

Delta-1 enhances marrow and thymus repopulating ability of human CD34⁺CD38⁻ cord blood cells

Kohshi Ohishi,¹ Barbara Varnum-Finney,¹ and Irwin D. Bernstein^{1,2}

¹Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, Washington, USA

²Department of Pediatrics, University of Washington, Seattle, Washington, USA

We investigated the effect of Notch signaling, a known regulator of cell fate in numerous developmental systems, on human hematopoietic precursors. We show that activation of endogenous Notch signaling in human CD34⁺CD38⁻ cord blood precursors with immobilized Delta-1 in serum-free cultures containing fibronectin and hematopoietic growth factors inhibited myeloid differentiation and induced a 100-fold increase in the number of CD34⁺ cells compared with control cultures. Immobilized Delta-1 also induced a multifold expansion of cells with the phenotype of common lymphoid precursors (CD34⁺CD7⁺CD45RA⁺) and promoted the development of cytoplasmic CD3⁺ T/NK cell precursors. IL-7 enhanced the promotion of T/NK cell differentiation by immobilized Delta-1, but granulocytic differentiation occurred when G-CSF was added. Transplantation into immunodeficient mice showed a substantial increase in myeloid and B cell engraftment in the marrow and also revealed thymic repopulation by CD3⁺ T cells due to cells being cultured for a longer period with immobilized Delta-1. These data suggest that Delta-1 can enhance myeloid and lymphoid marrow-repopulating ability and promote the generation of thymus-repopulating T cell precursors.

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Introduction

The hematopoietic system is maintained throughout the life of an organism by stem cells that are believed to self-renew and to give rise to precursor cells committed to the various hematopoietic lineages (1). Although numerous cytokines support the survival and proliferation of differentiating hematopoietic stem cell progeny, the factors that regulate self-renewal and differentiation have remained elusive (1). In other developmental systems, the Notch pathway is known to play a critical role in regulating cell-fate choices in numerous precursor types, often by inhibiting a specific differentiation pathway and permitting either self-renewal or differentiation along an alternate pathway (2).

We and others previously reported the expression of Notch-1 and Notch-2 in human CD34⁺ or CD34⁺Lin⁻ precursors (3–5) and of the Notch ligands Delta-1 and Jagged-1 in human bone marrow stromal cells and in human hematopoietic precursors (3, 6–8). Moreover, expression of a constitutively active, truncated form of Notch-1 in murine hematopoietic precursors led to increased stem cell numbers and produced an

immortal cell line that phenotypically resembles primitive hematopoietic precursors that, depending upon the cytokine context, can differentiate along the lymphoid and myeloid lineage (9, 10). While these findings point to a role for Notch signaling in the regulation of stem cell self-renewal, expression of activated Notch-1 in human CD34⁺ cord blood precursors induced only a modest increase in the number of progenitors (11). Furthermore, although activation of endogenous Notch receptors in murine hematopoietic precursors by soluble or cell membrane-expressed Notch ligands has led to increased numbers of hematopoietic precursors (7, 12–14), relatively little or no increase in the numbers of progenitors has been observed after incubation of human hematopoietic progenitors with soluble or cell membrane-expressed Notch ligands (3, 6, 15, 16).

In both gain-of-function and loss-of-function studies, Notch signaling has been shown to regulate lymphoid differentiation in mice by promoting T cell differentiation while inhibiting B cell differentiation, presumably due to an effect on common lymphoid precursors (15, 17–20). Further, Delta-1 expressed on the surface of S17 stromal cells was found to induce T cell rather than the expected B cell development by human CD34⁺ cord blood precursors (15).

We previously showed that immobilization of an engineered Notch ligand, Delta-1, was required for Notch activation and inhibition of C2 myoblast cell differentiation, and that Delta-1 in solution antagonized the effect of immobilized Delta-1 (21). We also observed that immobilized Delta-1, but not Delta-1 in solution, affects differentiation of human monocytes

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Address correspondence to: Irwin D. Bernstein, Fred Hutchinson Cancer Research Center, 1100 Fairview Avenue N., D2-373, Seattle, Washington 98109, USA. Phone: (206) 667-4886; Fax: (206) 667-6084; E-mail: ibernste@fhcrc.org.

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Nonstandard abbreviations used: phycoerythrin (PE); stem cell factor (SCF); thrombopoietin (TPO); flt-3 ligand (FL); Iscove's modified Dulbecco's medium (IMDM); growth factors (GF).

(4, 22). We therefore assessed the role of Notch signaling in primitive human hematopoietic precursors using immobilized Delta-1.

Here we show that incubation of CD34⁺CD38⁻ cells with immobilized Delta-1, together with fibronectin fragments and appropriate growth factors, induces a multiple-log increase in the number of human CD34⁺ cells and also promotes early T cell differentiation. In transplantation studies using immunodeficient mice, incubation of CD34⁺CD38⁻ cells with Delta-1 substantially enhances B cell and myeloid cell engraftment in the marrow and also leads to thymic reconstitution by human CD3⁺ T cells. These data suggest that Delta-1 enhances the self-renewal of *in vivo* myeloid and lymphoid marrow-repopulating cells and promotes the generation of thymus-repopulating T cell precursors.

Methods

Separation of CD34⁺CD38⁻ cells and immunofluorescence studies. Human cord blood samples obtained from normal full deliveries were separated by Ficoll-Hypaque, washed, incubated in ammonium chloride red blood cell lysis buffer, and suspended in PBS with 2% human type AB serum. Cells were stained with anti-CD34 antibody 12.8 (generated in our laboratory), followed by immunomagnetic bead-conjugated goat anti-mouse IgM (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), and purified according to the manufacturer's instructions. Cells were further incubated with FITC-conjugated anti-CD34 antibody and phycoerythrin-conjugated (PE-conjugated) CD38 antibody (both from Becton Dickinson Immunocytometry Systems, San Jose, California, USA), and isolated by FACS (Vantage; Becton Dickinson Immunocytometry Systems), excluding propidium iodide-positive (Sigma-Aldrich, St. Louis, Missouri, USA) dead cells. Immunofluorescence analysis was performed as previously described (4, 22), using FITC-labeled antibodies against CD7 (4H9), CD14, CD19, CD34, T cell receptor- $\alpha\beta$ (Becton Dickinson Immunocytometry Systems), and CD64 (Becton Dickinson Pharmingen, San Diego, California, USA), or PE-labeled antibodies against CD3, CD11b, CD14, CD34, T cell receptor- $\gamma\delta$ (Becton Dickinson Immunocytometry Systems), CD7 (8H8.1), CD15, CD25, CD56 (Immunotech, Marseilles, France), and CD45RA (Becton Dickinson Labware, San Diego, California, USA). FITC- or PE-conjugated isotype-matched antibodies were used as controls.

For cytoplasmic CD3 staining, cells were blocked with PBS containing 2% AB serum and incubated with PE-conjugated antibodies against CD7 or CD34 and streptavidin-conjugated tricolor (Caltag Laboratories Inc., Burlingame, California, USA) for 30 minutes at 4°C. After washing, cells were permeabilized in PermeaFix (Ortho Diagnostic Systems, Raritan, New Jersey, USA) for 20 minutes at room temperature, washed, and incubated with FITC-conjugated antibodies against CD3 (Becton Dickinson Pharmingen, San Diego, California, USA) for 30 minutes at 4°C (23).

Generation of Delta-1 construct. The extracellular domain of Delta-1, containing six myc tags (Delta1^{ext-myc}), and control medium were prepared as described (4, 22).

Cell cultures. Cord blood CD34⁺CD38⁻ cells were cultured in non-tissue culture treated culture plates (Becton Dickinson, Franklin Lakes, New Jersey, USA) containing serum-free medium (StemSpan; Stem Cell Technologies Inc., Vancouver, British Columbia, Canada), the designated cytokines, 20 μ g/ml of LDL (Sigma-Aldrich), and either Delta1^{ext-myc} or control medium. Culture plates were precoated with 10 μ g/ml of anti-myc antibody 9E10 F(ab')₂ or isotype-matched antibody 31A F(ab')₂ fragments as previously described (4, 22) with or without 5 μ g/ml of fibronectin fragment CH-296 (Takara Shuzo Co., Otsu, Japan). Cultures were initiated in 96-well non-tissue culture treated plates at 250–1,000 cells per well and replated to each well of similarly prepared 24-well plates containing fresh medium with the original concentration of cytokines and either Delta1^{ext-myc} or control medium. After 14 days, a portion of cultured cells was harvested and replated in 12-well plates to prevent overgrowth. Fresh medium with cytokines and either Delta1^{ext-myc} or control medium were added 3–4 days after replating. Cytokines were used at the following concentrations: 300 ng/ml of stem cell factor (SCF), 100 ng/ml of thrombopoietin (TPO), 300 ng/ml of flt-3 ligand (FL), 100 ng/ml of IL-6, 100 ng/ml of IL-7, 10 ng/ml of IL-3, 10 ng/ml of GM-CSF, and 10 ng/ml of G-CSF (all from PeproTech Inc., Rocky Hill, New Jersey, USA). Semisolid cultures were performed in 35-mm culture dishes (Corning Inc., Corning, New York, USA) containing Iscove's modified Dulbecco's medium (IMDM) with 40% IMDM-based methylcellulose (Stem Cell Technologies Inc.); 20% FCS; 2% BSA; 1 \times 10⁻⁴ mol/l 2-mercaptoethanol; SCF, FL, TPO, GM-CSF, and G-CSF, each at 50 ng/ml; and 2 U/ml erythropoietin (Amgen Inc., Thousand Oaks, California, USA) at 37°C. Colony-forming ability was assessed after 14–16 days of cultures.

RT-PCR. Total mRNA was extracted by Trizol (Invitrogen Life Technologies, Carlsbad, California, USA) according to the manufacturer's instructions. RT and PCR were performed using SuperScript one-step RT-PCR (Invitrogen Life Technologies) according to the manufacturer's instructions. RT-PCR was carried out in a 50- μ l reaction volume containing 15 ng of template RNA, recombinant Taq DNA polymerase, reverse transcriptase, 0.4 mM of dNTPs, 2.4 mM MgSO₄, and 10 μ M of sense and antisense primer. RT was performed at 45°C for 45 minutes, followed by heat inactivation at 94°C for 2 minutes. PCR amplification was carried out for 35 cycles of denaturation at 94°C for 15 seconds, annealing at 57°C for 20 seconds, and extension at 72°C for 1 minute in a PTC-200 Peltier Thermal Cycler (MJ Research Inc., Waltham, Massachusetts, USA). The absence of genomic CD3 ϵ gene was confirmed by amplification of mRNA without reverse transcriptase.

Oligonucleotide primer sequences were as follows (24): CD3ε 5' primer, 5'-AGT TGG CGT TGG GGG GCA AGT TGG TAA TGA AGA AA-3'; CD3ε 3' primer, 5'-CCC AGG AAA CAG GGA GTC GCA GGG GGA CTG GAG AG-3'; β-actin 5' primer, 5'-TAC CTC ATG AAG ATC CTC A-3'; β-actin 3' primer, 5'-TTC GTG GAT GCC ACA GGA C-3'.

Amplified products were electrophoresed on a 1% agarose gel and stained with ethidium bromide.

Real-time RT-PCR studies of HES-1 expression. Total mRNA was extracted using the Absolutely RNA RT-PCR Miniprep kit (Stratagene, La Jolla, California, USA) according to the manufacturer's instructions. Primers and probe of HES-1 were as follows (15): HES-1 forward primer, 5' TGG AAA TGA CAG TGA AGC ACC T 3'; HES-1 reverse primer, 5' GTT CAT GCA CTC GCT GAA GC 3'; HES-1 probe, CGC AGA TGA CGG CTG CGC TG 3'. Probes were labeled at the 5' end with the reporter dye molecule FAM and at the 3' end with the quencher dye molecule TAMRA (Applied Biosystems, Foster City, California, USA). The GAPDH gene was used as an endogenous control, and primers and JOE-labeled probe from TaqMan GAPDH Control Reagents (Applied Biosystems) were used. RT-PCR was performed using SuperScript one-step RT-PCR (Invitrogen Life Technologies Inc.) according to the manufacturer's instructions. Briefly, RT-PCR was carried out in a 50-μl reaction volume containing six replicates of a 10⁻¹-log dilution of RNA from 100 μg to 10 pg of template RNA, recombinant Taq DNA polymerase and SuperScript II Reverse Transcriptase, 0.4 nM of dNTPs, 50 mM MgSO₄, 200 nM of sense and antisense primer, and 100 nM of fluorescence-labeled probe. RT was performed at 45°C for 45 minutes, followed by heat inactivation at 94°C for 2 minutes. PCR amplification was carried out for 40 cycles of 1 minute at 60°C and 15 seconds at 95°C in an ABI PRISM 7700 Sequence Detector (Applied Biosystems). HES-1 RNA expression was normalized using GAPDH as the endogenous standard and neuroepithelioma cell line SK-N-MC (American Type Culture Collection, Manassas, Virginia, USA) as a positive standard (normalized target value). HES-1 expression was calibrated relative to values in control-cultured cells.

Transplantation of human hematopoietic cells into NOD/SCID or NOD/SCID β2m^{-/-} mice. Mice were bred and maintained at the Fred Hutchinson Cancer Research Center. NOD/SCID or NOD/SCID β2m^{-/-} mice at 8–10 weeks of age were γ-irradiated at 325 or 300 cGy, respectively, from a linear accelerator at an exposure rate of 20 cGy/min on the day prior to or the day of transplantation, and cells were injected via tail vein. When fresh CD34⁺CD38⁻ cells were transplanted, 1 × 10⁵ to 2 × 10⁵ irradiated (15 Gy) CD34⁻ cord blood cells were coinjected as carrier cells via tail vein. Thereafter, mice were given acidified water containing 100 mg/l ciprofloxacin. A mean volume of 12 μl of marrow was aspirated from both femurs at 3 and 6 weeks. At 9–12 weeks, mice were sacrificed, and cells were harvested from both femurs, both tibiae, and thymus and

analyzed for engraftment. Cells were incubated with ammonium chloride red blood cell lysis buffer, blocked with PBS containing 2% AB serum and anti-mouse CD16/32 antibody (FcγRII block, 2.4G2) (Becton Dickinson Pharmingen, San Diego, California, USA), and stained with FITC-conjugated CD45.1 (Becton Dickinson Immunocytometry Systems) or CD4 (Becton Dickinson Immunocytometry Systems), PE-conjugated anti-human CD3, CD8, CD19, CD33, or CD34 (Becton Dickinson Immunocytometry Systems), Peridinin chlorophyll protein-conjugated (PerCP-conjugated anti-human CD45 (Becton Dickinson, Sunnyvale, California, USA), or CyChrome-conjugated CD3 (BD

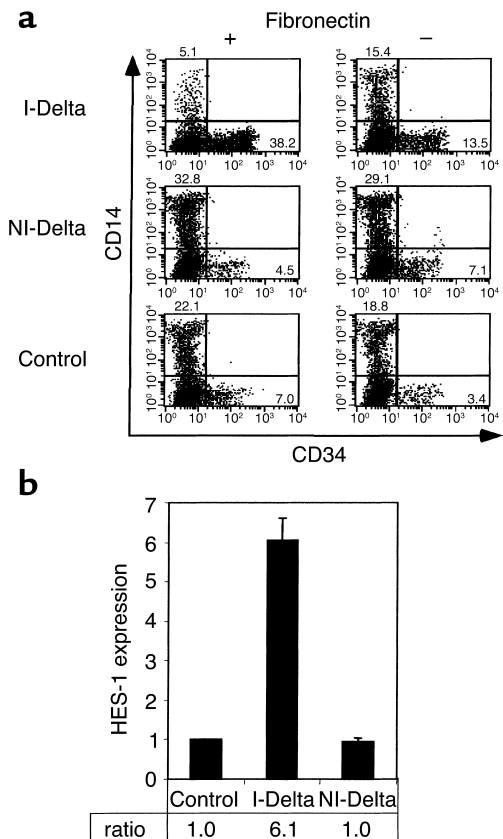


Figure 1 Immobilized Delta1^{ext-myc} specifically activated Notch signaling and effectively inhibited myeloid differentiation in the presence of fibronectin CH-296. (a) Culture plates were precoated either with anti-myc 9E10 antibody F(ab')₂ to immobilize ligand (I-Delta) or with isotype-matched 31A antibody F(ab')₂ (NI-Delta), with (+) or without (-) fibronectin fragment CH-296. CD34⁺CD38⁻ precursors (250 cells per well) were cultured for 2 weeks in serum-free medium containing SCF, FL, TPO, IL-6, and IL-3 (5GF) and either 1 μg/ml of Delta1^{ext-myc} or the same volume of control medium (Control), and analyzed by FACS for CD34 and CD14 expression. Data are representative of three independent experiments. Numbers in corners are the percentage of gated events within that quadrant. (b) CD34⁺ cells were cultured for 48 hours with 5GF, CH-296, and I-Delta1^{ext-myc}, NI-Delta1^{ext-myc}, or control medium, and expression of the Notch target gene HES-1 relative to that of GAPDH was assessed by quantitative RT-PCR. Expression of HES-1 mRNA was measured, and ratios were compared with those in control cells.

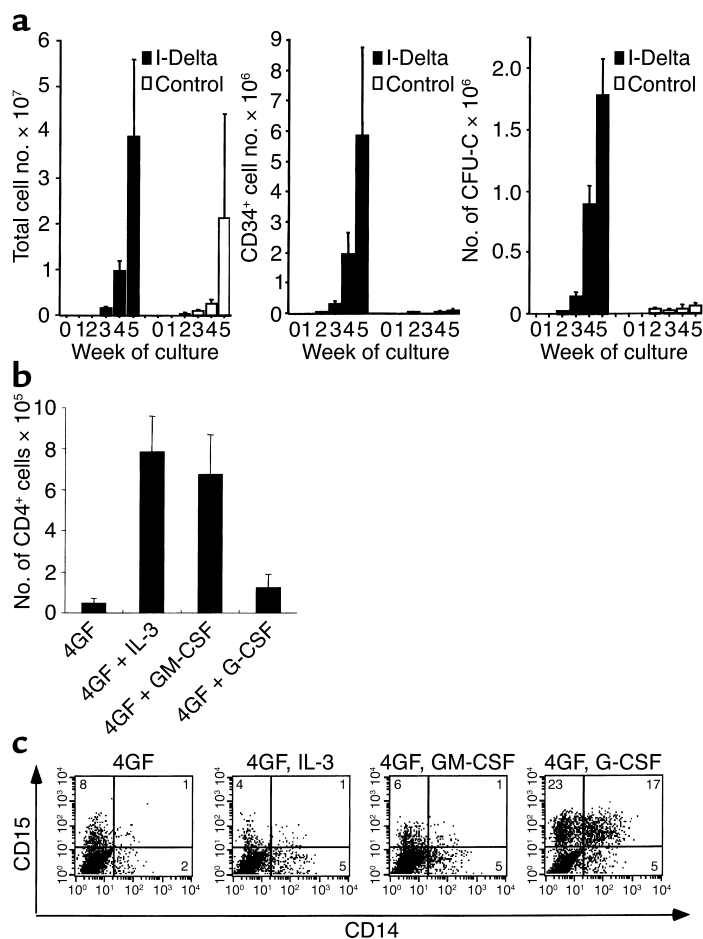


Figure 2
I-Delta^{1ext-myc} induced a multiple-log expansion of hematopoietic precursors with SCF, FL, TPO, IL-6, and IL-3. Culture plates were precoated with anti-myc 9E10 antibody F(ab')₂ and CH-296. (a) CD34⁺CD38⁻ cells (600 per well) were cultured with SCF, FL, TPO, IL-6, and IL-3 (5GF), CH-296, and either I-Delta^{1ext-myc} (I-Delta) or control medium (Control) for up to 5 weeks. Mean numbers (± SEM) of total cells (left panel), CD34⁺ cells (middle panel), and CFU (right panel) in six wells are shown. Data are representative of five independent experiments. (b and c) CD34⁺CD38⁻ cells (1,000 per well) were cultured for 2 weeks with I-Delta^{1ext-myc}, CH-296, and 4GF (SCF, FL, TPO, and IL-6), with or without IL-3 (10 ng/ml), GM-CSF (10 ng/ml), or G-CSF (10 ng/ml). Mean numbers (± SEM) of CD34⁺ cells in six wells (b) and expression of myeloid antigens CD15 and CD14 in cultured cells (c) are shown. Data are representative of four independent experiments. Numbers in corners in c are the percentage of gated events within that quadrant.

Pharmingen, San Diego, California, USA). Using FAC-Scan (Becton Dickinson Immunocytometry Systems), we assessed the presence of human cells by analyzing cells staining with anti-human CD45 but not with anti-mouse CD45.1, excluding 4',6-diamidino-2-phenylindole dihydrochloride-staining dead cells.

Statistical analysis. Student's *t* test or Wilcoxon rank-sum test was used to determine statistical significance.

Results

Delta1^{ext-myc} immobilized together with fibronectin fragment CH-296 effectively inhibits myeloid differentiation. CD34⁺CD38⁻ precursors, isolated by FACS, were cultured in serum-free culture medium containing five growth factors (5GF) at concentrations previously shown to optimally support the growth of primitive human hematopoietic precursors in serum-free medium: SCF and FL at 300 ng/ml, TPO at 100 ng/ml, IL-6 at 100 ng/ml, and IL-3 at 10 ng/ml (25–28). The Notch ligand construct Delta1^{ext-myc}, consisting of the extracellular domain of Delta-1 and six myc tags, was used to activate Notch signaling in cord blood precursors. To test whether immobilization is essential for an effect on human hematopoietic precursors, Delta1^{ext-myc} was either immobilized (I-Delta1^{ext-myc}) to the surface of culture plates via plastic-bound anti-myc antibody 9E10 F(ab')₂ fragments, or used in

solution (NI-Delta1^{ext-myc}) in culture plates coated with isotype-matched 31A control antibody F(ab')₂ fragments of irrelevant specificity. As a control for Delta1^{ext-myc}, conditioned medium was purified identically from cell lines not transfected with the construct (4, 22). The effect of plastic-bound fibronectin fragment CH-296 was also tested, because adhesion of hematopoietic precursors to fibronectin helps to maintain long-term reconstituting ability in vitro (29) and may enhance the attachment of precursors to immobilized Notch ligand.

When 2.5×10^2 CD34⁺CD38⁻ cells were cultured for 14 days with I-Delta1^{ext-myc}, NI-Delta1^{ext-myc}, or control medium, with or without CH-296, along with 5GF, total cell numbers in the cultures did not differ significantly. However, cultures with I-Delta1^{ext-myc} contained a higher proportion (13.5%) of CD34⁺ cells than cultures with NI-Delta1^{ext-myc} (7.1%) or control medium (3.4%) (Figure 1a). In the presence of CH-296, cultures containing I-Delta1^{ext-myc} showed a threefold increase in the proportion (38.3%) of CD34⁺ cells compared with cultures without CH-296, whereas cultures containing NI-Delta1^{ext-myc} or control medium showed little or no increase in the proportion of CD34⁺ cells with CH-296 (Figure 1a). Further, the proportion of cells expressing the myeloid differentiation antigen CD14 (5.2%) was considerably smaller in cultures with

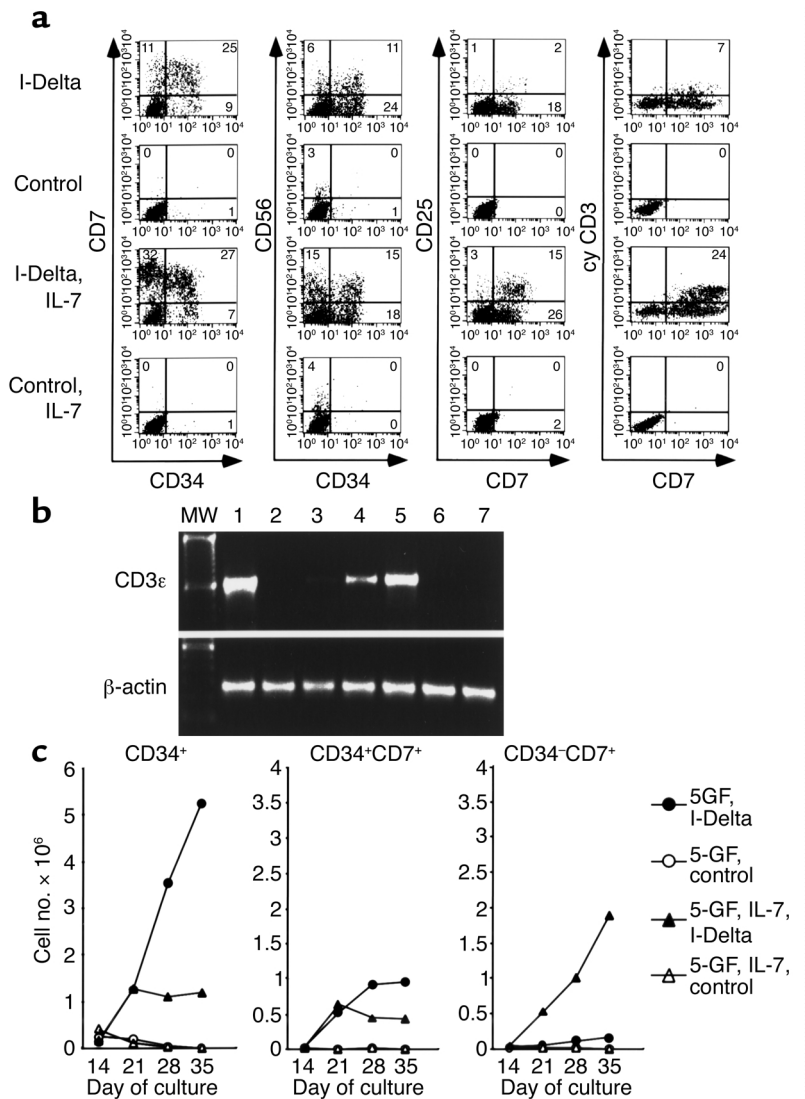
CH-296 and I-Delta1^{ext-myc} as than in cultures with NI-Delta1^{ext-myc} (32.8%), with control medium (22.1%), or with I-Delta1^{ext-myc} in the absence of CH-296 (15.4%) (Figure 1a). The presence of CH-296, however, did not affect the proportion of CD14⁺ cells in cultures containing NI-Delta1^{ext-myc} or control medium (Figure 1a). Similar observations were made for the expression of CD11b, CD15, and the high-affinity Fcγ receptor CD64 (data not shown). These results show that Delta1^{ext-myc} immobilized together with fibronectin effectively inhibits myeloid differentiation and maintains cord blood precursors at a less differentiated CD34⁺ stage in serum-free cultures.

To confirm that only I-Delta1^{ext-myc} induces Notch signaling in the presence of CH-296, we assessed the expression of HES-1, a target gene of Notch signaling (30, 31), using quantitative RT-PCR. Indeed, CD34⁺ cells cultured for 48 hours with 5GF, CH-296, and I-Delta1^{ext-myc} showed a sixfold increase in the expression of HES-1 mRNA in cultures containing I-Delta1^{ext-myc} compared with control cells, whereas no increase was seen in cultures containing NI-Delta1^{ext-myc} (Figure 1b).

I-Delta1^{ext-myc} induces a multiple-log expansion of cord blood precursors. To evaluate whether I-Delta1^{ext-myc} might further increase the number of precursors over an extended period of time, we incubated 6.0×10^2 CD34⁺CD38⁻ cells for 5 weeks with either I-Delta1^{ext-myc} or control medium together with CH-296 and 5GF. The total number of cells did not differ significantly in the two culture conditions (Figure 2a, left panel). However, in cultures with I-Delta1^{ext-myc}, the number of CD34⁺ cells increased over time, and by 5 weeks, $5.8 \times 10^7 \pm 1.3 \times 10^7$ CD34⁺ cells (mean \pm SEM) were generated (Figure 2a, middle panel), representing a 5-log expansion of CD34⁺ cells overall and a 2-log increase over that seen in cultures containing control medium ($6.1 \times 10^5 \pm 3.6 \times 10^5$ CD34⁺ cells, $P < 0.005$). Approximately 80% of CD34⁺ cells in cultures were CD38^{low/-}. In addition, cultures containing I-Delta1^{ext-myc} produced $1.7 \times 10^6 \pm 0.1 \times 10^6$ CFU at 5 weeks, representing a 2.6×10^3 -fold expansion of CFU and a 34-fold increase compared with control cultures ($5.3 \times 10^4 \pm 1.4 \times 10^4$ CFU, $P < 0.0001$) (Figure 2a, right panel). In three of five separate experiments, CD34⁺ cell numbers

Figure 3

I-Delta1^{ext-myc} promoted T/NK cell differentiation, which was enhanced by IL-7. Culture plates were pre-coated with anti-myc 9E10 F(ab')₂ and CH-296. (a) CD34⁺CD38⁻ precursors (400 cells per well) were cultured for 3 weeks in serum-free medium containing 5GF with or without IL-7 (100 ng/ml) and either 1 μg/ml of Delta1^{ext-myc} (I-Delta) or the same volume of control medium (Control). Data are representative of four independent experiments. Numbers in corners are the percentage of gated events within that quadrant. cy CD3, cytoplasmic CD3. (b) RNA was extracted from freshly obtained peripheral T cells (lane 1), freshly obtained peripheral granulocytes (lane 2), fresh CD34⁺CD38⁻ cells (lane 3), cells cultured for 3 weeks with I-Delta1^{ext-myc} (lane 4), cells cultured with I-Delta1^{ext-myc} and IL-7 (lane 5), cells cultured in control medium (lane 6), and cells cultured in control medium and IL-7 (lane 7). Equal amounts of RNA were subjected to RT-PCR, followed by electrophoresis on an agarose gel and ethidium bromide staining (see Methods). (c) CD34⁺CD38⁻ precursors (400 cells per well) were cultured for 5 weeks with 5GF, with or without IL-7 (100 ng/ml), and either I-Delta1^{ext-myc} or control medium. Cells were stained with FITC-conjugated CD7 (4H9) and PE-conjugated CD34 antibody. Mean numbers of CD34⁺ cells, CD34⁺CD7⁺ cells, and CD34⁺CD7⁺ cells in six wells are shown. Data are representative of three independent experiments. MW, molecular weight.



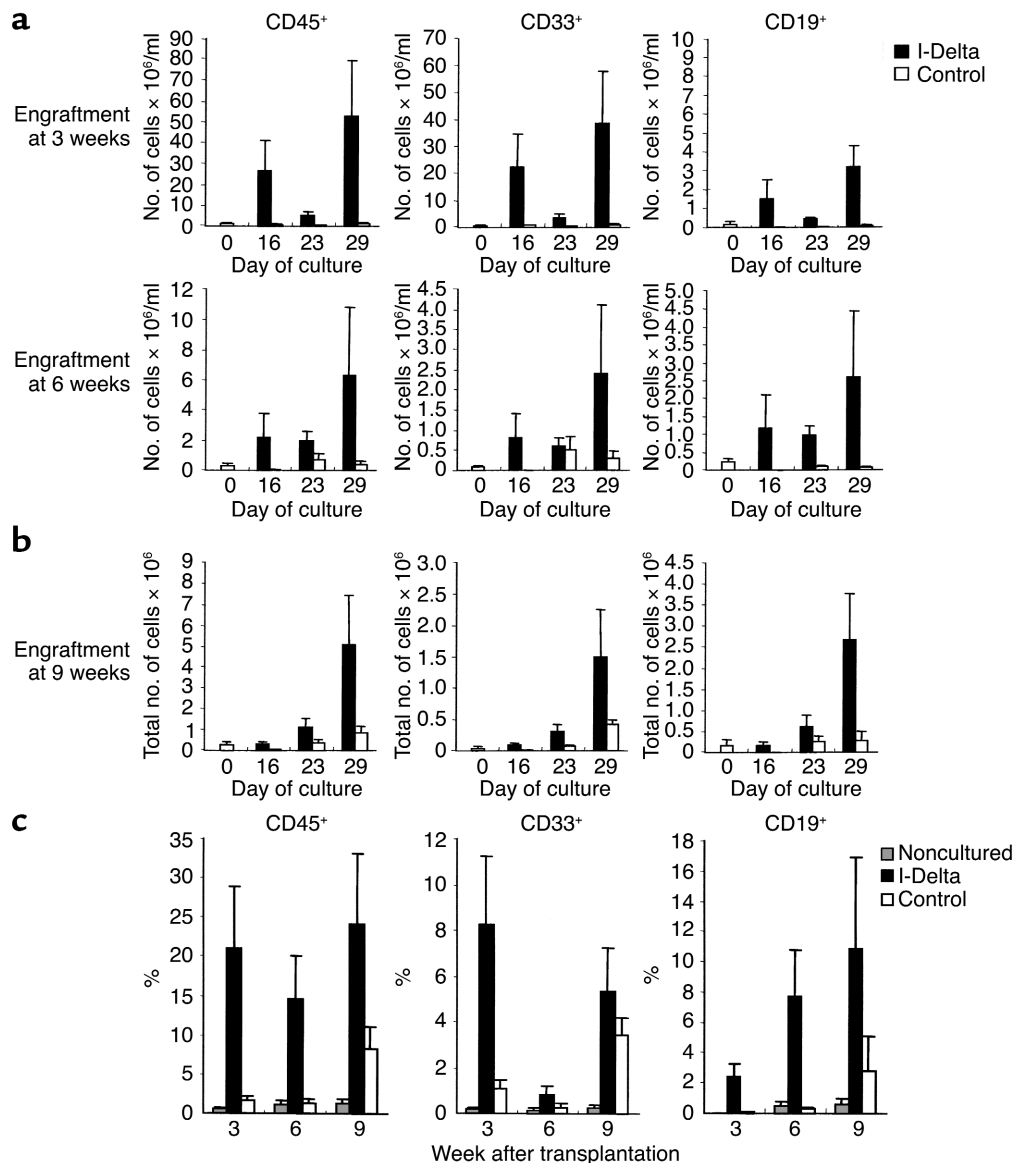


Figure 4

I-Delta1^{ext-myc} enhances engraftment of human cells in marrow of NOD/SCID $\beta 2m^{-/-}$ or NOD/SCID mice. (a and b) We transplanted 4.7×10^3 fresh CD34⁺CD38⁻ cells along with 2×10^6 irradiated (15 Gy) CD34⁺ cord blood cells (day 0), or all the progeny of 4.7×10^3 CD34⁺CD38⁻ cells cultured for 16, 23, or 29 days with Delta1^{ext-myc} (I-Delta) or control medium (Control), into sublethally irradiated NOD/SCID $\beta 2^{-/-}$ mice ($n = 7$). At 3 and 6 weeks, marrow was aspirated from both knee joints (a), and at 9 weeks, mice were sacrificed and marrow was harvested from both femurs and both tibiae (b). We assessed human cell engraftment by counting the number of cells, in aspirated marrow, that stained with Peridinin chlorophyll protein-conjugated (PerCP-conjugated) anti-human CD45 and PE-conjugated anti-human CD33, CD19, or CD34, excluding cells staining with FITC-conjugated anti-mouse CD45.1 and DAPI-staining dead cells (see Methods). Mean (\pm SEM) engraftment of human cells in mice in each group is shown. Data are representative of four different experiments. (c) We transplanted 5×10^3 fresh CD34⁺CD38⁻ cells along with 2×10^6 irradiated (15 Gy) CD34⁺ cord blood cells (Noncultured), or all the progeny of 5×10^3 CD34⁺CD38⁻ cells cultured for 21 days with I-Delta1^{ext-myc} (I-Delta) or control medium (Control), into sublethally irradiated NOD/SCID mice ($n = 10$). At 3 and 6 weeks, marrow was aspirated from both knee joints, and at 12 weeks, mice were sacrificed and marrow was harvested from both femurs and both tibiae. Mean percentage (\pm SEM) of human cells in marrow is shown.

steadily increased for 6 weeks, at which time cultures were voluntarily ended (data not shown). These data indicate that cord blood precursors undergo a multiple-log expansion in the presence of I-Delta1^{ext-myc} together with fibronectin and 5GF.

Effect of cytokine combinations on precursor cell expansion due to I-Delta1^{ext-myc}. In initial experiments, we found that a combination of three growth factors (3GF), including

SCF, FL, and TPO, which substantially expand primitive human hematopoietic precursors (25, 32), were also essential for precursor cell expansion due to I-Delta1^{ext-myc} (data not shown). When IL-6 was used in addition to 3GF, CD34⁺CD38⁻ cells incubated for 19–20 days with cytokines in the presence of I-Delta1^{ext-myc} and CH-296 showed a 2.8-fold increase ($P < 0.05$) in the number of CD34⁺ cells in three independent experiments.

Addition of IL-3 or GM-CSF to the 4GF combination (SCF, FL, TPO, and IL-6) substantially increased the numbers of CD34⁺ cells ($P < 0.00005$ and $P < 0.0005$, respectively; Figure 2b). Although the addition of G-CSF to the 4GF showed only a modest increase in the number of CD34⁺ cells ($P < 0.05$), it did induce a substantially greater proportion (40%) of granulocytes expressing CD15 (Figure 2c). These data suggest that expansion of CD34⁺ cells due to I-Delta1^{ext-myc} and CH-296 is optimal in the presence of SCF, FL, TPO, IL-6, and IL-3 (5GF), but that in the presence of G-CSF, I-Delta1^{ext-myc} permits myeloid differentiation.

Immobilized Delta1^{ext-myc} enhances early T/NK differentiation. I-Delta1^{ext-myc} along with CH-296 and 5GF also induced a higher proportion of CD7⁺ cells compared with cultures containing NI-Delta1^{ext-myc} or control medium (data not shown), suggesting that Delta-1 may promote early lymphoid differentiation. In a subsequent study, immunofluorescence analysis of additional antigens associated with lymphoid differentiation revealed that a substantial portion of the CD7⁺ cells expressed CD34 (Figure 3a). These CD34⁺CD7⁺ cells also expressed CD45RA but little or no Thy-1 (data not shown), a phenotype (CD34⁺CD7⁺CD45RA⁺Thy^{low/-}) associated with common lymphoid precursors from cord blood (33, 34). A small portion of the CD7⁺ cells also expressed cytoplasmic CD3, a phenotype of T/NK cell precursors (35, 36) (Figure 3a). Expression of cytoplasmic CD3 was confirmed by RT-PCR of CD3ε mRNA (Figure 3b). In addition, cells expressing the NK cell marker CD56 (Figure 3a) were also generated. Thus, I-Delta1^{ext-myc} appears to induce CD34⁺CD38⁻ cells to differentiate along the lymphoid lineage, promoting the development of common lymphoid precursors, but also further directing their differentiation along the T/NK cell lineage. This is consistent with studies demonstrating promotion of T cell differentiation and inhibition of B cell differentiation by Notch signaling (15, 18–20).

Addition of IL-7, a cytokine known to support lymphoid differentiation (37, 38), to the cultures led to enhanced generation of CD34-CD7⁺ cells (Figure 3a) and of CD7⁺ cells that coexpressed the low-affinity IL-2α receptor (CD25; Figure 3a). No cells expressing surface CD3, T cell receptor-αβ or -γδ, or CD19 were detected (data not shown). Further, in IL-7-containing cultures,

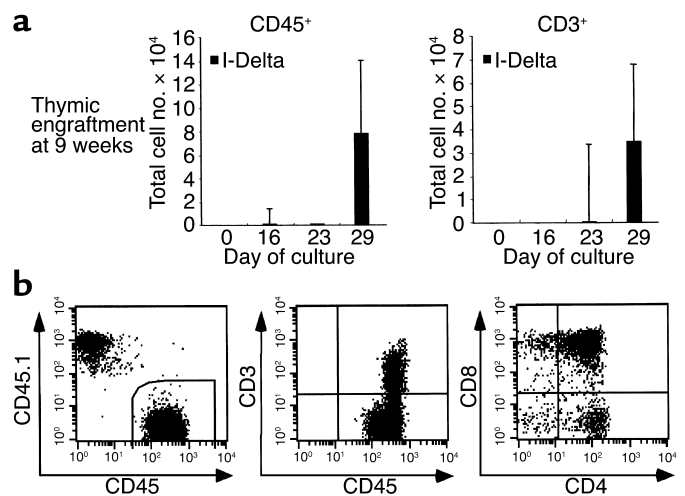
a deterioration in the expansion of CD34⁺ cells was observed after 3 weeks, and at 5 weeks there were five-fold fewer CD34⁺ cells but 11-fold more CD34⁺CD7⁺ cells compared with cultures lacking IL-7 (Figure 3c). In cultures containing control medium, few cells expressed CD34 or antigens associated with T cell differentiation in the presence or absence of IL-7 (Figure 3, a and c). These data indicate that IL-7 enhances the promotion of CD34⁺ cell differentiation along the T/NK cell lineage by I-Delta1^{ext-myc}.

I-Delta^{ext-myc} expands in vivo myeloid and lymphoid repopulating precursors. To test whether Delta1^{ext-myc} affects cells with in vivo repopulating ability, 4.7×10^3 CD34⁺CD38⁻ cells were cultured for 16, 23, or 29 days with I-Delta1^{ext-myc} or control medium together with CH-296 and 5GF. Following culture, all of the progeny derived from the starting population were injected into sublethally irradiated NOD/SCID β2m^{-/-} mice. Marrow from injected mice was aspirated at 3 and 6 weeks to assess marrow engraftment by short-term and relatively long-term reconstituting cells, respectively (39), and mice were sacrificed at 9 weeks to assess marrow engraftment and thymic reconstitution. Human cells were detected by staining with anti-human CD45 antibody and by excluding cells staining with anti-mouse CD45.1 antibody.

Injection of cells cultured with I-Delta1^{ext-myc} into immunodeficient mice resulted in engraftment of a greater number of CD45⁺ human cells in the aspirated marrow at 3 and 6 weeks after transplantation compared with the number in mice that received noncultured or control-cultured cells (Figure 4a). For example, transplantation of cells cultured for 29 days with 5GF and Delta-1 resulted in a 55-fold increase at 3 weeks ($P < 0.005$) and a 20-fold increase at 6 weeks ($P < 0.0005$) in the human cell engraftment compared with the number seen in noncultured cells (day 0), and a 43-fold increase at 3 weeks ($P < 0.05$) and a 16-fold increase at 6 weeks ($P < 0.005$) compared with that seen in control-cultured cells. At 3 weeks, engrafted human cells due to transplantation of

Figure 5

I-Delta1^{ext-myc} enhanced engraftment of human CD45⁺ or CD3⁺ T cells in the thymus of NOD/SCID β2m^{-/-} mice. We transplanted 4.7×10^3 CD34⁺CD38⁻ cells or all the progeny of 4.7×10^3 CD34⁺CD38⁻ cells cultured for 16, 23, or 29 days with 5GF, I-Delta1^{ext-myc}, and CH-296 into NOD/SCID β2m^{-/-} mice, and the thymus was harvested after 9 weeks. (a) Total numbers of human CD45⁺ and CD45⁺CD3⁺ cells in thymus were assessed. (b) The proportion of cells expressing CD45 in thymic cells (left panel), CD3 in thymic engrafted CD45⁺ cells (middle panel), and CD4 and/or CD8 in thymic engrafted CD3⁺ cells (right panel) are shown. Data are representative of three independent experiments.



noncultured cells or cells cultured with I-Delta1^{ext-myc} or control were mainly CD33⁺ myeloid cells (51% ± 2%, 71% ± 36%, and 72% ± 36%, respectively, mean ± SEM), and only 15% ± 7%, 7% ± 2%, and 16% ± 7%, respectively, were CD19⁺ B cells. However, at 6 weeks, the CD19⁺ B cell population represented 64% ± 11% and 41% ± 4% of the engrafted human cells due to transplantation of noncultured cells or cells cultured with I-Delta1^{ext-myc}, respectively, but only 10% ± 6% due to transplantation of control-cultured cells, while 24% ± 11%, 38% ± 9%, and 80% ± 9%, respectively, were CD33⁺ myeloid cells (Figure 4a).

At 9 weeks, assessment of total marrow contents from both femurs and both tibiae after sacrifice of mice showed that engraftment of human cells from cells cultured with I-Delta1^{ext-myc} was again greater than seen with control-cultured cells (Figure 4b). For example, transplantation of cells cultured for 29 days with 5GF and Delta1^{ext-myc} resulted in a 21-fold increase ($P < 0.05$) and a sixfold increase ($P < 0.05$) in engraftment compared with noncultured (day 0) or control-cultured cells, respectively. Similar to the findings at 6 weeks, 58% ± 3% and 56% ± 4% of the engrafted human cells in mice that received noncultured cells or cells cultured with I-Delta1^{ext-myc} were CD19⁺ B cells, but only 22% ± 13% were CD19⁺ B cells due to control-cultured cells, while engraftment of CD33⁺ myeloid cells was 22% ± 4%, 28% ± 2%, and 63% ± 16%, respectively. These data show that immobilized I-Delta1^{ext-myc} enhances short-term myeloid repopulating ability as well as relatively long-term myeloid and B cell repopulating ability in the marrow.

I-Delta1^{ext-myc} also enhances in vivo marrow-repopulating ability in NOD/SCID mice. Since it is possible that the enhanced marrow-repopulating ability due to I-Delta1^{ext-myc} resulted from mature progenitors able to grow in NOD/SCID $\beta 2m^{-/-}$ mice but not in conventional NOD/SCID mice (39), we also tested repopulating ability in the latter strain. We found that injection of cells cultured for 21 days with I-Delta1^{ext-myc} led to greater marrow reconstitution by CD45⁺ cells, consisting of both myeloid and B cells, from 3 to 12 weeks compared with noncultured cells and control-cultured cells (Figure 4c). Cells cultured with I-Delta1^{ext-myc} resulted in a 38-fold increase in the percentage of human CD45⁺ cells in marrow at 3 weeks ($P < 0.0005$), a 12-fold increase at 6 weeks ($P < 0.05$), and an 18-fold increase at 12 weeks ($P < 0.001$), respectively, compared with noncultured cells. Cells cultured with ligand led to a 13-fold increase in the percentage of human CD45⁺ cells in marrow at 3 weeks ($P < 0.001$), an 11-fold increase at 3 weeks ($P < 0.05$), and a threefold increase at 12 weeks ($P < 0.05$), respectively, compared to control-cultured cells. At 12 weeks, mice were sacrificed and the total CD45⁺ cell numbers in marrow were determined. We found that injection of cells cultured with I-Delta1^{ext-myc} resulted in engraftment of $1.0 \pm 0.6 \times 10^6$ cells, which was also significantly greater than the number resulting from noncultured cells ($6.8 \pm 3.7 \times 10^4$, $P < 0.01$) or

control-cultured cells ($2.5 \pm 2.0 \times 10^5$, $P < 0.05$). Similar results have been obtained in two additional experiments. Thus, it is unlikely that the observed enhanced marrow repopulation due to I-Delta1^{ext-myc} solely resulted from expansion of more mature progenitors only detectable in the NOD/SCID $\beta 2m^{-/-}$ mice strain of immunodeficient mice.

I-Delta1^{ext-myc} promotes the development of thymus-repopulating T cell precursors. Evaluation of thymic engraftment due to cells cultured with I-Delta1^{ext-myc} revealed reconstitution of the thymus by human CD3⁺ T cells at 9 weeks. This engraftment was greater following infusion of cells incubated in vitro for more than 25 days. After injection of all the progeny of 4.7×10^3 CD34⁺CD38⁻ cells cultured for 29 days with I-Delta1^{ext-myc} into NOD/SCID $\beta 2m^{-/-}$ mice, $7.8 \times 10^4 \pm 6.2 \times 10^4$ human CD45⁺ cells were detected in the thymus (24.9% ± 28.9% of thymic cells), 25.4% ± 18.0% of which were CD3⁺ T cells (Figure 5a). CD3⁺ cells mostly expressed both CD4 and CD8 (74%) (Figure 5b). In contrast, no or few (less than 200) human CD45⁺ cells and no CD3⁺ cells were engrafted in the thymus after infusion of 4.7×10^3 noncultured CD34⁺CD38⁻ cells (day 0), or the progeny of 4.7×10^3 CD34⁺CD38⁻ cells cultured for 16, 23, or 29 days with control medium (data not shown). Similar results were obtained in two additional experiments. These data suggest that, in addition to promoting expansion of myeloid and lymphoid marrow-repopulating cells, incubation with I-Delta1^{ext-myc} for relatively longer periods promotes the development of precursors capable of reconstituting thymus with CD3⁺ T cells.

Discussion

In this study, we report that ligand-induced Notch activation in cultured CD34⁺CD38⁻ cord blood precursors inhibited myeloid differentiation and induced a multiple-log expansion of CD34⁺ precursor cells. Notch activation also caused generation of cells with the phenotype of common lymphoid precursors (CD34⁺CD7⁺CD45RA⁺) (33, 34) as well as cells with cytoplasmic CD3 indicative of T/NK differentiation (35, 36). Thus, in addition to promoting T cell differentiation by common T and B cell precursors (15, 18–20), Notch signaling also promotes differentiation along the lymphoid lineage by multipotent CD34⁺CD38⁻ precursors (40, 41). These findings therefore represent, to our knowledge, the first successful utilization of defined regulatory molecules to promote lymphoid differentiation from nonmutant, multipotent hematopoietic precursor cells in the absence of stromal cells.

We also found that ligand-induced Notch activation in cultured CD34⁺CD38⁻ cord blood precursors substantially enhanced the generation of cells capable of in vivo myeloid and B cell marrow repopulation in immunodeficient mice. Previous studies have shown that Notch activation in murine hematopoietic precursors promotes lymphoid differentiation at the

expense of myeloid differentiation (10) as well as T cell differentiation at the expense of B cell differentiation (15, 17–20). Hence, while the generation of seemingly committed lymphoid precursors occurred at the expense of myeloid ones during in vitro culture with Notch ligand, cultures continued to contain cells capable of myeloid repopulation. We speculate, therefore, that induction of Notch signaling in cultures of pluripotent stem cell precursors leads to enhanced self-renewal as well as lymphoid differentiation, and that the observed myeloid repopulation resulted from increased numbers of pluripotent precursors. Moreover, since Notch signaling induced lymphoid precursors to undergo T/NK differentiation, enhanced B cell repopulation observed in these experiments was also likely derived from pluripotent precursors.

In addition, only cells cultured with Notch ligand reconstituted the thymus of immunodeficient mice with CD3⁺ cells, most of which were CD4⁺ and CD8⁺. Neither uncultured CD34⁺CD38⁻ cord blood precursors nor cells cultured with control medium reconstituted the thymus with CD3⁺ cells. These results suggest that a more committed T cell precursor capable of thymic reconstitution may be generated during in vitro culture of CD34⁺CD38⁻ precursors with Notch ligand. Since substantial numbers of human cells other than CD3⁺ T cells were co-engrafted in the thymus, the possibility remains that these human cells are also required for the observed development of CD3⁺ T cells within the thymus.

This study also indicates that the effect of Notch signaling on cell fate is dependent on the context of stimulating cytokines. I-Delta1^{ext-myc} in the presence of 4GF (SCF, FL, TPO, and IL-6) with or without GM-CSF and IL-3 inhibited myeloid differentiation and promoted early T cell differentiation, which was further enhanced with IL-7. Conversely, terminal myeloid differentiation occurred after the addition of G-CSF to 4GF and I-Delta1^{ext-myc} or in the absence of I-Delta1^{ext-myc}.

Only immobilized Delta1^{ext-myc}, but not Delta1^{ext-myc} in solution, activated HES-1, a target gene of Notch, and affected the differentiation of hematopoietic precursors. This is consistent with our previous findings that immobilization is required for Delta1^{ext-myc} to activate Notch and inhibit differentiation in C2 myoblasts (21), and that only immobilized Delta1^{ext-myc} affects human monocyte differentiation (4, 22). The effect of immobilized Delta1^{ext-myc} on precursors was further enhanced with fibronectin in serum-free cultures, possibly because adherence to fibronectin-coated culture plates enhances precursor binding to immobilized Delta1^{ext-myc}. Other laboratories recently reported that comparable Delta-1 constructs in solution showed an effect on CD34⁺ cell numbers, albeit a quantitatively less substantial one (3, 6), but no effect on T cell differentiation was noted. Further studies evaluating the possible advantages of immobilizing constructs used by other laboratories, and of using fibronectin, might be of interest.

In clinical bone marrow transplantation, the frequent inability to find human stem cell donors fully or partially matched with the recipient at loci encoding histocompatibility antigens remains a problem. Although cryopreserved cord blood cells present a readily available alternative, such a source often provides inadequate numbers of stem cells for prompt reestablishment of hematopoiesis in adults, and slow recovery has been associated with lethal infection (42, 43). Thus, the significant expansion of myeloid and lymphoid cells from cord blood stem cells observed in the present study points to the promise of enhancing the utility of human stem cell transplantation.

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