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Notch signals contribute to preserve the multipotentiality of human $CD34^+CD38^-CD45RA^-CD90^+$ hematopoietic progenitors by maintaining T cell lineage differentiation potential

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Notch signals are critical for T-cell development, limiting the differentiation potential of multipotent progenitors arriving in the thymus via the bloodstream. Notch ligands Delta-like and Jagged are expressed in the bone marrow and, consequently, a role in the regulation of early events of adult hematopoiesis has been proposed. However, mice with disruptions in the Notch pathway do not show gross defects in the hematopoietic stem cell compartment, limiting Notch effects at later stages of development. In this study, we identify cord blood $CD34^+CD38^-CD45RA^-CD90^+$ cells, a recently described population of hematopoietic stem cells, as one of the earliest targets of Notch in human hematopoiesis. Upon Notch activation, $CD34^+CD38^-$ cells are blocked in their differentiation at the $CD34^+CD38^-CD45RA^-CD90^+$ stage. Importantly, population and clonal analysis demonstrate that Delta-like–1 exposure does not affect lymphoid vs myeloid decisions. However, Notch signaling is required before lymphoid commitment to preserve T-cell potential of $CD34^+CD38^-CD45RA^-CD90^+$ cells. Our experiments also show that in terms of differentiation potential, $CD34^+CD38^-CD45RA^-CD90^+$ cells cultured in the presence of Notch signals, resemble cells directly isolated from cord blood. These results could have implications for translational efforts in the design of strategies aimed to accelerate immune reconstitution after transplantation. © 2012 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.

Under physiological conditions, adult hematopoietic stem cells (HSC) reside in bone marrow (BM)–specialized niches where they receive signals that tightly regulate their ability to undergo self-renewal and multipotential differentiation.

HSC activity has been detected in human $Lin^-CD34^+CD90^+$ and $CD34^+CD38^-$ cell populations [1,2]. However, in comparison to the mouse, fewer studies have addressed the hierarchical organization of the human hematopoietic system. Recently, it has been proposed that differential expression of CD45RA and CD90 on $CD34^+CD38^-$ cells can be useful to distinguish HSC ($CD34^+CD38^-CD45RA^-CD90^+$) cells from multipotential ($CD34^+CD38^-CD45RA^-CD90^-$) or more committed progenitors ($CD34^+CD38^-CD45RA^+CD90^-$) [3,4]. Developmental steps downstream of multipotent progenitors (MPP) are less clear and, although the existence of common myeloid and lymphoid progenitors [5] has been used to postulate a strict separation between lymphoid and myeloerythroid lineages, examples in humans and mice accumulate to challenge this hierarchical model of hematopoiesis [6–8].

Several developmentally conserved pathways, such as Notch, Hedgehog, and Wnt, have been implicated in regulating HSC function [9]. Notch signals have been shown to be essential for the emergence of definitive HSC during fetal life [10]; however, their role in adult hematopoiesis is still debatable. In BM, Notch ligands are expressed by

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stromal cells and can activate Notch receptors present in HSC [11]. Specifically, some components of the hematopoietic niche, such as osteoblasts and endothelial cells, express Delta-1ike–1 (DL1) and Jagged proteins [12], and experiments using soluble forms of this ligand have demonstrated its effect on the expansion and marrow repopulating ability of human immature hematopoietic cells [13,14]. Also from a genetic approach, gain-of-function experiments in both human and mice suggested that Notch signaling increases self-renewal and blocks differentiation of hematopoietic progenitors [15,16]. However, recent studies using dominant negative forms of Mastermind-like–1 or RBP/j have shown stable reconstitution of irradiated hosts and a normal frequency of long-term HSC, which strongly argues against an obligate role for Notch signaling in adult HSC maintenance [17]. Less controversial is its role in regulating lymphoid differentiation. In the thymus, Notch signals are critical for T-cell development, where they, acting presumably on MPP, induce a T-cell program while repressing differentiation to myeloid and other lymphoid lineages [18,19].

In this study, we investigate the effects of Notch activation by DL1 on the earliest human hematopoietic progenitors identified in cord blood (CB). We describe $CD34^+CD38^-CD45RA^-CD90^+$ cells as a new target of Notch activity in the regulation of human hematopoiesis. At this early stage of hematopoietic development, Notch can play a dual role, preserving the multipotentiality of $CD34^+CD38^-CD45RA^-CD90^+$ progenitor cells and favoring specification of the T-cell lineage.

Materials and methods

Cell source, flow cytometry analysis, and cell sorting

CB samples were provided by the Blood and Tissue Bank (Barcelona, Spain) in accordance with the ethical committee of Institut de Recerca Hospital Vall d'Hebron. Informed consent was obtained from all mothers. Mononuclear cells were isolated by density gradient (Lymphoprep; Axis-Shield PoC AS, Oslo, Norway) and $CD34^+$ cells were enriched using magnetic-activated cell sorting (Miltenyi Biotech, Bergisch-Gladbach, Germany). Unless otherwise stated, 4 to 5 CB units were pooled in each experiment.

Cell-surface staining was performed using the following anti-human antibodies: CD34-allophycocyanin (APC), CD38–fluorescein isothiocyanate (FITC), CD90-phycoerythrin (PE) (BD Pharmingen, Franklin Lakes, NJ, USA), CD45RA-Alexa700, anti-IL7Rα-biotin (BioLegend, San Diego, CA, USA), and CD7-biotin (Caltag; Invitrogen, Carlsbad, CA, USA). Streptavidin–Alexa 405 was purchased from Invitrogen. Sorting was performed in either FACSAria (BD Biosciences) or MoFlo (Beckman-Coulter, Brea, CA, USA) cell sorters. Data were analyzed using FACS Express 3.0 (De Novo Software, Los Angeles, CA, USA).

In vitro expansion

$CD34^+CD38^-$ cells (typically $1-1.2 \times 10^4$ cells per well in 12-well plates) were cocultured for 8 days with confluent OP9 or OP9-DL1 murine stromal cell lines (kindly provided by Dr. Hergen Spits, Department of Cell Biology and Histology, University of Amsterdam, Amsterdam, The Netherlands) using minimum essential medium–α (Invitrogen) supplemented with 20% Fetalclone I (Hyclone; ThermoFisher Scientific, Waltham, MA, USA), 1% antibiotic-antimycotic (Invitrogen) in the presence of 10 ng/mL human stem cell factor (SCF), thrombopoietin (TPO), and fms-like tyrosine kinase receptor–3 (Flt3L) (Immuno-Tools, Friesoythe, Germany). Medium and cytokines were replaced every 3 days. When indicated, the γ-secretase inhibitors N-[N-(3,5-difluorophenacetyl)-L-alanyl]-(S)-phenylglycine t-butyl ester (DAPT) (Sigma-Aldrich, St Louis, MO, USA) and (S,S)-2-[2-(3,5-Difluorophenyl)acetylamino]-N-(5-methyl-6-oxo-6,7-dihydro-5H-dibenzo[b,d]azepin-7-yl)propionamide (DBZ) (Merck, Darmstadt, Germany) were added to the culture.

Differentiation assays

Sorted populations were cocultured in 24-well plates containing confluent stromal cells in minimum essential medium–α supplemented with different cytokine combinations to promote differentiation to the different lineages: Flt3L, SCF, and TPO for myeloid cells; Flt3L, SCF, TPO, and interleukin (IL)-15 (from ProSpec, Ness-Ziona, Israel) for natural killer (NK) cells and Flt3L, SCF, TPO and IL-7 for B cells. All the cytokines were used at 10 ng/mL. Cells were cultured on OP9 cells during 19 to 21 days with weekly changes of medium, stroma, and cytokines. TPO was added only during the first week. To induce T-cell differentiation, cells were cultured for 28 to 30 days on OP9-DL1 with Flt3L and IL-7 at 5 ng/mL each. Analysis was performed by flow cytometry in a FACSCalibur (BD Biosciences) after labeling with CD15-FITC (Immunotech) and CD14-PE (BioLegend) to identify myeloid cells; CD56-FITC (BD Biosciences) for NK cells; CD10-APC (BioLegend) and CD19-PE (Pharmingen) for B cells; and CD8-FITC (BD Biosciences), CD3-PE (BioLegend), and CD4-APC (Pharmingen) for T cells.

Clonogenic differentiation assays

Once the first phase of expansion in the presence of stroma had been completed, $CD34^+CD38^-CD45RA^-CD90^+$ cells were sorted in a 96-well plate at one cell per well and cultured in HPO1 serum-free medium (Maco Biotech cell culture; Maco-Pharma, Mouvaux, France) supplemented with 50 ng/mL each of SCF, TPO, Flt3L, and IL-6. After 8 days, proliferating wells (>10 cells) were scored as positive and divided into two wells. To obtain T cells, one half was added into a well containing irradiated OP9-DL1 and medium supplemented with 5 ng/mL each of Flt3L and IL-7 during 28 days. The other half was added on to a well containing irradiated OP9 cells and medium with 10 ng/mL each of Flt3L, SCF, IL-7, and IL-15 during 21 days to obtain myeloid, NK, and B cells. IL-15 was added only from the second week. Analysis was performed by flow cytometry in a FACSCalibur after labeling with CD14-PE (myeloid cells), CD56-FITC (NK cells), CD19-PE (B cells), and CD8-FITC, CD3-PE, and CD4-APC (T cells).

Statistics

Statistical analysis using Student's t test for paired and unpaired data was performed with GraphPad Prism software (La Jolla, CA, USA). A p value ≤ 0.05 was considered statistically significant ($^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

Results

Notch activation blocks differentiation of human hematopoietic progenitors at the $CD34^+CD38^-CD45RA^-CD90^+$ stage

In the BM microenvironment, HSC are exposed to Delta-like and Jagged proteins [12]. In order to examine the role of DL1 signals in the expansion and differentiation of human hematopoietic progenitors, we cocultured $CD34^+CD38^-$ cells purified from human CB with a stromal cell line (OP9) or with its equivalent overexpressing DL1 (OP9-DL1). Compared to OP9 cultures, we observed that Notch activation led to a strong reduction in the number of nucleated cells (Fig. 1A). In this situation, we also

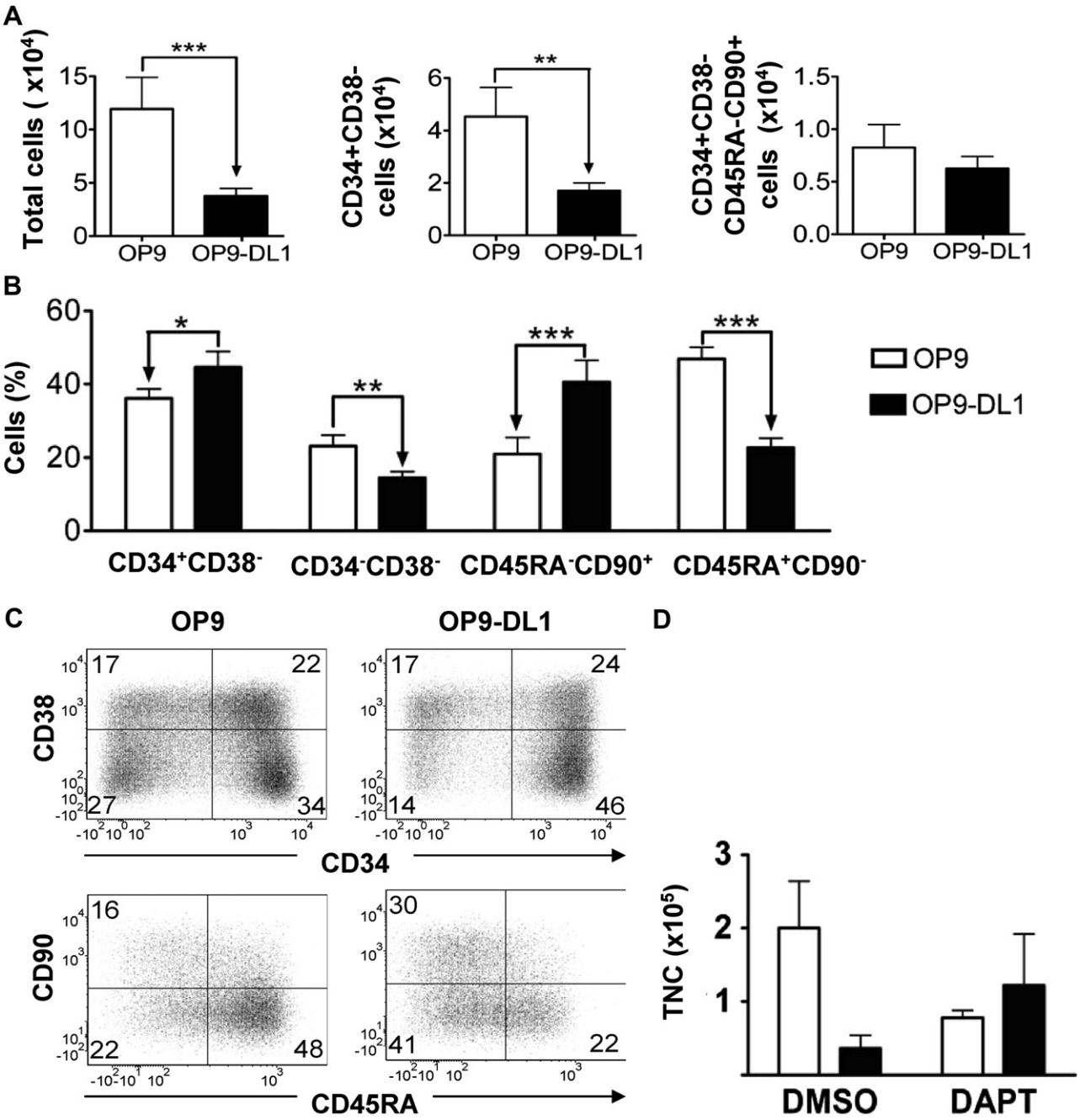

Figure 1. Notch signaling regulates expansion and differentiation of $CD34^+CD38^-$ CB cells. Human $CD34^+CD38^-$ cells were cultured on OP9 or OP9-DL1 cells for 8 days. Cultures were initiated with 10^4 purified cells. (**A**) Absolute numbers of total nucleated cells, $CD34^+CD38^-$ cells and the $CD34^+CD38^-CD45RA^-CD90^+$ subpopulation after 8 days of culture are shown. (**B**) Chart represents percentages of the indicated populations. CD45RA/CD90 percentages are shown after gating on $CD34^+CD38^-$ cells. (**C**) Representative flow cytometry plots showing expression of CD34/CD38 (upper panel) and CD90/CD45RA after gating on $CD34^+CD38^-$ cells (lower panel). (**D**) Cells were cultured as before in the presence of solvent (dimethyl sulfoxide [DMSO]) or DAPT (10 μM). Total number of cells recovered from the cultures is indicated.

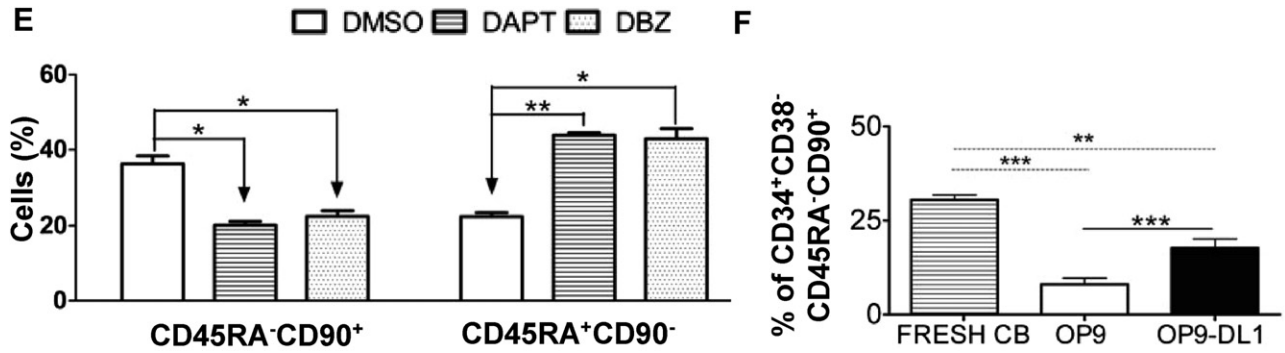

Figure 1. (*Continued*) (**E**) Cells were cultured in the presence of DMSO, DAPT (10 μM) or DBZ (100 nM). After 8 to 10 days, flow cytometry was performed. Percentage of CD45RA/CD90 subsets after gating on $CD34^+CD38^-$ cells is indicated. Error bar represents standard error of mean (SEM) (n = 3). (**F**) Percentage of $CD34^+CD38^-CD45RA^-CD90^+$ cells in the cultures at the beginning (fresh CB) or after 8 days in contact with OP9 cell lines. Error bar represents SEM (n = 10).

noticed a small but significant increase in the percentage of $CD34^+CD38^-$ cells, whereas $CD34^-CD38^-$ cells prevailed in the absence of Notch activating signals (Fig. 1B).

CD45RA and CD90 markers have been used to define different HSC subpopulations [3]. As shown in Figure 1B, Notch activation caused an increase in the percentage of $CD34^+CD38^-CD45RA^-CD90^+$ cells. In contrast, the percentage of the most differentiated $CD34^+CD38^-CD45RA^+CD90^-$ cells was higher in OP9 cultures. The relative increase of $CD34^+CD38^-$ and $CD34^+CD38^-CD45RA^-CD90^+$ populations suggests that hematopoietic progenitors are less differentiated in OP9-DL1 cultures.

To demonstrate Notch involvement in the effects described, cells were cultured in the presence of either DAPT or DBZ, γ-secretase inhibitors widely used to prevent activation of Notch pathway. Noticeably, both inhibitors reverse to some extent the observed decrease in the total number of cells recovered from cultures done in the presence of DL1 signals (Fig. 1D and data not shown). In this setting, the percentage of $CD34^+CD38^-CD45RA^-CD90^+$ cells after coculturing $CD34^+CD38^-$ progenitors with OP9-DL1 cells is clearly reduced in comparison with cultures done in the presence of the vehicle (Fig. 1E).

At the initiation of the cultures, approximately one third of the cells plated were $CD34^+CD38^-CD45RA^-CD90^+$ (31.4% ± 4.0%). After 8 days in culture, this percentage had decreased remarkably, with significant differences between the two cocultures. In OP9-DL1 cultures, $CD34^+CD38^-CD45RA^-CD90^+$ cells represented 17.8% ± 8.5% of the cells recovered from the plates, whereas they were only 8.0% ± 5.8% in the OP9 cultures (Fig. 1F). In contrast, more mature $CD34^-CD38^+$ and $CD45RA^+CD90^-$ cells were over-represented in OP9 vs OP9-DL1 cultures. In absolute terms, no significant differences were found between the number of $CD34^+CD38^-CD45RA^-CD90^+$ cells recovered in cultures made under these two experimental conditions, with a similar two- to threefold increase over the input. These data indicate a preferential enrichment in $CD34^+CD38^-CD45RA^-CD90^+$ cells in the OP9-DL1 cultures. Taken together, these data suggest that upon Notch activation, $CD34^+CD38^-$ cells block their differentiation at the $CD34^+CD38^-CD45RA^-CD90^+$ stage.

Notch activation maintains multilineage potential of $CD34^+CD38^-CD45RA^-CD90^+$ cells

To study the effect of Notch activation on the differentiation potential of $CD34^+CD38^-CD45RA^-CD90^+$ cells present in the culture, after 8 days on stroma these cells were sorted and placed under specific conditions to stimulate development of myeloid and lymphoid lineages.

Regardless of whether cells had received DL1 signals, purified $CD34^+CD38^-CD45RA^-CD90^+$ cells were able to differentiate to all the lineages tested. However, some differences were noted; $CD34^+CD38^-CD45RA^-CD90^+$ cells harvested from OP9-DL1 cocultures generated a significantly higher percentage of T cells than those obtained from cultures unexposed to Notch activating stroma, whereas the opposite was true for B and NK cells and no definitive trend was perceptible for myeloid cells (Fig. 2A and Supplementary Figure E1; online only, available at www.exphem.org). However, inclusion of γ-secretase inhibitors during the first phase of the culture minimized the development of a T-cell program from the $CD34^+CD38^-CD45RA^-CD90^+$ cells harvested from OP9-DL1 cultures (Fig. 2B). Interestingly, cells with erythroid features were also raised from progenitors isolated from both culture conditions (Supplementary Figure E1; online only, available at www.exphem.org).

Although these results are suggestive of the multilineage potential of $CD34^+CD38^-CD45RA^-CD90^+$ cells, with this analysis it is not possible to rule out the presence of a mixture of lineage-committed progenitors generated during the culture. Thus, we designed a two-phase clonal assay (Fig. 3A). $CD34^+CD38^-CD45RA^-CD90^+$ cells obtained after culturing hematopoietic progenitors on OP9 or OP9-DL1 stroma

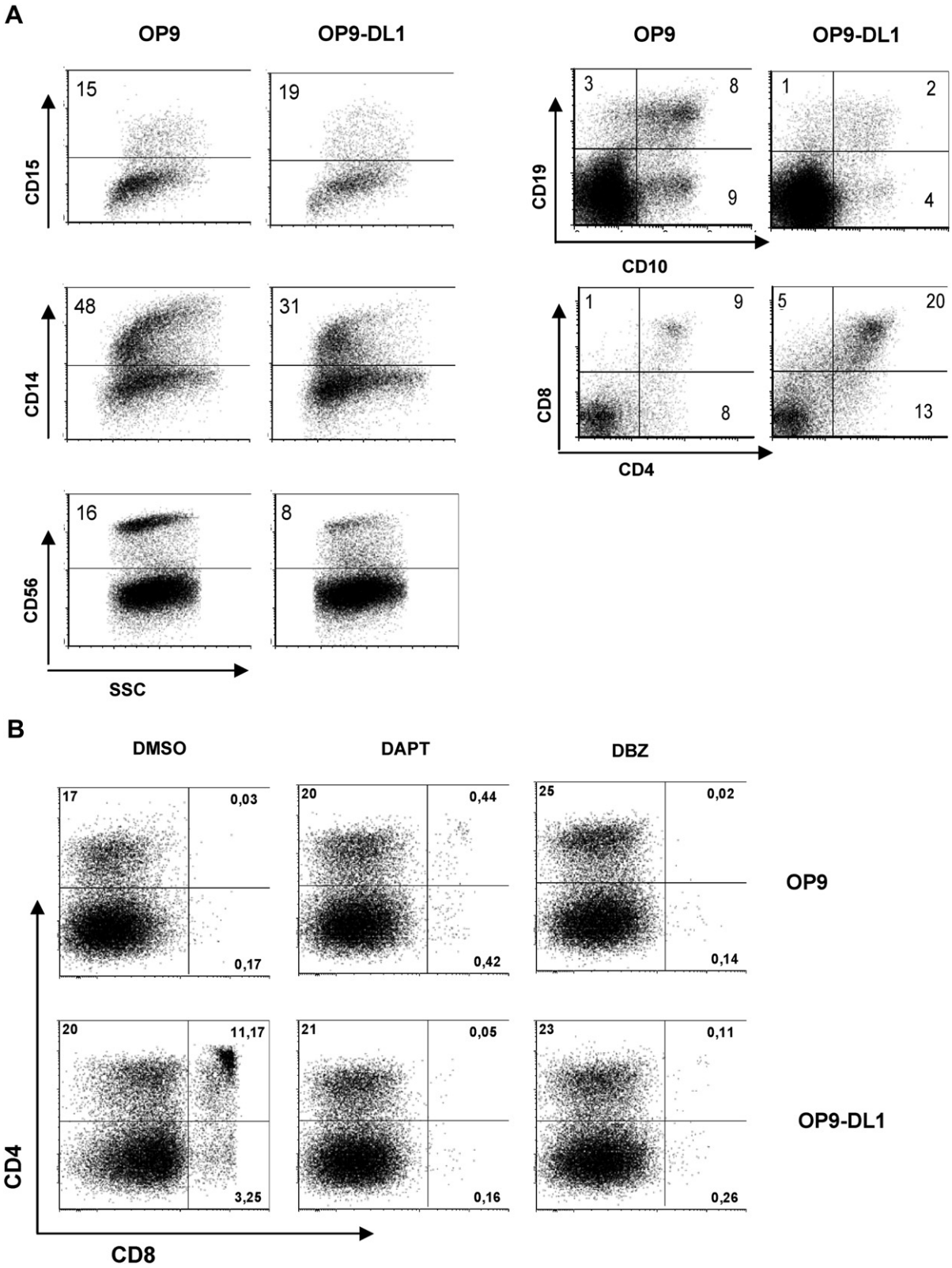

Figure 2. Lympho-myeloid potential of $CD34^+CD38^-CD45RA^-CD90^+$ cells. Human $CD34^+CD38^-$ cells were cultured on OP9 or OP9-DL1 monolayers. After 8 days, cells were harvested and sorted $CD34^+CD38^-CD45RA^-CD90^+$ cells were incubated in defined conditions to allow development of different lineages (see Material and Methods). (**A**) The phenotype was assessed after labeling with CD15 and CD14 for myeloid cells, CD56 for NK cells, CD10 and CD19 for B cells, and CD8 and CD4 for T-cell development. Plots are representative of three independent experiments. (**B**) $CD34^+CD38^-$ cells were cultured as before in the presence of either solvent or gamma secretase inhibitors. After 8 days, sorted $CD34^+CD38^-CD45RA^-CD90^+$ cells were cultured in T-cell differentiation conditions. Charts represent flow cytometry plots of a representative experiment (n = 3) showing the expression of CD4 and CD8 markers.

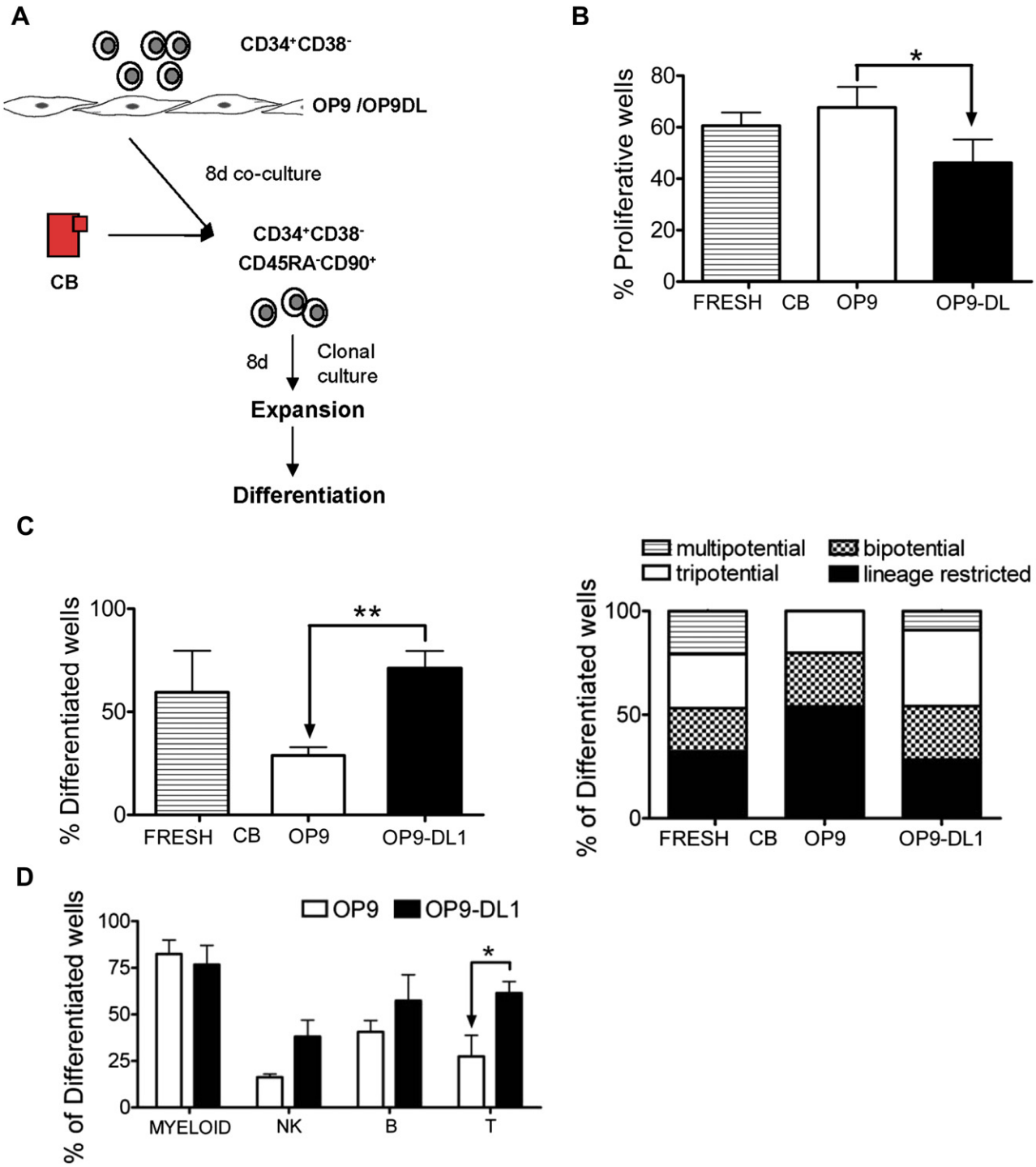

Figure 3. Notch pathway maintains multilineage potential of $CD34^+CD38^-CD45RA^-CD90^+$ cells. (**A**) Experimental design to evaluate the ability of $CD34^+CD38^-CD45RA^-CD90^+$ cells to proliferate and differentiate. $CD34^+CD38^-CD45RA^-CD90^+$ cells, either directly isolated from CB or obtained from cultures of $CD34^+CD38^-$ cells on OP9 or OP9-DL1 monolayers, were individually sorted into serum-free medium with cytokines and cultured for 8 days. Wells with positive growth were then tested for differentiation. More than 480 wells were analyzed. (**B**) Wells were scored as proliferation-positive when >10 cells were present in the well. Percentage of positive wells is represented as mean of the results from three independent experiments. (**C**) The content of wells with positive growth was divided and cultures were set to allow development of mature blood cells (see Material and Methods). Wells were considered positive for differentiation when mature cells originated from a single colony could be identified in at least one condition. Chart shows the percentage of colonies able to originate a differentiated progeny and represents the mean and standard error of mean (SEM) of three independent experiments. In the right panel, the distribution of wells containing mature cells is shown, according to the ability of the original colony to generate progeny of the different lineages. Wells containing T cells, B cells, NK cells, and myeloid cells are labeled as multipotential, whereas clones that originate cells of two or three different lineages are designated as bi- or tripotential and they are marked as lineage restricted if only one cell type is detected. (**D**) Percentage of wells containing mature cells of the indicated lineage. Data represent the mean of independent experiments. Error bar represents SEM.

(hereafter referred to as $CD34^+CD38^-CD45RA^-CD90^+$/OP9 and /OP9-DL1 cells, respectively) were single-cell sorted and seeded onto individual wells containing serum-free medium and cytokines. Freshly isolated $CD34^+CD38^-CD45RA^-CD90^+$ cells were used for comparison. After 8 days, wells with >10 cells were scored as positive for proliferation. After evaluating >480 wells in three independent experiments, we observed a significantly lower frequency of positive wells in those seeded with $CD34^+CD38^-CD45RA^-CD90^+$/OP9-DL1 cells (46.7% ± 9.0% vs 67.7% ± 7.0% in the case of wells seeded with $CD34^+CD38^-CD45RA^-CD90^+$/OP9 cells). We then investigated the ability of these differentially raised $CD34^+CD38^-CD45RA^-CD90^+$ cells to generate mature progeny. The content of each positive well was divided to culture the cells in defined conditions for the generation of myeloid, NK, B, and T cells. $CD34^+CD38^-CD45RA^-CD90^+$/OP9-DL1 cells gave rise to more wells containing at least one type of mature cell (71.2% ± 8.3% vs 28.8% ± 4.0% in cultures derived from $CD34^+CD38^-CD45RA^-CD90^+$/OP9 cells). Noticeably, although $CD34^+CD38^-CD45RA^-CD90^+$/OP9-DL1 cells had a differentiation potential similar to that of freshly isolated CB progenitors, in terms of proliferation these uncultured cells more closely resembled those purified from OP9 cocultures (Fig. 3B, C).

According to the differentiation potential of the single-sorted cells, it was possible to classify the wells into four groups; 10% of $CD34^+CD38^-CD45RA^-CD90^+$/OP9-DL1 cells could generate mature cells of the four lineages tested (Fig. 3C right panel, and Supplementary Figure E2; online only, available at www.exphem.org), whereas none of the $CD34^+CD38^-CD45RA^-CD90^+$/OP9 cells produced a four-lineage progeny. In addition, $>50\%$ of cells arising from cultures performed without Notch ligands restricted their differentiation potential to only one cell type, whereas in the presence of DL1, this percentage was $<25\%$. Again, the differentiation pattern of the $CD34^+CD38^-CD45RA^-CD90^+$/OP9-DL1 cells resembled that of cells directly isolated from CB. Detailed analysis of the different cell types present in the wells revealed that although a previous contact with a Notch ligand did not influence development of mature myeloid or B cells, the percentage of T-cell–containing wells was significantly higher ($p < 0.05$) in the plates seeded with $CD34^+CD38^-CD45RA^-CD90^+$/OP9-DL1 cells (Fig. 3D). Also, in this later situation, there were more wells with NK content, although without reaching statistical significance. In summary, these experiments suggest that Notch signals are critical to preserve differentiation potential, and specifically the ability to generate T-cell progenitors.

T-cell lineage specification program on $CD34^+CD38^-CD45RA^-CD90^+$ cells: early signs of commitment via DL1

Current views of T-cell development sustain that different cell types in the BM and CB have the potential to give rise to T cells [6,20,21]. Once in the thymus, activation of the Notch pathway will restrict the developmental potential of these hematopoietic progenitors to the T-cell lineage [19].

Results from our differentiation assays (Figs. 2, 3D) indicate that Notch activation during the first stage of culture, while favoring T-cell commitment, does not suffice to block development of non-T-cell–committed progenitors, suggesting that Notch may be acting on multilineage progenitors to promote T-cell specification. With this in mind, we investigated the possibility that these cells could already express surface markers characteristic of T lymphoid progenitors.

In CB, the acquisition of CD7 by $CD34^+CD38^-$ HSC has been proposed to distinguish primitive from more committed human lymphoid progenitors and also from pluripotent stem cells [21,22]. We analyzed CD7 expression in cells harvested after culturing $CD34^+CD38^-$ progenitors on stromal monolayers. Regardless of the population studied, the percentage of cells expressing CD7 was higher under Notch-activating conditions (Fig. 4A). This effect was already evident at the most immature $CD34^+CD38^-CD45RA^-CD90^+$ stage, where approximately 20% of the cells expressed CD7 and the percentage was even higher (70%) among the $CD34^+CD38^-CD45RA^+CD90^-$ cells, the most differentiated subset (Fig. 4C). This pattern is not observed in the absence of DL1-mediated signals, where the percentage of $CD7^+$ cells was similar in all the CD45RA/CD90 populations. Moreover, induction of CD7 expression was not seen when cultures were performed in the presence of a γ-secretase inhibitor (data not shown). Interestingly, in fresh CB, CD7 is preferentially expressed in the $CD45RA^+CD90^-$ population with few $CD7^+$ cells in the $CD45RA^-CD90^+$ subset. In summary, these results suggest that DL1 induces a specification program toward T-cell lymphoid lineage that can start as early as the $CD34^+CD38^-CD45RA^-CD90^+$ stage.

In addition to Notch, other signals are required to complete T-cell specification and expansion of committed progenitors. Constitutive activation of Notch1 in human hematopoietic progenitors increases expression of the IL-7 receptor (IL7Rα) [23], and IL-7 has been proposed to play a role in MPP differentiation into lymphoid progenitors [24,25]. To investigate whether the effects of Notch might be due to IL-7–mediated expansion of a subset of progenitors, IL7Rα expression was determined by flow cytometry. Few cells expressed IL7Rα within the $CD34^+CD38^-CD45RA^-CD90^+$ population, and in none of the situations assessed could we observe significant differences between the different $CD34^+CD38^-$ subsets compared (Fig. 4B). These observations indicate that differential IL7Rα expression does not account for the Notch-mediated expansion of T lymphoid progenitors observed in the differentiation assays.

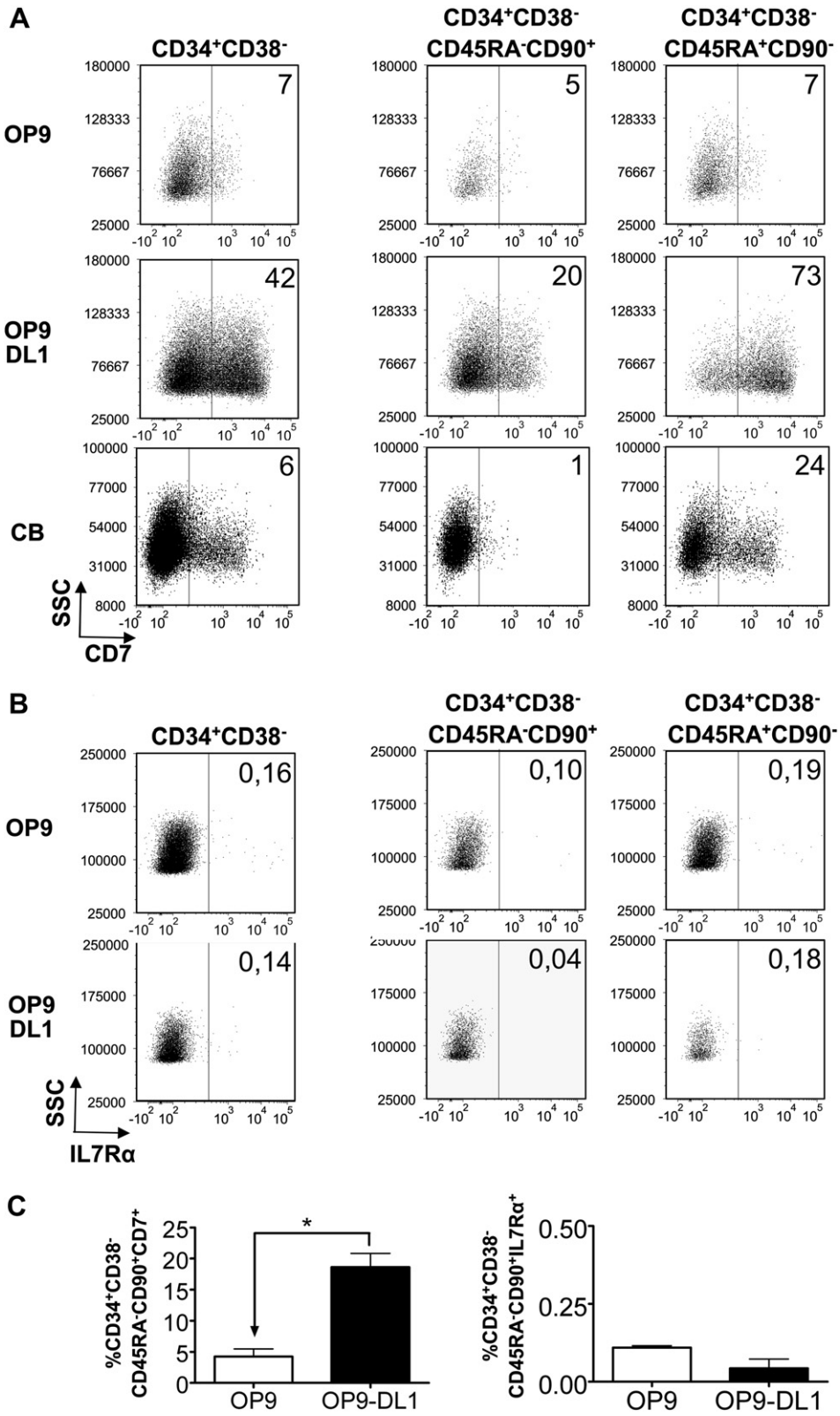

Figure 4. DL1 signals induce signs of early lymphoid commitment of $CD34^+CD38^-CD45RA^-CD90^+$ cells. Human $CD34^+CD38^-$ CB cells were cultured on OP9 or OP9-DL1 monolayers for 8 days. Cells were then harvested and the expression of CD7 (**A**) and IL7Rα (**B**) analyzed by flow cytometry. Different gated populations are shown. In (**A**), equivalent populations of fresh CB are included for comparison. Plots are representative of three independent experiments. (**C**) Percentage of CD7 or IL7Rα expressing $CD34^+CD38^-CD45RA^-CD90^+$ cells in OP9 or OP9-DL1 cultures. Error bar represents standard error of mean.

Discussion

There is abundant literature describing how Notch signals affect proliferation and cell fate decisions at different stages of hematopoietic development, including HSC self-renewal and differentiation [15,16] or T/B lineage choice in common lymphoid progenitors [18,19]. Our studies identify $CD34^+CD38^-CD45RA^-CD90^+$ cells as a new target of Notch in human hematopoiesis. Furthermore, we have found that it is possible to preserve T-cell developmental potential on this population of MPP by using culture conditions designed to activate Notch by DL1, a Notch ligand that is expressed in the BM microenvironment [12,13,26].

Analysis of these cultures reveals that Notch-activating signals inhibit differentiation of $CD34^+CD38^-$ cells, and identify the $CD34^+CD38^-CD45RA^-CD90^+$ population as one of the earliest points at which this blockade occurs. Although population assays do not evidence differences in the expansion of $CD34^+CD38^-CD45RA^-CD90^+$ cells, studies done at the clonal level clearly demonstrate that $CD34^+CD38^-CD45RA^-CD90^+$ cells exposed to DL1 exhibit a lower proliferative response than cells cultured in the absence of Notch activation. A role of Notch in the regulation of cell proliferation has been suggested previously by studies showing up-regulation of Hes1 in nonproliferating cells, such as quiescent fibroblasts or neural and hematopoietic stem cells [27–29]. Despite these differences, after culture, $CD34^+CD38^-CD45RA^-CD90^+$ cells still retain the capacity to give rise to all the lineages tested, including mature myeloid and erythroid cells (Supplementary Figure E1; online only, available at www.exphem.org). These data strongly argue in favor of Notch acting on very early progenitors and not only on committed precursors.

Moreover, $CD34^+CD38^-CD45RA^-CD90^+$/OP9-DL1 cells, despite having lower proliferative activity, displayed a higher capacity for differentiation than $CD34^+CD38^-CD45RA^-CD90^+$/OP9 cells. This might be explained by exhaustion of this competence in these later cells during the successive expansion rounds [30] or alternatively by a direct effect of Notch on the differentiation potential of $CD34^+CD38^-CD45RA^-CD90^+$ cells similar to that reported in other developmental and differentiation systems [31–33]. Despite that Notch activation could result in a reduced number of cell divisions during the first phase of the culture, the absolute number of $CD34^+CD38^-CD45RA^-CD90^+$ cells recovered from cultures done in the presence of either OP9 or OP9-DL1 cells was similar. Consequently, the effects we observed when we studied the proliferation and differentiation potential of $CD34^+CD38^-CD45RA^-CD90^+$ cells might be due to a mark imposed by Notch activation during the first stage of the cultures.

In terms of differentiation potential, $CD34^+CD38^-CD45RA^-CD90^+$/OP9-DL1 cells resemble MPP isolated from fresh CB. In contrast, $CD34^+CD38^-CD45RA^-CD90^+$/OP9 cells appear to lose this feature, becoming more restricted to a single lineage. Careful analysis of the cell types generated from individual cells allowed us to conclude that T cells, and to a lower extent NK cells, appear more frequently in colonies arising from $CD34^+CD38^-CD45RA^-CD90^+$/OP9-DL1 cells. Strikingly, although it was possible to find wells containing T cells only, this occurred more often when the original cell placed in the well came from cultures unexposed to DL1. At this stage, Notch would act preserving early progenitor's potentialities rather than blocking development of non–T lineages.

Notch receptors and their ligands are important regulators of T-cell commitment [34]. Using the OP9-DL1 system, CD7 expression has been defined as one of the first indications of T-cell development [35]. Very relevantly, Awong et al. have recently shown that $CD34^+CD45RA^+CD7^+$ cells, generated in vitro using this system, have thymus-reconstituting ability in vivo. They also described the appearance of two $CD34^+CD45RA^+CD7^+$ subpopulations characterized by differential expression of CD5. Although both of them can originate T cells in culture, they show a different ability to enter and reconstitute a mouse thymus in vitro [36]. Our data indicate that upon Notch activation, CD7 is already detected at the $CD34^+CD38^-CD45RA^-CD90^+$ stage, and the percentage is increased in the most mature $CD34^+CD38^-CD45RA^+CD90^-$ cells, recently characterized as lymphomyeloid progenitors [6]. This pattern of expression is similar to that seen in freshly isolated CB cells and confirms the sequential relationship between these two stages of development. However, the percentage of $CD34^+CD38^-CD45RA^-CD90^+$ cells expressing CD7 increases after in vitro culture, which suggests that DL1 signals could be inducing early T-cell specification in MPP. Although additional work is needed to define the intrinsic characteristics and function of these particular cells, recent studies support this view, showing that although the lack of CD7 expression in the $CD34^+CD38^-$ subset of CB cells identifies an immature progenitor capable of generating both lymphoid and myeloerythroid cells, its presence defines progenitors more committed to the T-cell lineage [21]. In contrast to these findings, IL7Rα expression was not detected in any of the $CD34^+CD38^-$ subpopulations analyzed and was instead restricted to more mature cells (Supplementary Figure E3; online only, available at www.exphem.org).

Besides their role in the thymus as regulators of lineage commitment and development, Notch receptors are important for homeostasis of the BM stem cell pool. Qualitative or quantitative differences of Notch ligands at these two locations can explain this duality. The importance of quantitative aspects of Notch signaling has already being noted during Drosophila development, where different thresholds of Notch activation are required for different functions [37]. Likewise, low doses of Notch ligands appear to operate in

steady state in BM, while continuous activation of this pathway is required to sustain T-cell identity in the thymus [18,19]. It has been reported that culturing human hematopoietic progenitors with low densities of DL1 promoted the expansion of SCID-repopulating cells, whereas higher amounts induced cell death. Noticeably, an increase in generation of $CD7^+$ thymus-repopulating T-cell precursors was observed in all the cases [38]. Early expression of CD7 in $CD34^+CD38^-CD45RA^-CD90^+$/OP9-DL1 suggests an initial T-cell lineage specification in progenitors with a multilineage potential. Interestingly, cells expressing CD7 were able to differentiate to the myeloid lineage, indicating that the state of these cells is not fully determined (Supplementary Figure E4; online only, available at www.exphem.org). To note, a $CD34^+CD1^-CD7^+$ population isolated from human thymus is also capable of generating myeloid cells [39].

In humans, recovery of T-cell compartment after transplantation is hampered by aging, probably due to age-related changes in the thymic stroma [40]. Also, HSC isolated from aged mice have compromised their T-cell potential when transplanted into young recipients, whereas myelopoiesis is not affected [41]. Our results indicated that, cultured in the absence of Notch-activating signals, MPP can lose their ability to generate T cells. It is likely that changes in the density or quality of the signals provided by the niche in the BM (e.g., Notch) can alter the developmental potential of hematopoietic progenitors.

T-cell lymphopenia after transplantation is behind the great incidence of potentially fatal viral infections that these patients suffer. Establishment of $CD34^+CD38^-CD45RA^-CD90^+$ cells as a new target of Notch pathway and as the earliest population susceptible to showing certain degrees of T-cell specification might open the possibility to design ways to accelerate the reconstitution of the lymphoid compartment after hematopoietic transplantation [42,43].

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Conflict of interest disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

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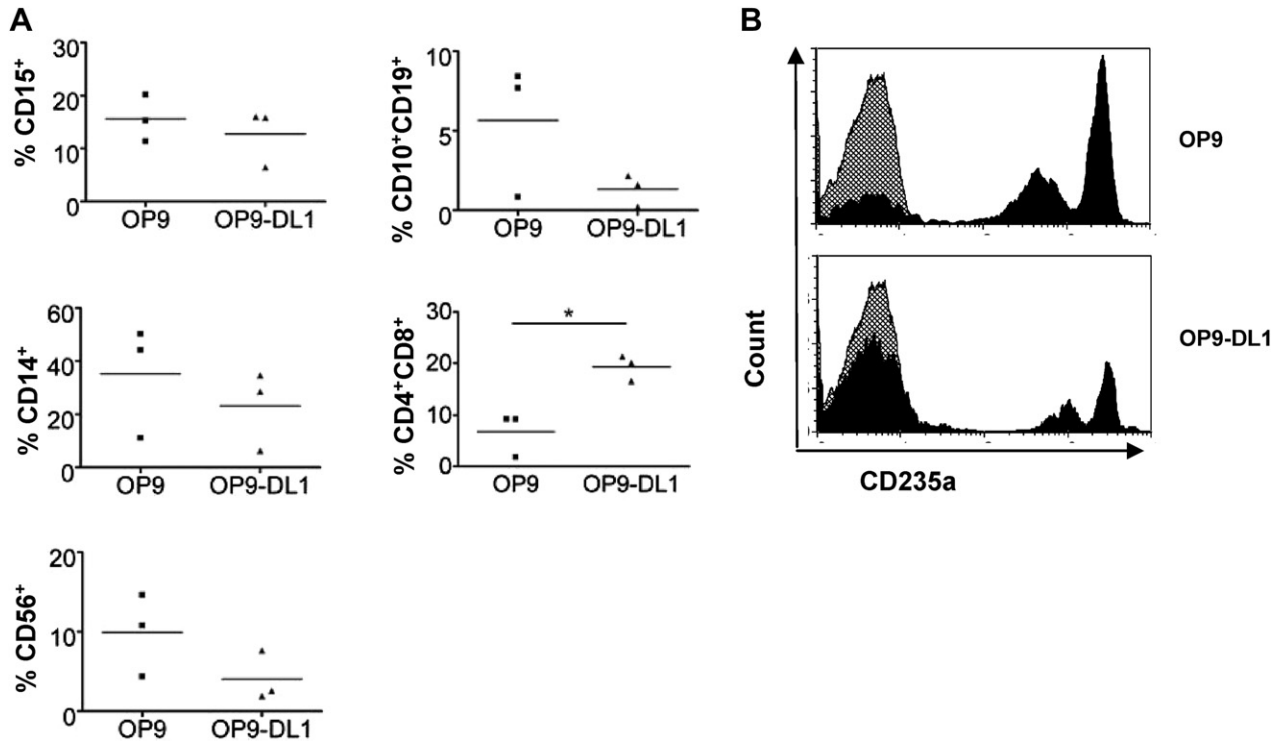
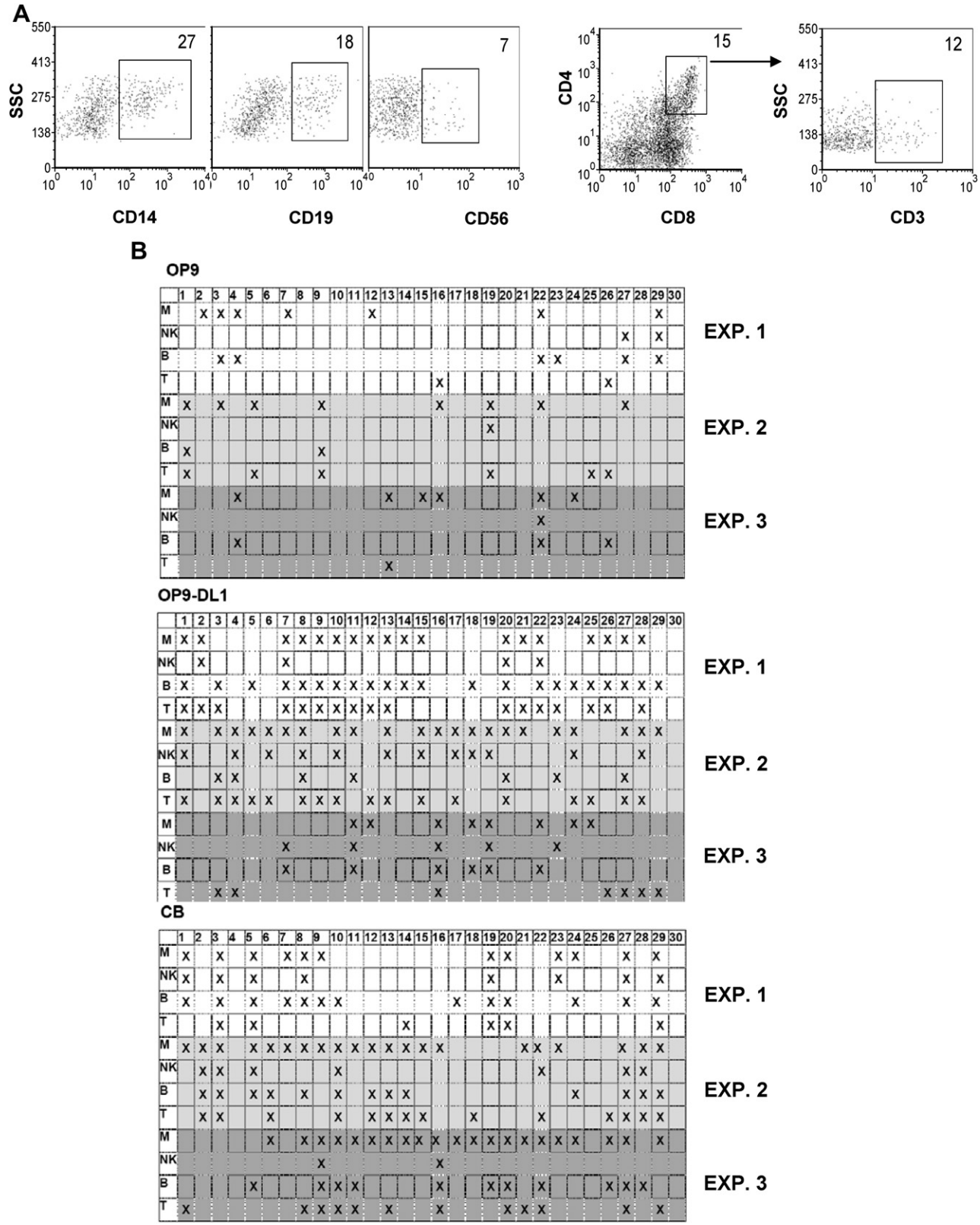
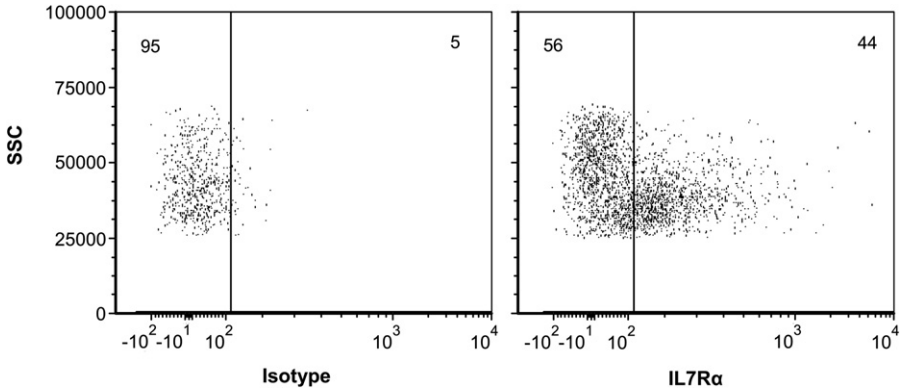


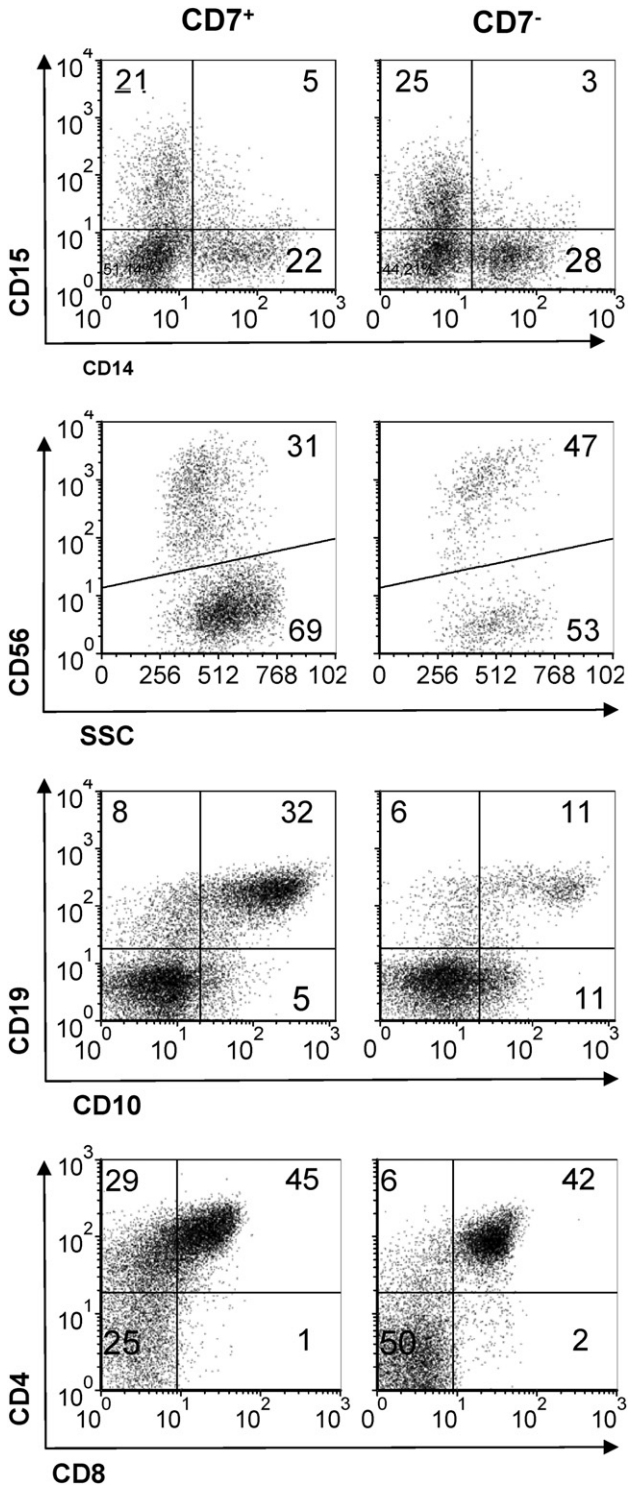
Supplementary Figure E1. Lympho-myeloerythroid potential of $CD34^+CD38^-CD45RA^-CD90^+$ cells. Human $CD34^+CD38^-$ cells were cultured on OP9 or OP9-DL1 monolayers. After 8 days, cells were harvested and sorted $CD34^+CD38^-CD45RA^-CD90^+$ cells were incubated in defined conditions to allow development of different lineages (see Material and Methods). (**A**) Chart represents the percentage of cells expressing the indicated markers (n = 3). (**B**) Erythroid potential was evaluated by the analysis of CD235a expression on cells differentiated in the presence of erythropoietin. Charts represent flow cytometry plots of a representative experiment showing isotype control vs CD235a staining (black).



Supplementary Figure E2. Notch pathway activation maintains multilineage potential of $CD34^+CD38^-CD45RA^-CD90^+$ cells. $CD34^+CD38^-CD45RA^-CD90^+$ cells harvested from the cultures of $CD34^+CD38^-$ cells on OP9 monolayers were sorted and individually placed in conditions designed to expand them. Clones with a positive growth were induced to differentiate by culturing them in specific conditions (see Material and Methods). Freshly isolated CB $CD34^+CD38^-CD45RA^-CD90^+$ cells were included as control. (**A**) An example of differentiation potential of an isolated clone is shown. (**B**) Summary of the results from three independent experiments (30 individual clones/experiment) analyzing developmental potential of the expanded colonies. Identification of clones with the indicated potential is marked.



Supplementary Figure E3. IL7Rα expression in CD34$^-$ cells from cord blood. CB CD34$^-$ fraction was stained with a specific anti-IL7Rα antibody and analyzed by flow cytometry.



Supplementary Figure E4. Lympho-myeloid potential of $CD34^{+}CD38^{-}CD45RA^{-}CD90^{+}$ CD7 cells. Human $CD34^{+}CD38^{-}$ cells were cultured on OP9 or OP9-DL1 monolayers. After 8 days, cells were harvested and sorted $CD34^{+}CD38^{-}CD45RA^{-}CD90^{+}$ $CD7^{+/-}$ cells were incubated in defined conditions to allow development of different lineages (see Material and Methods). (**A**) The phenotype was assessed after labeling with CD15 and CD14 for myeloid cells, CD56 for NK cells, CD10 and CD19 for B cells, and CD8 and CD4 for T-cell development. Plots are representative of three independent experiments.