}$–specific *TCR* gene-modified (*AURKA-TCR*) or non–gene-modified (NGM) CD8$^+$ T cells (n = 4 group). Subsequently, 5 weekly infusions of the respective CD8$^+$ T-cell populations (5×10^6 cells per infusion) were administered intravenously. Tumor growth was monitored every 5 days. (B) Winn assay: survival curve. Treatment with AURKA$_{207-215}$–specific *TCR* gene-modified (*AURKA-TCR*) CD8$^+$ T cells significantly prolonged survival ($P < .005$). (C) Therapeutic adoptive transfer model. NOG mice (n = 4 per group) were inoculated with 5×10^6 of GANMO-1 cells. Intravenous administration of either 5×10^6 AURKA$_{207-215}$–specific *TCR* gene-modified (*AURKA-TCR*) or non–gene-modified (NGM) CD8$^+$ T cells commenced on the same day and was continued weekly thereafter. Therapeutic infusions of AURKA$_{207-215}$–specific *TCR* gene-modified CD8$^+$ T cells significantly suppressed tumor growth ($P < .02$). Error bars represent SDs.

Although adoptive antileukemia/lymphoma therapy with redirected T cells using tumor antigen-specific *TCR* or *CAR* gene transfer remains in its infancy, emerging evidence supports the development of such therapeutic options.

A number of preclinical and clinical studies of tumor antigen-specific *TCR* gene therapy have underscored the fact that appropriate antigen selection is essential to minimize the likelihood of on-target adverse events mediated by redirected T cell recognition of normal tissues expressing self-derived specificities.[39] This concept is further supported by a recent study of NY-ESO-1–specific *TCR* gene transfer.[19] In this report, objective clinical responses were observed in 5 of 11 patients with metastatic melanoma and 4 of 6 patients with metastatic synovial cell sarcoma without any toxicity related to engineered T cell activity.[19] Thus, the exploration of novel tumor antigens to identify safe and effective targets for *TCR* gene therapy is warranted, especially in the context of hematologic malignancies.

Previously, we reported a significant correlation between the overexpression of *AURKA* mRNA and the aggressiveness of lymphoma cells.[13] Furthermore, we found that *AURKA* mRNA is overexpressed in a large proportion of freshly isolated human leukemia cells.[6] However, in normal tissues, *AURKA* mRNA expression is largely limited to the testis.[13] Subsequently, we identified an immunogenic nonamer epitope derived from AURKA that was presented in the context of HLA-A*0201.[6] In the present study, we set out to examine the feasibility of redirected T cell–based adoptive immunotherapy for the treatment of human leukemia using a TCR derived from an HLA-A*0201–restricted AURKA$_{207-215}$–specific CD8$^+$ T-cell clone (AUR-2). Expression of this TCR in CD8$^+$ T cells conferred antileukemia reactivity both in vitro and in a xenogeneic mouse model of human leukemias. Furthermore, CD4$^+$ T cells could be redirected using this TCR to recognize the same HLA-A*0201–restricted AURKA$_{207-215}$ epitope. This represents a potentially important advantage, as the same TCR could redirect both helper (CD4$^+$) and cytotoxic (CD8$^+$) functions within the transduced T-cell population, which might sustain the antileukemia response in vivo after adoptive transfer.

Redirected CD8$^+$ T cells expressing the *TCR* cloned from AUR-2 displayed similar levels of functional sensitivity to the parental CTL clone. In vitro, AURKA$_{207-215}$–specific *TCR* gene-transduced CD8$^+$ T cells were able to lyse HLA-A*0201$^+$ human leukemia line GANMO-1 cells, which overexpress *AURKA* mRNA, and freshly isolated leukemia cells from HLA-A*0201$^+$ patients. This antileukemia reactivity was implemented through recognition of the endogenously processed AURKA$_{207-215}$ epitope presented in the context of HLA-A*0201. Importantly, these AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells did not lyse HLA-A*0201$^+$ normal PBMCs, mitotic PHA-lymphoblasts or cord blood CD34$^+$ cells; these data suggest that on-target adverse effects would be minimal in clinical applications. Furthermore, we demonstrated the efficacy of AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells in vivo by showing the inhibition of leukemia cell growth in a xenograft mouse model. As many hematopoietic progenitor cells actively proliferate and will therefore have enhanced AURKA expression levels, these cells may become targets for AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells in vivo, as is the case with selective AURKA inhibitors.[40] However, our observations suggest that redirected CD8$^+$ T cells targeting AURKA may not cause severe bone marrow failure, although further studies are needed to substantiate this point.

Notably, AURKA is overexpressed in the fraction of bone marrow cells that encompasses myeloid leukemia stem cells.[6,41] Recently, targeting leukemia stem cells has been highlighted as a treatment strategy to prevent disease progression in a durable fashion.[42] Monoclonal antibodies that target leukemia stem cell surface antigens have been proposed for this purpose. Examples of such molecules include CD123 (IL3Rα)[43] and TIM-3.[44] Cellular immunotherapy targeting antigens that are preferentially overexpressed in leukemia stem cells has also been proposed. In this regard, WT1 appears to be a particularly attractive candidate.[45] Indeed, we have cloned an HLA-A*2402–restricted WT1$_{235-243}$–specific *TCR* gene into our unique si-*TCR* vector to address the potential of this approach.[46] With respect to AURKA, we previously described that the CD34$^+$CD38$^-$ fraction of bone marrow mononuclear cells from CML patients expressed high levels of *AURKA* mRNA and that these cells were susceptible to AURKA-specific CTL-mediated lysis.[6] Thus, redirected T cell–based immunotherapy targeting AURKA might be able to suppress leukemia stem cells. Furthermore, such an approach may be synergistic with the administration of selective AURKA inhibitors, for example in the treatment of relapsed leukemia after allogeneic hematopoietic stem cell transplantation.

Strategic options to achieve better clinical responses in the field of *TCR* gene transfer are much needed. The manipulation of helper CD4$^+$ T cells is one such approach.[24,47,48] To date, the adoptive transfer of redirected CD4$^+$ T cells concurrently with CD8$^+$ T cells expressing the same tumor-specific TCR gene has not been described; however, this is an intriguing notion that could enhance the antitumor reactivity of such adoptive transfer approaches in vivo. In recognition of this possibility, we found that AURKA$_{207-215}$ *TCR*-transduced CD4$^+$ T cells displayed Th1 cytokine production in response to the HLA-A*0201/AURKA$_{207-215}$ epitope in vitro. The effects of such activity in vivo, however, remain to be clarified. Another approach to combined immunotherapy employs peptide vaccination. Indeed, vaccination with the relevant peptide has been

shown to enhance the antitumor functionality of infused gene-modified T cells.[49] The feasibility of this combination strategy using $AURKA_{207-215}$ peptide vaccination is currently under investigation.

In summary, we have demonstrated the feasibility of antileukemia adoptive therapy using AURKA-specific *TCR* gene transfer. As AURKA is also overexpressed in diverse solid tumors,[4] the potential clinical applications of this approach are widespread. Further studies are therefore warranted to investigate the safety and utility of this novel therapy in the clinic.

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Authorship

Contribution: K.N. and T.O. performed the research and wrote the paper; H.F. designed and performed the research, wrote and edited the paper, and provided financial support; J.A., T.S., J.M., H.S., J.J.M., and E.I. discussed and interpreted the experimental results and provided materials; K.K., E.G., and D.A.P. made and supplied the tetramers and edited the paper; and M.Y. discussed and interpreted the experimental results, edited the paper, and provided financial support.

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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).

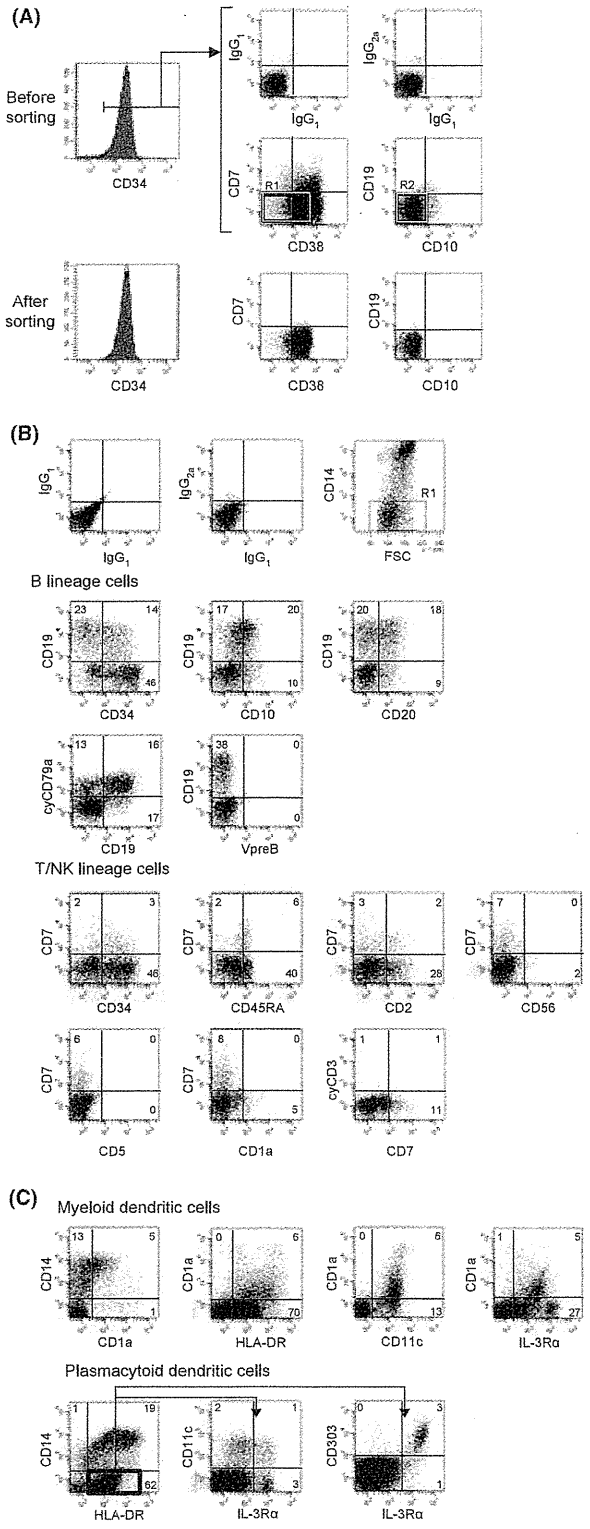

Fig 1. Expression of lymphoid-lineage antigens in cells cultured with telomerized stromal cells. (A) Phenotype of the $CD38^{low/-}CD19^-CD10^-CD7^-$ cell fraction before and after sorting of immunomagnetically-enriched $CD34^+$ cells. (B) $CD34^+CD38^{low/-}CD19^-CD10^-CD7^-$ cells (5×10^3 cells/well) 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, $CD14^-HLA\text{-}DR^+$ 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 $CD1a^+HLA\text{-}DR^+CD11c^+$ myeloid (Fig. S2A) and $CD14^-HLA\text{-}DR^+CD11c^-IL\text{-}3R\alpha^{high}CD303^+$ 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

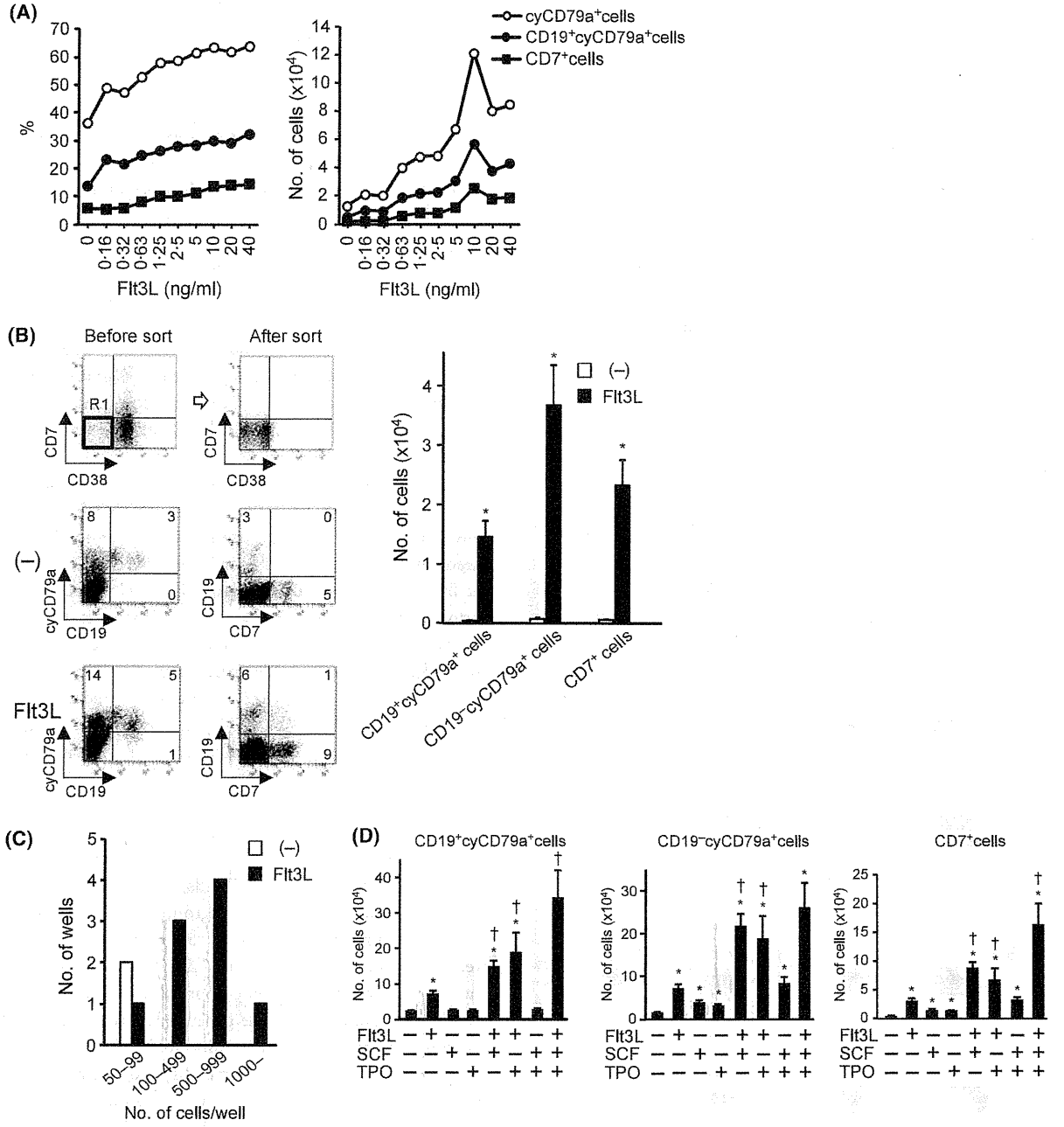

Fig 2. Effects of flt3L, SCF and TPO on lymphopoiesis. The effects of flt3L on $CD34^+CD38^{low/-}CD19^-CD10^-CD7^-$ (1×10^4 cells/well) (A) and $CD34^+CD38^-CD19^-CD10^-CD7^-$ (1×10^4 cells/well) (B) cultured on telomerized stromal cells. The percentage and number of $CD19^+cyCD79a^+$, $CD19^-CD79a^+$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

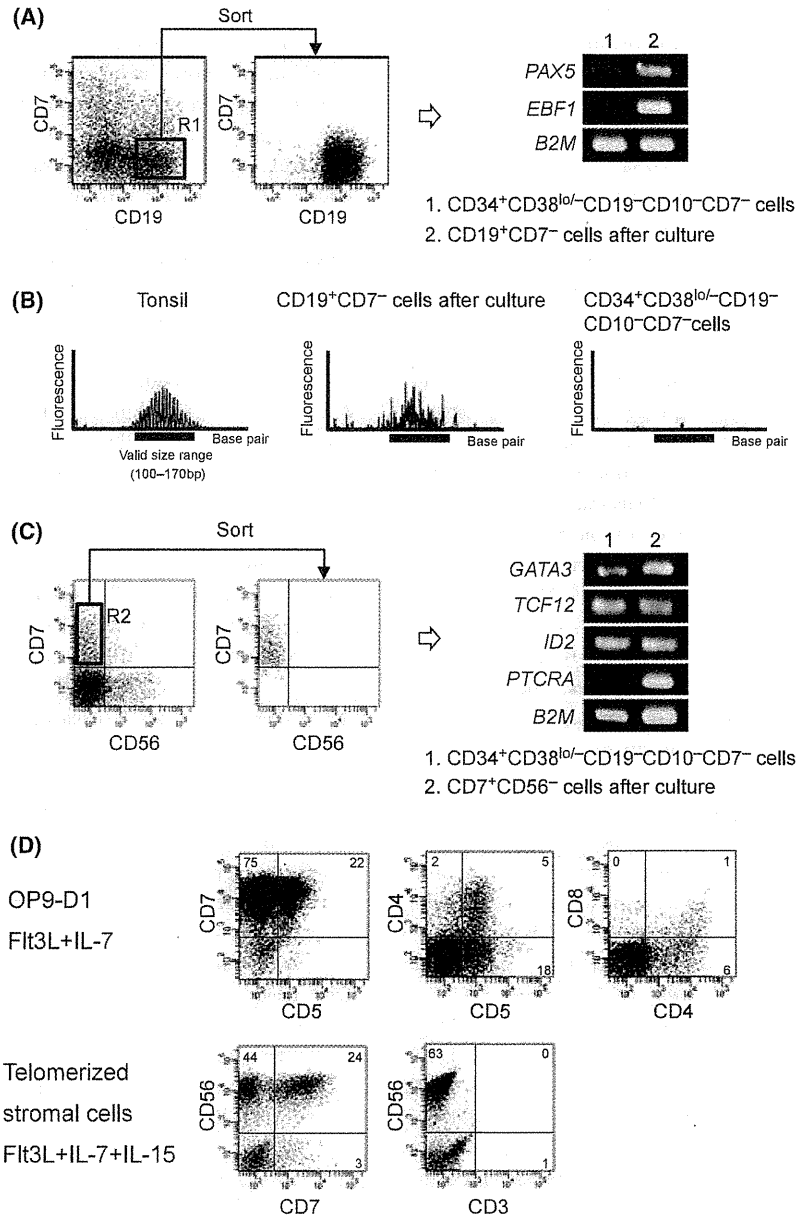

Fig 3. Characteristics of generated $CD19^+$ and $CD7^+CD56^-$ cells. (A) Gene expression of *PAX5*, *EBF1* and *B2M* in $CD34^+CD38^{low/-}CD19^-CD10^-CD7^-$ and $CD19^+CD7^-$ cells. (B) VDJ rearrangements of *IGH@* genes. (C) Gene expressions of *GATA3*, *TCF12*, *PTCRA* and *B2M* in $CD34^+CD38^{low/-}CD19^-CD10^-CD7^-$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

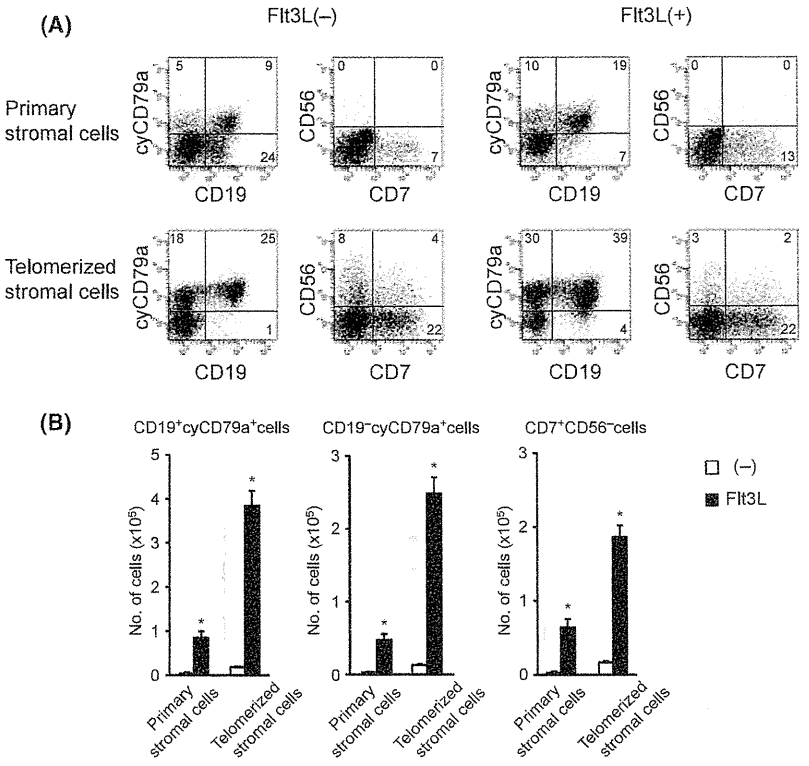

Fig 4. B and T/NK lymphopoiesis occurs on primary human stromal cells. $CD34^+CD38^{low/-}CD19^-CD10^-CD7^-$ cells (1×10^4 cells/well) were cocultured with primary or telomerized stromal cells, with or without flt3L, and analysed for the cell phenotypes (A) and numbers (B). The phenotypes after excluding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

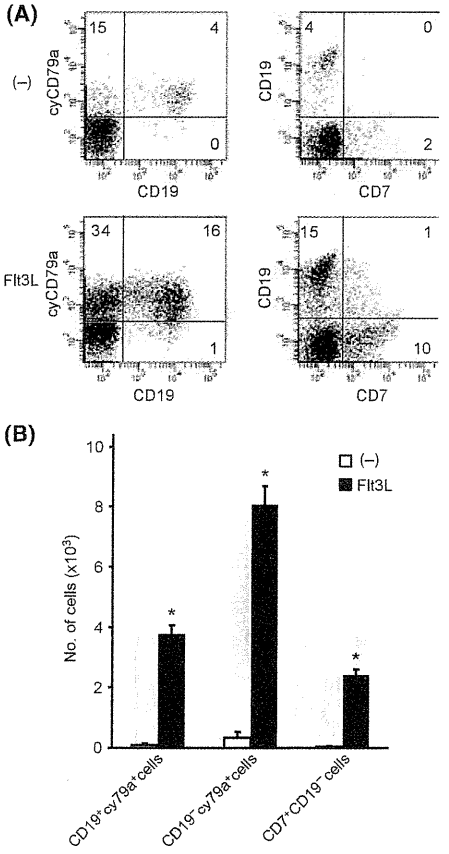

Fig 5. B and T/NK lymphopoiesis can occur separately from stromal cells. $CD34^+CD38^{lo/-}CD19^-CD10^-CD7^-$ cells (1×10^4 cells/well) were cultured separately from telomerized stromal cells using cell culture inserts in the presence or absence of flt3L. The phenotype (A) and number (B) of the cultured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

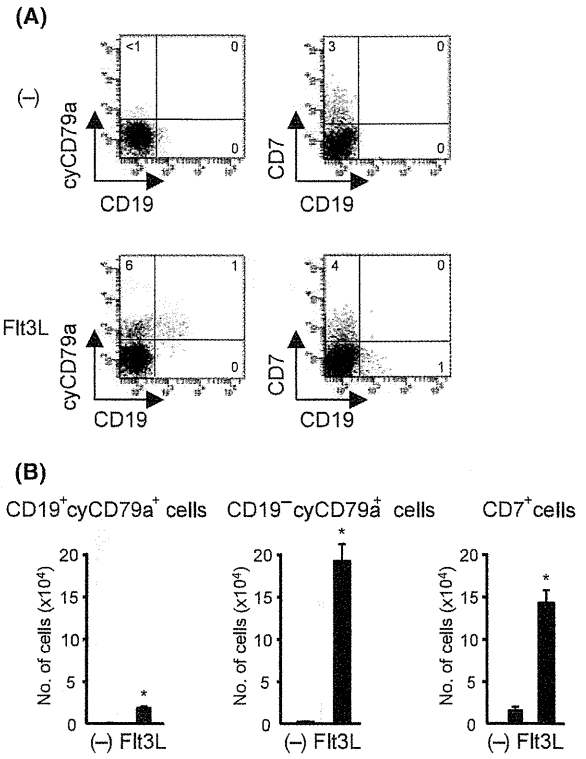

Fig 6. Effect of flt3L in serum-free cultures. $CD34^+CD38^{low/-}CD19^-CD10^-CD7^-$ cells (1×10^4 cells/well) were cultured with telomerized stromal cells in serum-free cultures with or without flt3L, and the phenotype (A) and the number (B) of $CD19^+cyCD79a^+$, $CD19^-cyCD79a^+$ and $CD7^+$ cells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