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Identification of essential genes in human lymphopoiesis

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Abbreviations

APC	allophycocyanin
bHLH	basic helix-loop-helix
BM	bone marrow
BMP	bone morphogenetic protein
CD	cluster of differentiation
cDNA	copy DNA
CLP	common lymphoid progenitor
CMLP	common myelo-lymphoid progenitor
CMP	common myeloid progenitor
CSL	$\underline{C}$BF1, $\underline{S}$uppressor of Hairless, $\underline{L}$ag-1
DAPT	7 (N-[N-(3,5-difluorophenyl)-l-alanyl]-S-phenyl-glycine t-butyl ester)
DC	dendritic cell
DL1	Delta-like-1
DNA	desoxyribonucleic acid
DP	double positive
EGFP	enhanced green fluorescent protein
ELP	early lymphoid progenitor
EPO	erythropoietin
ETP	early thymic progenitor
FACS	fluorescence activated cell sorter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FL	Flt3/Flk-2 ligand
FTOC	fetal thymus organ culture
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	granulocyte/macrophage colony-stimulating factor
GMP	granulocyte/monocyte progenitor
GO	gene ontology
HES	hairy enhancer of split
HLA	human leukocyte antigen
HPRT	hypoxanthine phosphoribosyl transferase
HSC(s)	hematopoietic stem cell(s)
ICN	intracellular domain of Notch-1
Ig	Immunoglobulin
IL	interleukin
IRES	internal ribosomal entry site
ISP	immature single positive
Lin	lineage
LSK	$Lin^- Sca-1^{hi} c-Kit^{hi}$
LT-HSC	long-term repopulating hematopoietic stem cell
MAML	Mastermind-like
MEP	megakaryocyte/erythrocyte progenitor
MHC	major histocompatibility complex
MPP	multipotent progenitor
mRNA	messenger RNA

NK	natural killer
NOD	nonobese diabetic
PBS	phosphate buffered saline
PE	phycoerythrin
RAG	recombination activating gene
RNA	ribonucleic acid
RNAi	RNA interference
RT-PCR	reverse transcription polymerase chain reaction
SCF	stem cell factor
SCID	severe combined immunodeficiency
SD	standard deviation
siRNA	small interfering RNA
SP	single positive
SRC	SCID repopulating cell
ST-HSC	short-term repopulating hematopoietic stem cell
T-ALL	T cell acute lymphoblastic leukemia
TCR	T-cell receptor
Th	T helper cell
TNF	tumor necrosis factor
TPO	thrombopoietin

General introduction and state of the art

1. Introduction

The bloodstream provides every cell of the body with nutrients and oxygen and removes their waste products. Blood consists of plasma, the watery phase, and suspended blood cells. Red blood cells (or erythrocytes), which comprise 40-50 % of the blood volume, are responsible for the oxygen transport. Platelets (or thrombocytes) trigger blood clotting in damaged tissues. White blood cells (or leukocytes) are the immune cells that protect the body against tumor cells and invading micro-organisms. Many types of leukocytes exist, each with a specialized function in the immune system. B cells and T cells are lymphoid cell types that mediate acquired immune responses against pathogens. Granulocytes and monocytes are myeloid cells that mediate inflammation and carry out innate immune responses. NK cells are lymphoid cells involved in innate immunity. Dendritic cells are specialized for the presentation of antigen to T cells.

Most blood cells have limited life-spans and need to be replaced continuously throughout life. The turnover of blood cells in an average man (weighing 70 kg) is estimated to be close to 1 trillion cells per day (1). All blood cells ultimately derive from a small pool of hematopoietic stem cells (HSCs) in the bone marrow by a highly orchestrated process termed **hematopoiesis** (derived from the Greek words "haima", blood and "poiein", to make). Hematopoietic stem cells have two defining properties. They are multipotential, meaning they have the capacity for differentiation and stepwise maturation into all known blood cell lineages, and they can generate additional HSCs through the process of self-renewal. Along the path of differentiation, progenitor cells gradually lose their self-renewal activity, and become more and more specialized until they irreversibly commit to a certain blood cell lineage. Maturing cells are continuously released into the circulation.

Hematopoiesis already begins in the yolk sac of the embryo, and during embryonic development the site of blood formation changes sequentially from yolk sac to fetal liver to bone marrow. After birth, the bone marrow is the dominant site of hematopoiesis and the number of HSCs is maintained relatively constant under normal conditions (homeostasis). In the event of bleeding or infection, the production of blood cells can be readily increased.

The development of T lymphocytes does not take place in the bone marrow, but in a specialized organ lying above the heart, the thymus, which is seeded with bone marrow precursors that migrate to the thymus via the bloodstream. The molecular mechanisms

regulating T-lymphopoiesis are not yet completely understood. One signal transduction pathway that has been shown to be critically important for thymopoiesis is signalling through the transmembrane receptor Notch. A better understanding of the processes that regulate T-cell development and hematopoiesis in general is necessary to develop effective therapeutic strategies against diseases such as leukemia, hereditary or acquired immune deficiencies and autoimmune diseases, which are caused by defects in the hematopoietic differentiation program. Moreover, this knowledge could be used to enhance restoration of the T-cell compartment after myeloablative therapy and bone marrow transplantation.

2. Characterization and isolation of hematopoietic stem cells

It has been known for more than 50 years that bone marrow transplantation can restore hematopoiesis in lethally irradiated mice. It was also recognized soon that a small population of clonogenic, multipotent, self-renewing stem cells in the bone marrow is responsible for the immune reconstitution (2). Transplantation of putative HSCs into lethally irradiated or genetically immune-deficient mice is the accepted gold standard for analysing stem cell activity. Injection of even a single hematopoietic stem cell has been shown to be sufficient for long-term reconstitution of lethally irradiated mice (3). The assay is also successfully used for characterizing human hematopoietic stem and progenitor cells. NOD/SCID mice are most often used for studying human hematopoiesis in vivo, and engrafting human cells are termed SCID repopulating cells (SRCs) (4). As an alternative to murine xenotransplantation models, the group of Zanjani created chimeric sheep by injecting human HSCs intraperitoneally into pre-immune fetal sheep (5-7).

The development of monoclonal antibodies (8) and the multiparameter fluorescence activated cell sorter (FACS) technology (9) enabled the phenotypic characterization of HSCs and their isolation from the bone marrow. In the **mouse**, all HSC activity in the bone marrow was found to reside in a small population (0.05% of total bone marrow) of cells expressing low or undetectable levels of hematopoietic lineage cell surface markers ($Lin^{lo/-}$), high levels of Sca-1 and c-Kit, and low levels of Thy1.1 ($Lin^{lo/-}$ $Sca-1^{hi}$ $c-Kit^{hi}$ $Thy1.1^{lo}$) (10-12). This population was further separated into cells with short-term repopulating activity (ST-HSCs: Lin^{lo}) and cells with long-term repopulating activity (LT-HSCs: Lin^-). While both populations confer radioprotection, only the latter can provide reconstitution beyond 10 weeks (13). Later the compartment containing "true" stem cells (LT-HSCs) was narrowed down further by the finding that expression of the cytokine receptor Flk-2/Flt3 (CD135) (14, 15) and/or CD27 (16) within the Lin^- $Sca-1^{hi}$ $c-Kit^{hi}$ (LSK) stem cell compartment is accompanied by loss of

self-renewal capacity. In contrast to human HSCs, LT-HSCs from normal adult mice lack expression of CD34 (3) but express the CD38 antigen (17). Yang et al. recently showed that LSK CD34$^+$Flt3$^-$ bone marrow cells are ST-HSCs capable of rapidly reconstituting myelopoiesis and thus protecting mice from lethal myeloablation (18). Kiel et al. recently discovered that LT-HSCs and non-selfrenewing progenitors can be distinguished on the basis of differential expression of 3 SLAM family receptors (19). HSCs are CD150$^+$CD244$^-$CD48$^-$, while multipotent progenitors are CD244$^+$CD150$^-$CD48$^-$ and most restricted progenitors are CD48$^+$CD244$^+$CD150$^-$.

The first surface marker identified on **human** hematopoietic stem and progenitor cells is the sialomucin CD34 (20). The CD34$^+$ population is very heterogeneous and contains both stem cells and lineage-committed progenitors. The most primitive hematopoietic cells in the bone marrow were found in a small (only 1-10%) subset of CD34$^+$ cells that do not express CD38 (21). In addition to the bone marrow, these HSCs are also present in umbilical cord blood, placenta and peripheral blood. Transplantation experiments in immune-deficient mice showed that the Lin$^-$CD34$^+$CD38$^-$ cell fraction is highly enriched with SCID repopulating cells with long-term repopulation capacity (LT-SRC), while Lin$^-$CD34$^+$CD38$^+$ cells lack self-renewal and can only contribute to short-term, transient engraftment (ST-SRC) (22, 23). The Lin$^-$CD34$^+$CD38$^-$ fraction is still very heterogeneous with regard to cell surface marker expression. Other surface markers expressed on hematopoietic stem cells include CD90 (Thy-1) (24), KDR (VEGFR2) (25) and CD133 (26). In contrast to murine long-term reconstituting HSCs, human CD34$^+$CD38$^-$ SRCs express Flt3/Flk-2 (27) and only low levels of c-Kit (CD117) (28, 29).

In addition to methods exploiting surface marker expression, techniques based on the distinctive ability of stem cells to efflux fluorescent dyes such as the mitochondrion-binding dye Rhodamine 123 (Rh-123) (30-32) and the DNA-binding dye Hoechst 33342 (33) have been used to isolate murine and human long-term repopulating HSCs. Efflux of Rh-123 is mediated by the transmembrane carrier protein encoded by the multidrug-resistance gene *MDR1*, which is highly expressed on human Rh-123^{dull} multipotent HSCs (34). Analogously, Hoechst-low cells, also referred to as 'side population' cells because of their typical location in the lower left quadrant of the Hoechst red vs. Hoechst blue flow cytometric dot plots, overexpress the transporter ABCG2 (also known as Bcrp1) (35, 36) which was shown to be responsible for the efflux of Hoechst (37). The physiological function of these two pumps present on HSCs remains to be determined. Pearce et al. recently showed that the majority of murine 'side population' cells possess the primitive Lin$^-$ Sca-1^{hi} c-Kit^{hi} Thy-1^{lo} Flt3$^-$ cell

surface phenotype and represent a small subset of BM cells with this phenotype (38). The remaining 'side population' cells were mostly Lin⁻ Sca-1^{hi} c-Kit^{hi} Thy-1⁻ and may represent a previously undescribed, extremely rare, Thy-1-negative stem cell population (38). Another promising strategy to isolate hematopoietic stem cells is on the basis of their high cellular expression of the enzyme aldehyde dehydrogenase (ALDH) (39).

Although the utility of CD34 as marker for human HSCs is well established and purified CD34$^+$cells have been successfully used for many clinical transplantations, human CD34⁻ cell populations with long-term multilineage engraftment potential have also been detected (40-42). These Lin⁻CD34⁻CD38⁻ cells give rise to CD34$^+$ cells in vivo (41) and in vitro (43, 44), suggesting they might be upstream of CD34$^+$ cells in the hematopoietic hierarchy. Because SCID-repopulating activity in the Lin⁻CD34⁻CD38⁻ population is restricted to those cells expressing CD133 (45) and CD133 is also expressed on CD34^{bright} cells (26), CD133 might be a better choice for HSC enrichment.

3. Models of hematopoiesis

Knowledge about the hematopoietic differentiation pathway is achieved by rigorous purification of HSC and progenitor cell populations by surface phenotype and evaluation of their full differentiation capacity, ideally at the single cell level. This showed that the differentiation from multipotential hematopoietic stem cells to mature specialized blood cells is a stepwise process in which each successive stage loses self-renewal and developmental potential. Lineage commitment is considered to be an irreversible step, meaning that a cell that has made a certain lineage decision and loses the potential to develop into a specific lineage, never regains that potential.

The symmetry between B cell and T cell development, and the observation that IL-7R$\alpha^{-/-}$ mice are deficient in both T and B lymphocytes, led to the hypothesis that both lymphoid cells are closely related and might share a common ancestor. Kondo et al. were the first to isolate a murine BM cell population with the potential to generate all lymphoid lineage cells (B, T and NK cells), but no myeloid cells (46). Bipotent B/T potential was shown in clonal assays. These **common lymphoid progenitors** (CLPs) are Lin⁻ Sca-1^{low} c-Kit^{low} Thy-1.1⁻ and express high levels of the α chain of the IL-7 receptor (IL-7Rα^+). CLP also have uniformly high expression of the Flt3 cytokine receptor (47) and have lost self-renewal capacity. Analogously, a **common myeloid progenitor** cell (CMP) (Lin⁻ Sca-1⁻ c-Kit$^+$ IL-7Rα⁻ FcγR^{lo} CD34$^+$), generating all myeloid cell types but devoid of any lymphoid potential, was isolated from murine BM (48). Granulocyte/macrophage lineage-restricted progenitors

(GMPs) (Lin$^-$ Sca-1$^-$ c-Kit$^+$ IL-7Rα^- FcγR^{hi} CD34$^+$), and megakaryocyte/erythrocyte lineage-restricted progenitors (MEPs) (Lin$^-$ Sca-1$^-$ c-Kit$^+$ IL-7Rα^- FcγR^{lo} CD34$^-$) were also isolated from the murine BM and were shown to derive from CMPs in *in vitro* cultures (48).

The identification of CLP, CMP, GMP and MEP led Weissman and co-workers to propose a model for hematopoietic development, which is still prevailing (See Figure 1). This model suggests that the first lineage commitment step of HSCs leads to a strict separation of myeloid and lymphoid differentiation pathways. Later it was shown that dendritic cells can be derived from both CLP and Flt3$^+$ CMP (49-51).
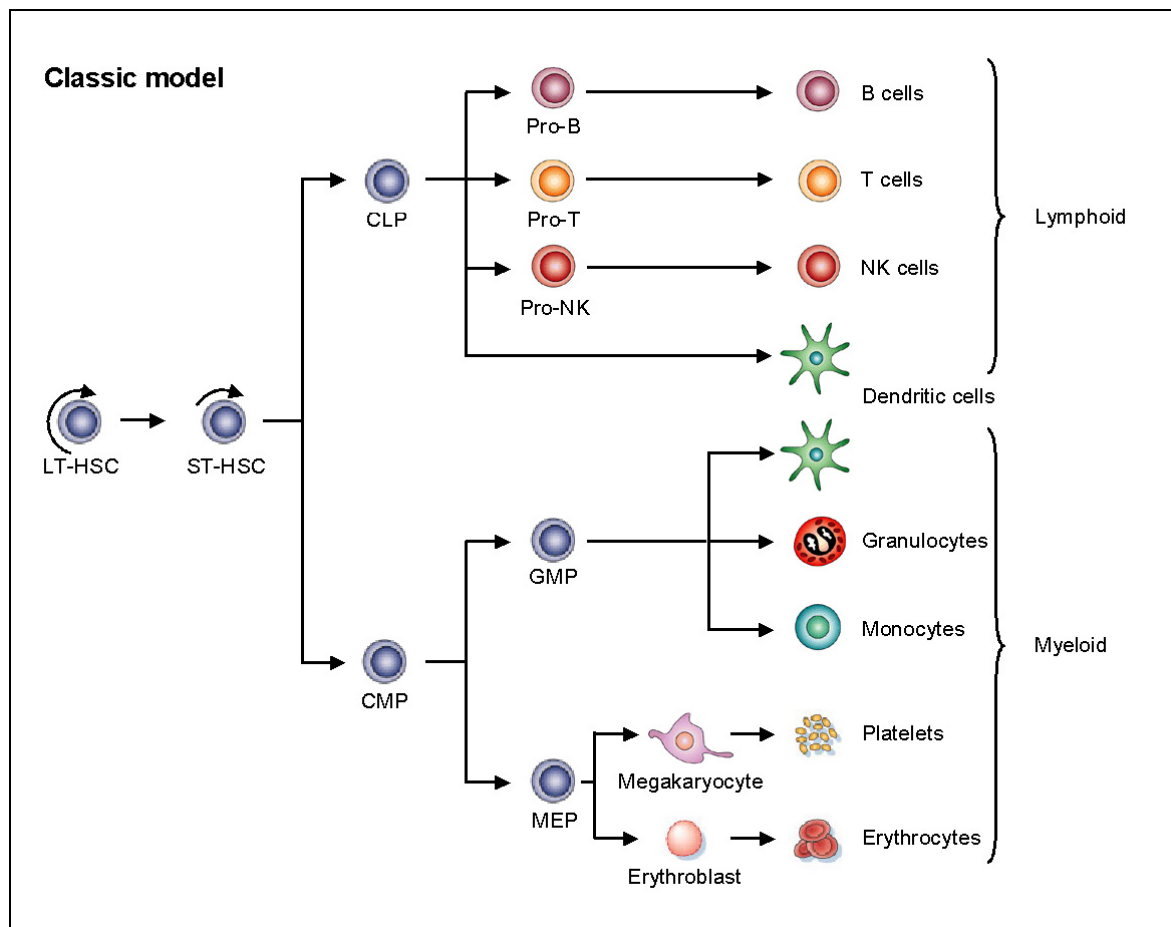
Figure 1. Schematic overview of the classic model of hematopoiesis. Adapted from Akashi et al. (48) and Reya et al. (52). LT-HSC, long-term self-renewing HSC; ST-HSC, short-term self-renewing HSC (curved arrows indicate self-renewal); CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulocyte/macrophage progenitor; MEP, megakaryocyte/erythrocyte progenitor.

In human, CLPs have been described in adult bone marrow and umbilical cord blood. Bone marrow Lin$^-$CD34$^+$CD38$^+$CD10$^+$ cells give rise to B, NK and dendritic cells on a clonal level and to T cells at high frequencies (53). Cord blood CD34$^+$CD38$^-$CD7$^+$ cells give rise to

B, NK and dendritic cells (54). Both are devoid of myeloerythroid differentiation capacity. The human counterparts of CMP, GMP and MEP have also been isolated from bone marrow and cord blood (55).

According to the classic model, B and T cells are both derived from the CLP in the bone marrow, implicating that the CLP migrates to the thymus to give rise to T cells. However, the identification of the earliest thymic progenitors (ETPs) by the group of Bhandoola (56) challenged this concept and demonstrated that bone marrow CLPs are in fact not physiological T-cell progenitors. ETPs will be discussed in greater detail in the section on T-cell seeding progenitors.

The classic differentiation scheme is also being challenged by the isolation of progenitor populations that don't fit in. Using their multilineage progenitor (MLP) assay, a clonal assay which permits T-, B- and myeloid-lineage development from single progenitors, Katsura et al. investigated the developmental potential of individual cells in various subpopulations of murine fetal liver cells. In this assay single progenitors are cultured together with a deoxyguanosine-treated fetal lobe in the presence of SCF, IL-3 and IL-7. Unipotent myeloid, B- and T-progenitors and multipotent myeloid/B/T progenitors were routinely found, as well as bipotent myeloid/B and myeloid/T progenitors. On the contrary, bipotent T/B bipotent progenitors, corresponding with the CLP, were never found (57). These findings suggested that in the murine fetal liver, the lineage restriction of multipotent progenitors to unipotent B and T progenitors occurs through the bipotent myeloid/B and myeloid/T progenitors, respectively. Accordingly, the relationship between the B and myeloid lineages or between the T and myeloid lineages is closer than that between the T and B lineages. By adding EPO to the cytokine mixture, also erythroid development could be studied (MLP-METB assay). This assay showed that most multipotent progenitors were M/T/B tripotent progenitors, but a small proportion also generated erythroid cells and were thus M/E/T/B progenitors, which correspond to HSCs. M/E progenitors, corresponding to the CMP, were also detected. M/T/B cells represent a common progenitor for M/T and M/B progenitors, and is called the common myelo-lymphoid progenitor (CMLP). These data also suggest that erythroid potential is lost at an early stage before branching towards T and B progenitors.

As M/B and M/T progenitors have not yet been identified in the adult murine bone marrow, and conversely, CLP are not present in the fetal liver, the possibility exists that lineage commitment differs between adult and fetus. However, recently Hou et al. identified a B-cell progenitor (CD34$^+$CD19$^+$CXCR4$^-$) with full myeloid differentiation capacity in human

bone marrow (58), suggesting that also in the adult, myeloid and lymphoid differentiation potential might not always segregate as early as previously assumed.

On the contrary, new data suggest that megakaryocyte/erythroid potential might be lost much earlier than assumed. Adolfsson et al. identified murine bone marrow Lin$^-$Sca-1$^+$c-Kit^{hi}CD34$^+$Flt3$^+$ (LSKFlt3$^+$) cells with combined B, T, granulocyte and monocyte potential, but without megakaryocyte/erythroid differentiation potential, which they should possess according to the conventional model of the hematopoietic hierarchy (59). These data suggest that megakaryocyte/erythroid differentiation potential is lost earlier than granulocyte/monocyte potential when HSCs differentiate to lymphocyte progenitors. This in fact correlates well with the findings of Katsura et al. in the fetal liver. A schematic overview of the models proposed by Katsura et al. and Adolfsson et al. is presented in Figure 2.

Katsura model

HSC
MMEP   CMLP
MEP   Monocytes   MB   MT
B cells   Monocytes   T cells

Adolfsson model

LT-HSC
ST-HSC
MEP   LMPP
GMP   CLP
B cells   T cells
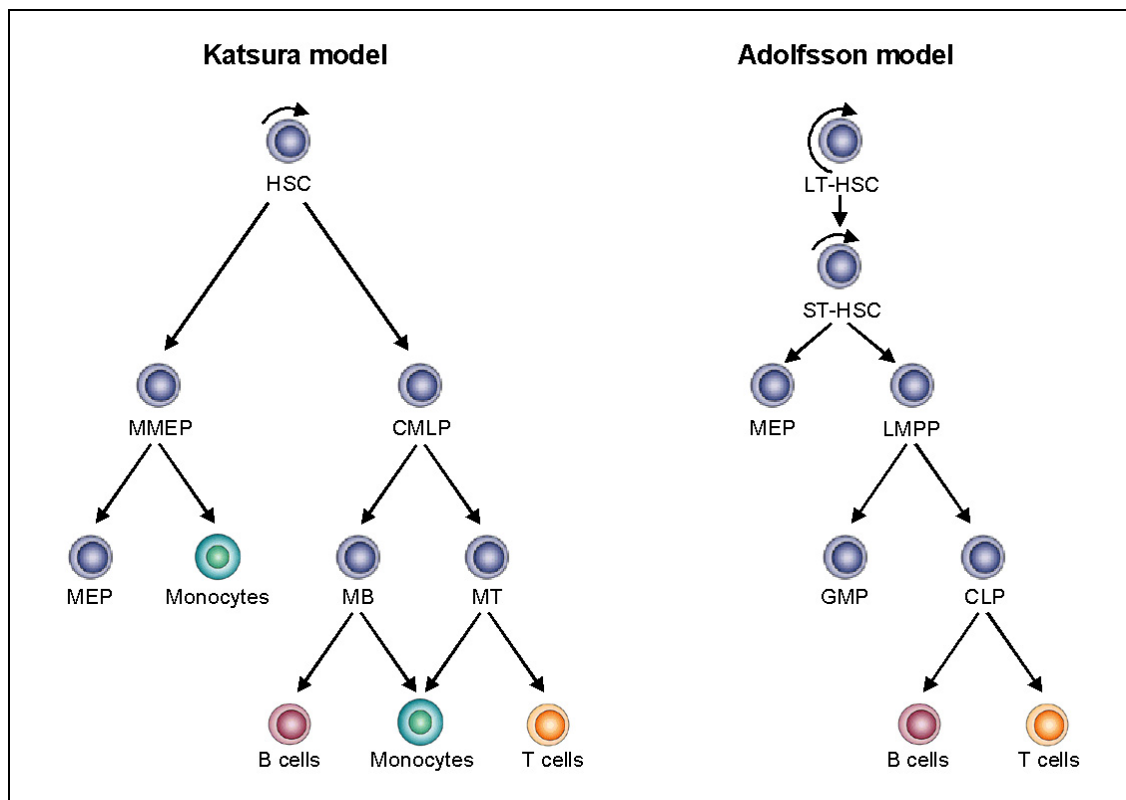
Figure 2. Schematic overview of the alternative hematopoietic differentiation models proposed by Katsura et al. (57) and by Adolfsson et al. (59). HSC, hematopoietic stem cell; MMEP, monocyte/megakaryocyte/ erythrocyte progenitor; MEP, megakaryocyte/erythrocyte progenitor; CMLP, common myelo-lymphoid progenitor; MB, monocyte/B-cell progenitor; MT, monocyte/T-cell progenitor; LT-HSC, long-term self-renewing HSC; ST-HSC, short-term self-renewing HSC (curved arrows indicate self-renewal); LMPP, lymphoid-primed multipotent progenitor; GMP, granulocyte/monocyte progenitor; CLP, common lymphoid progenitor.

4. T-cell differentiation

While other lymphoid lineages (B and NK cells) are generated in the bone marrow, T-cell development occurs almost exclusively in the thymus, which provides the specialized microenvironment necessary for T-cell development. T-lymphopoiesis in the thymus is replenished continuously by hematopoietic precursors that migrate from the bone marrow to the thymus via the blood (60). This small population of precursors generates large numbers of T cells with diverse T-cell receptor (TCR) specificities. Two major subtypes of T lymphocytes can be distinguished on the basis of antigen receptor expression. Conventional TCR-$\alpha\beta$ T cells recognize peptide antigens bound to MHC molecules, while TCR-$\gamma\delta$ T cells are not MHC-restricted and can recognize soluble protein. $\gamma\delta$ T cells constitute only a small proportion (1-5%) of the lymphocytes circulating in the blood and peripheral organs, but they comprise up to 50% of T cells within epithelial-rich tissues such as the skin, intestine and reproductive tract (61). Their biological role has not been elucidated so far. In addition to these two major T-cell lineages, also 2 types of 'unconventional' T cells develop in the thymus. NKT cells, which express many surface receptors normally expressed by NK cells, and $CD4^+CD25^+$ regulatory T cells, which are involved in preventing autoimmune diseases.

4.1. Stages of T-cell differentiation

T-cell precursors enter the thymus at the cortico-medullary junction and then migrate in a defined pattern within the thymus during differentiation (62). Thymocytes pass through distinct developmental stages that can be discriminated on the basis of the differential expression of cell surface markers. Both in human and mice, the earliest thymic progenitors are negative for the T-cell marker CD3 and the co-receptors CD4 and CD8. The murine $CD4^-CD8^-$ double-negative (DN) thymocyte subpopulation can be further subdivided into 4 consecutive developmental subsets by the surface expression of CD44 and/or CD25 (IL-2 receptor α chain) (63). These are sequentially the DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3 ($CD44^-CD25^+$) and DN4 ($CD44^-CD25^-$) stage. In human T-cell development, 3 distinct DN stages can be recognized based on the expression of CD34, CD38 and CD1a. The most immature human thymocytes are $CD34^+CD38^-CD1a^-$, followed by the $CD34^+CD38^+CD1a^-$ and $CD34^+CD38^+CD1a^+$ stages (64). An overview of the corresponding stages in murine and human T-cell development is presented in Figure 3.

Figure 3. Stages of T-cell differentiation. The surface phenotypes of corresponding stages in murine and human T-cell development are shown and critical decisions during murine T-cell development are indicated with arrows. See text for details. Figure adapted from (64).

During the first stages (DN1 through DN3), rearrangement of the T-cell receptor (TCR) δ and γ genes takes place. Thymocytes that successfully rearranged both γ and δ genes differentiate to mature TCR-$\gamma\delta$ cells. In TCR-$\alpha\beta$ lineage precursors, rearrangement of the TCR-β chain gene starts in the DN2 stage and continues in the DN3 stage. A successfully rearranged (in-frame) β-chain is expressed at the cell surface together with a surrogate α chain (pTα) and CD3 components in the pre-TCR complex. Only cells expressing a functional pre-TCR complex escape programmed cell death and differentiate further to the DN4 stage. This first checkpoint is known as β-selection. During human T-cell development, a few cells undergo β-selection already in the $CD34^+CD38^+CD1a^+$ stage, before CD4 is expressed (64). A larger proportion is β-selected in the $CD4^+$ISP stage (65), and a third group upregulate CD4 and CD8α before initiating and completing TCR rearrangements (66). In the next stage, thymocytes express one co-receptor at the cell surface, namely CD8 in the mouse and CD4 in humans. These immature single positive (ISP) thymocytes proliferate rapidly in response to signals received through the pre-TCR and start rearranging the TCR-α gene segments. The resulting double positive (DP) thymocytes, expressing both CD4 and CD8, comprise circa 90% of the total thymocyte population. The earliest DP cells are CD3$^-$, while late DP cells express CD3 at the cell surface. After rearranging the TCR α gene, a unique functional TCR is expressed and DP thymocytes undergo positive and negative selection

based on the affinity of their αβ TCR with peptide-MHC complexes on thymic epithelial cells. Positive selection encompasses the elimination of thymocytes with a TCR that does not recognize self-MHC molecules, while negative selection eliminates thymocytes with a TCR that recognizes self-antigens. Surviving thymocytes develop into mature CD3^{+}CD4^{+} helper or CD3^{+}CD8^{+} cytotoxic single positive (SP) T cells that are released into circulation. Mature T cells express high levels of the TCR and CD3.

4.2. TCR gene rearrangements

The T-cell receptor is a heterodimer consisting of disulfide-linked α and β polypeptide chains (in TCR-αβ lineage cells) or γ and δ chains (in TCR-γδ lineage cells), with each chain containing a constant domain and a variable domain, the latter being responsible for binding a peptide-MHC complex. The variable domains of the β and δ chains are encoded by several variable (V), diversity (D) and joining (J) gene segments, while the α and γ chain genes are composed of V and J gene segments. During T-cell development the TCR gene segments are joined together during a process called V(D)J recombination. This process is initiated by a specialized heterodimeric endonuclease formed from the products of the recombination-activating genes *RAG-1* and *RAG-2*, which recognizes the heptamer and nonamer recombination signal sequences (RSS) that flank the TCR gene segments and produces a double-strand break between the RSSs and flanking coding segments. Coding segments are then joined by components of the nonhomologous endjoining pathway. Since gene segments can be joined in multiple combinations, a diverse T-cell receptor repertoire is created. Additional diversity is generated by the addition of nucleotides at the junctions between V, D, and J segments by the enzyme TdT (terminal deoxynucleotidyl transferase) and by the deletion of nucleotides by exonucleases. As the number of nucleotides added or deleted at the junctions is random, the reading frame of the coding sequences beyond the joint is often disrupted, leading to the expression of a non-functional protein. Such rearrangements are therefore called non-productive. TCRβ rearrangement proceeds in an ordered fashion, witch Dβ to Jβ rearrangement preceding Vβ to DJβ rearrangements.

4.3. Nature of the thymus-seeding progenitor

The DN1 thymic subset which contains the most immature T-lineage progenitors, is heterogeneous and also contains precursors for B, NK and dendritic cells (67). DN2 stage thymocytes have lost B-cell potential but retain NK and dendritic cell potential, while DN3

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thymocytes are fully committed to the T-cell lineage (68). Based on c-Kit and IL-7Rα expression, the DN1 subset can be subdivided into c-Kit$^{neg/low}$IL-7Rα$^+$ and c-Kit^{hi}IL-7Rα$^{neg/low}$ cells. Only the latter possess measurable T-lineage potential and are thus bona fide early T-lineage progenitors (ETPs) (56). ETPs were shown to have also B-cell and even some myeloid differentiation potential. Most recently, Porritt et al. detected 5 DN1 subpopulations based on the expression of CD24 and CD117 (69). All of them could give rise to T cells, but only CD117$^+$CD24$^-$ and CD117$^+$CD24$^+$ cells displayed the kinetics of differentiation, proliferative capacity and other traits of canonical T-cell progenitors. These 2 subpopulations only possess T- and NK-cell potential, while B-cell potential of the DN1 subset is derived from the other DN1 subpopulations.

These data indicate that T-lineage commitment likely occurs after entry of the BM-derived progenitors in the thymus. The nature of the progenitors that seed the thymus is still a matter of debate. Many bone marrow populations have been identified that are able to generate T cells when introduced intravenously into irradiated recipient mice: HSCs, multipotent progenitors that have lost the capacity to self-renew (MPP) (14, 15), including CD62L$^+$ cells (70) and early lymphoid progenitors (ELPs) (71), and lymphoid-committed progenitors such as the CLPs described by Kondo et al. (46) and by Martin et al. (72).

The CD62L (L-selectin)-expressing Lin$^-$ Sca-1$^+$ c-Kit$^+$ Thy-1.1$^-$ bone marrow fraction was shown to contain robust T-lineage progenitor activity, with minor B-lineage and myeloid potential, similar to the thymic ETP (70). ELPs (early lymphoid progenitors) are immature lymphoid progenitors in the Lin$^-$ Sca-1^{high} c-Kit^{high} IL-7Rα$^-$ bone marrow fraction that were isolated from Rag1/GFP knock-in mice on the basis of *Rag1* locus activation (GFP$^+$) (71). ELPs differ from stem cells by their expression of Flk2/Flt3 and CD27 and are proposed to be upstream of the CLP in the hematopoietic hierarchy.

In addition to the CLP identified by Kondo et al. (46), a second type of common lymphoid progenitor, referred to as CLP-2, was isolated by Martin et al. from the bone marrow of mice expressing human CD25 as a reporter gene under the control of pre-TCR α regulatory sequences (72). These cells, which are Lin$^-$hCD25$^+$c-Kit$^-$CD19$^-$B220$^+$, were shown to contain bipotent B/T progenitors in clonal assays. After injection of Lin$^-$ BM cells into recipient mice, the only subset that had colonized the thymus efficiently at early time points (2 days) after injection showed the surface markers of the bone marrow-derived CLP-2 population (B220$^+$CD4$^-$c-Kit$^-$), and not of the CLP-1 population (B220$^-$CD4$^-$c-Kit^{lo}), suggesting that CLP-2 cells represent thymic immigrants. As CLP-2 cells could be derived

from CLP-1 in short-term culture, they were thought to represent the missing link between bone marrow CLP-1 and intrathymic T-cell precursors.

However, there is consensus that the thymus is not seeded by bone marrow CLPs. For instance, while Ikaros-deficient mice lack BM CLPs, they have normal numbers of early thymic precursors (ETPs) in their thymi, which suggests that ETPs are not derived from BM CLPs (56). Moreover, ETPs phenotypically resemble BM HSCs more closely than BM CLPs and they differ from CLPs in the efficiency and kinetics with which they generate B cells and maintain thymopoiesis. In contrast to CLPs, ETPs possess some myeloid potential, which suggests that the thymus is seeded by a multipotent progenitor closely related to HSCs instead of a lymphoid committed progenitor (56). The finding that BM CLPs are not physiological direct precursors of T cells is confirmed by the observation that CLPs are not present in the peripheral blood of adult mice (73). The only progenitors in the blood with potent T-lineage potential are multipotent progenitors with the LSK phenotype (Lin^- $Sca-1^{hi}$ $c-Kit^{hi}$), and like the analogous population in bone marrow, they contain both HSCs and non-renewing MPPs, including ELPs and $CD62L^+$ cells.

By creating CCR9/EGFP knock-in mice, Benz and Bleul recently showed that a Lin^- $CD25^-c-Kit^{hi}EGFP^+IL-7R\alpha^{-/lo}$ population with strong T-cell differentiation potential is present in the adult bone marrow, peripheral blood and thymus (74). The thymic counterpart of this population corresponds to the ETP population, and thymic ETPs with the highest $EGFP^+$ expression were shown to be the most immature T-cell precursors and to generate T cells with similar kinetics as the $EGFP^+$ cells in the blood, which suggests that this population represents the thymus repopulating cell that travels to the thymus via the blood. B-cell differentiation capacity in the ETP population was contained entirely in the most immature $EGFP^{hi}$ subpopulation. Both $EGFP^{hi}$ and $EGFP^{lo}$ ETPs could give rise to myeloid cells *in vitro*, indicating that upon entry in the thymus, B-cell potential is lost before myeloid potential. The thymic $Lin^-CD25^-c-Kit^{hi}EGFP^{hi}$ population, named 'thymic multipotent precursor' (TMP), was shown to contain progenitors with tripotent B/T/DC differentiation potential, indicating that thymic precursors exist that enter the thymus as multipotent progenitors and commit to the T-cell lineage only within the thymic microenvironment.

5. Regulation of hematopoiesis

5.1. General mechanisms

Multipotential hematopoietic stem and progenitor cells display low-level 'promiscuous' expression of several lineage-specific genes before commitment and differentiation to a particular lineage, a phenomenon known as 'lineage priming' (75, 76). Lineage specification involves the upregulation or activation of lineage-appropriate sets of genes and the repression of the inappropriate genes of alternate lineages, which is regulated by lineage-determining transcription factors.

A vast number of transcription factors involved in hematopoiesis have been identified. Many of those were first discovered as genes involved in chromosomal translocations associated with leukemias (77). The role of specific transcription factors in HSC fate decisions has been determined by retroviral overexpression experiments and by gene targeting of the ortholog genes in mice. Some transcription factors were shown to be indispensable for the development of multiple hematopoietic lineages, such as SCL/Tal-1, a master regulator of hematopoiesis without which no blood cells are formed (78, 79) and PU.1, which is essential for the development of B and T lymphocytes, monocytes, and granulocytes (80).

How transcription factors regulate lineage specification and commitment is best exemplified by the regulatory network involved in B-cell lineage development. Gene targeting studies identified several transcription factors that are essential for early B-cell development, including Pax5 (BSAP), EBF (early B cell factor) and the bHLH proteins encoded by the *E2A* gene (81-84). These transcription factors act sequentially to direct lymphoid progenitors to the B-cell fate. EBF is activated by E2A (85), and together these 2 transcription factors induce the transcription of several B-lineage specific genes and of another key transcription factor, Pax5 (86). EBF and E2A also control D-J recombination of the immunoglobulin heavy chain gene (87). As a result, B-cell development is arrested before rearrangement of the immunoglobulin heavy chain gene in the absence of EBF and *E2A* proteins (82-84). In contrast, Pax5-deficient progenitors express the early B-lineage specific genes, have undergone DJ rearrangements at the IgH locus but are arrested at the pro-B stage (81, 88). Pax5 is involved in regulating V-DJ rearrangement (88) and activates transcription of additional B-lineage genes such as CD19 and BLNK. Remarkably, while wild type pro-B cells are committed to the B-lymphoid lineage, Pax5-deficient pro-B cells are multipotent and have the ability to develop into all myeloid and lymphoid lineages (except B cells), both *in vitro* (89) and *in vivo* (89, 90). Thus Pax5 critically determines B-lineage commitment. In

agreement with the 'lineage priming' model, multipotent Pax5$^{-/-}$ pro-B cells express multiple genes specific to non-B lineages, which are repressed upon retroviral transduction of Pax5 (89). Therefore, an important function of Pax5 in B-lineage commitment is to repress the transcription of lineage-inappropriate genes, resulting in the suppression of alternative lineage choices.

Multiple studies show that apparently committed progenitors can be redirected to other lineages by ectopic expression of a single instructive lineage-specific transcription factor. For instance, enforced expression of GATA-1, a major erythroid lineage-affiliated transcription factor (91), can induce differentiation of committed granulocyte/monocyte progenitors and common lymphoid progenitors into megakaryocyte/erythroid lineage cells (92, 93). Also, enforced expression of C/EBPα and C/EBPβ in committed B cells leads to their rapid and efficient reprogramming into macrophages (94). Not only the mere expression of a transcription factor, but also the level at which it is expressed may influence lineage choice and differentiation. For instance, expression of low levels of PU.1 in HSCs induce B-cell generation, while high levels of the same transcription factor lead to macrophage development (95).

The primary events or mechanisms leading to the activation or deactivation of lineage-specific transcription programs are obscure. Both intrinsic and extrinsic mechanisms have been proposed and are evidenced by experimental data (96-98). According to the stochastic model, expression of transcription factors is stochastic and cell-autonomous. In this model, cytokines and growth factors secreted by stromal cells in the bone marrow merely permit the survival and induce proliferation of progenitors that are already 'programmed' to differentiate down a certain pathway. According to the instructive model, external signals, such as binding of cytokines to their cognate receptors on hematopoietic stem and progenitor cells, directs lineage commitment decisions. These external signals are 'translated' in the differentiating cell by intracellular signalling pathways ultimately leading to activation or deactivation of lineage-determining transcription factors. The existence of instructive cytokine signalling was first shown by Kondo et al. (99). Bone marrow CLPs that were transduced with the receptors for IL-2 (interleukin-2) or GM-CSF (granulocyte/macrophage colony-stimulating factor) generated macrophages and granulocytes when cultured with IL-2 and GM-CSF respectively. These authors also showed that receptors for GM-CSF and M-CSF are expressed at low levels on primitive HSCs, are absent on CLPs and are upregulated after myeloid linage induction by IL-2, suggesting that downregulation of cytokine receptors that drive myeloid cell development is a critical step in lymphoid commitment. During recent years, evidence has

accumulated for an instructive role for IL7 receptor signalling in specification of the B-cell lineage (reviewed in (100)).

Other extrinsic signalling mechanisms involved in the control of cell fate decisions include receptor-ligand interactions between HSCs and stromal cells (e.g. Notch signalling) and interactions of HSCs with the BM extracellular matrix.

5.2. Regulation of T-lymphoid development

The thymic microenvironment provides the signals necessary for the commitment of BM-derived hematopoietic progenitor cells to the T-cell lineage. As will be discussed in detail later, signals delivered by Delta-ligands of the Notch transmembrane receptor are essential for the induction of T-cell commitment.

Several cytokines and growth factors secreted by thymic stromal cells are also essential for T-cell differentiation. An indispensable role for IL-7 in early thymocyte development is shown by the dramatic loss of thymocytes and mature T cells in mice lacking either IL-7 (101) or IL-7 receptor α (102). Inhibition of IL-7 receptor signalling during FTOC with human CD34$^+$ fetal liver stem cells by the addition of IL-7 neutralizing antibodies or antibodies that block the human IL-7Rα chain also resulted in a profound reduction in human thymic cellularity (103). Contrary to the instructive role of IL-7 signalling in B-cell development, IL-7 signalling during T-cell development serves to promote the survival of early thymocytes, as ectopic expression of the anti-apoptotic protein Bcl-2 can rescue thymopoiesis in IL-7Rα-deficient mice (104, 105). Stem cell factor (SCF), the ligand for the receptor tyrosine kinase c-Kit, is also essential for the expansion of early thymocytes (106, 107). A recent study by Massa et al. provides evidence for a link between c-Kit signalling and Notch-signalling. First, upon culturing Pax5$^{-/-}$ pro-B cells on OP9 stromal cells expressing the Notch ligand Delta-like-1, cell-surface expression of c-Kit is rapidly upregulated on these progenitors, suggesting that expression of the c-Kit gene is under direct control of Notch signalling (108, 109). The same was observed with bone marrow EPML (early progenitors with myeloid and lymphoid potential, a multipotent but 'B-cell biased' population (110)). Moreover, inhibition of c-Kit signalling using either an anti-c-Kit antibody or a chemical inhibitor of c-Kit signalling confirmed that c-Kit signalling is essential for Notch-induced T-cell development (109). A role for TNF-α in T-cell development is suggested by the observation that preincubation of human CD34$^+$ adult bone marrow cells in the presence of TNF-α promotes T-cell differentiation in FTOC (111). TNF-α was shown to induce the

upregulation of IL-7Rα expression. Additionally, administration of TNF-α to NOD/SCID mice before transplantation of human cells could augment T-lymphopoieisis *in vivo* (112).

A number of studies have demonstrated a role for morphogens during T-cell development (113). Morphogens are secreted factors that control cell fate specification in many developing tissues by creating a concentration gradient. Different concentrations of the morphogen cause nearby cells to choose a different cell fate. Morphogens secreted by thymic stromal cells and involved in T-cell development include bone morphogenetic proteins (BMPs), Wnt proteins and Hedgehog proteins. BMPs negatively regulate T-cell development, as exogenous BMPs added to FTOCs block T-cell development at the DN1 stage (114). Binding of Wnt proteins on Frizzled family transmembrane receptors on developing thymocytes results in the stabilization of β-catenin, which forms a complex with transcription factors of the T-cell factor (TCF) family. The complex between β-catenin and TCF proteins is required for the transcriptional activation of target genes. TCF-1 is required for early T-cell development (115, 116) and Wnt-mediated TCF-1 activation is also required for thymocyte proliferation and differentiation beyond the immature single positive (ISP) stage (117). Two mammalian Hedgehog proteins are secreted in the thymus: Sonic Hedgehog (Shh) and Indian Hedgehog (Ihh) (118). Hedgehog proteins bind to the Patched (Ptch) receptor, which, in the absence of Hh, inhibits the downstream signal transducer smoothened (Smo) (119). Signal transduction via Smo leads ultimately to the nuclear localization and activation of the Gli family of transcription factors. Target genes of Hg signalling include Ptch, Bcl-2, Wnt and BMPs (120). Shh-deficient embryos display a diminished proliferation at the DN1 and DN2 stages of T-cell development and a partial block at the DN-to-DP transition. High levels of exogenous Shh also result in a developmental block at this stage, indicating that the concentration of Shh is important (118, 121). Using conditional knock-outs of *Smo* and chemical antagonists of Smo, Hedgehog signalling was recently shown to be an essential positive regulator of adult T-cell progenitor survival, proliferation and differentiation (122).

Chemokines (chemotactic cytokines) produced by the thymic stromal cells are important for T-cell development in several ways. First, several chemokines are thought to be involved in the homing of BM progenitors from the blood into the thymus (123). In concert with adhesion molecules such as selectins and integrins, chemokines are also responsible for regulating the ordered migration of maturing thymocytes through the thymus. Thymocyte progenitors enter the thymus at the cortico-medullary junction, migrate to the subcapsular zone of the outer cortex during the DN stages of T-cell development and move to the inner

cortex as they become DP cells. Following positive selection, SP thymocytes enter the medulla where they undergo negative selection and further maturation (62). Thymocytes differentially express chemokine receptors at discrete maturational stages, while their chemokine ligands are produced by particular stromal cells. Chemokine signalling is thus important for positioning developing thymocytes in the microenvironment necessary for each stage of T-cell development (113, 124). In addition to this important function, chemokine receptor signalling may deliver signals for lymphocyte proliferation or survival.

6. Notch-1 signalling

6.1. The Notch signalling pathway

Signalling through the Notch transmembrane receptor is evolutionarily conserved and regulates cell-fate decisions in many cell types throughout vertebrate and invertebrate development (125, 126). In mammals 4 isoforms of Notch have been identified (Notch-1-4) (127-131), and these can be activated by 5 ligands: Delta-like-1, -3 and -4 (132-134), which are homologues of the *Drosophila* Delta prototype, and Jagged-1 and -2 (135-137), homologues of *Drosophila* Serrate.

Notch is a heterodimeric transmembrane receptor consisting of noncovalently associated extracellular and transmembrane subunits. Binding with a ligand on a neighbouring cell initiates 2 proteolytic cleavages, resulting in the release of the intracellular domain of the Notch receptor (ICN) (138) (See Figure 4). The first cleavage, extracellularly close to the transmembrane domain, is mediated by TACE, a member of the ADAM (a disintegrin and metalloprotease domain) family of metalloproteases (139). The second cleavage occurs in the transmembrane domain and is mediated by a multiprotein complex with γ-secretase activity containing Presenilin, Nicastrin, APH-1, and PEN-2 proteins (140, 141). After cleavage, ICN translocates to the nucleus where it binds the transcription factor CSL (for CBF1/RBP-Jk in mammals, Suppressor of Hairless in *Drosophila,* Lag-1 in *C. elegans*) and displaces the co-repressors which are associated with CSL (142). In addition, ICN recruits Mastermind-like (MAML) proteins (143), which in turn recruit transcriptional co-activators such as p300, leading to transcriptional activation of specific target genes (144) that are repressed in the absence of ICN.
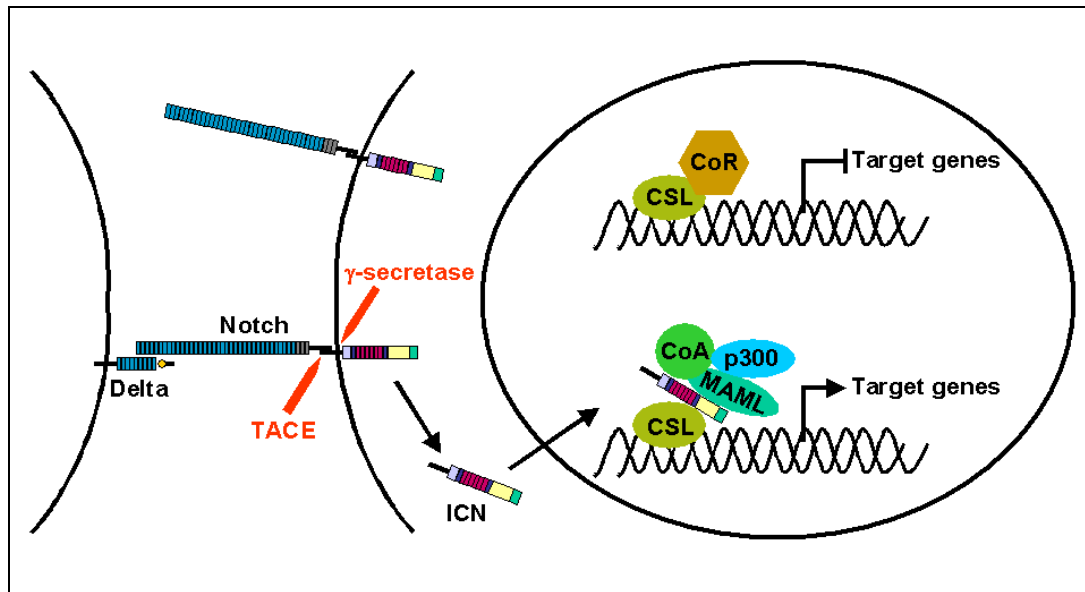
Figure 4. Schematic overview of the Notch signalling pathway. See text for details. CoR, co-repressors; CoA, co-activators.

Notch signalling can be disrupted at every step of the pathway: by γ-secretase inhibitors, by knock-out of CSL and by dominant-negative MAML, truncated mutants that bind ICN but are unable to recruit co-activators (145, 146).

6.2. Notch-1 signalling in hematopoiesis

Mammalian Notch-1 was first identified as a gene involved in a chromosomal translocation with the TCR-β gene in a small subset of human T cell acute lymphoblastic lymphomas (T-ALL), causing dysregulated expression of constitutively active truncated forms of Notch-1 (127). An important role for Notch-1 in hematopoiesis was further suggested by the observation that Notch-1 is expressed in human CD34^{+} BM precursors (147) and in precursors and peripheral blood cells of both myeloid and lymphoid lineages (148). Notch ligands are expressed on BM and fetal liver stromal cells, on thymic epithelial cells and on hematopoietic cells ((148) and references therein). These data suggested that Notch-1 signalling functions in multiple lineages and at various stages of maturation in the hematopoietic cascade. Notch-1 knock-out mice have many developmental defects causing them to die in utero (149). Also the generation of HSCs was shown to be impaired in these Notch-1^{-/-} embryos (150). Constitutive activation of Notch-1 signalling in murine HSCs, either by retroviral overexpression of the active form of Notch-1 (ICN) (151, 152) or by ligand-binding (153), induces self-renewal and favors lymphoid over myeloid differentiation (152, 153). However, an essential physiological role for Notch-1 signalling in stem cell self-

renewal was recently ruled out by Mancini et al. (2005), who showed that inducible knock-out of Notch-1 or its ligand Jagged-1 does not impair HSC self-renewal or differentiation (154).

6.3. Notch-1 signalling in T-cell development

The best-documented function of Notch-1 signalling in lymphopoiesis is its essential role in T- versus B-lineage decision. The development of mouse BM progenitors with an induced inactivation of Notch-1 is arrested at or before the most immature thymocyte precursor stage (DN1), and instead these cells develop into B cells in the thymus of reconstituted mice (155, 156). The same phenotype is obtained when Notch-1 signalling is inactivated by knock-out of the CSL transcription factor (157) or by overexpression of dominant-negative Mastermind-like (146). Conversely, the expression of a constitutive active form of Notch-1 in mouse BM precursors leads to a block in B-cell development and ectopic T-cell development in the bone marrow (158). Similarly, activation of Notch-1 signalling in hematopoietic precursors by co-culturing them on stromal cells expressing the Delta-like-1 ligand directs them to the T-cell fate (159). Even embryonic stem cells are forced to T-cell development in this culture system (160). These studies collectively showed unequivocally that signalling through the Notch-1 receptor is essential for T-cell commitment. Overexpression of Lunatic Fringe (161), Deltex-1 (162) and Nrarp (163) have also been shown to inhibit T-cell development and hence were identified as negative regulators of Noch-1 signalling.

Several studies using ICN-transgenic mice suggested additional functions for Notch-1 signalling at later stages during T-cell development. Notch-1 signalling would influence the αβ versus γδ lineage decision (164), the CD4 versus CD8 lineage decision (165, 166), the maturation and/or survival of DP thymocytes (167-169) and would inhibit positive selection of DP cells by interfering with TCR signal strength (170). However, these alleged functions of Notch-1 could not be confirmed in loss-of-function studies. Mice in which the Notch-1 gene is inactivated in all thymocytes beyond the DN3 (CD25^{+}CD44^{-}) stage, have normal numbers of all thymocyte subsets, indicating that Notch-1 signalling does not influence the CD4 versus CD8 lineage commitment, maturation or survival (171). Inactivation of the CSL transcription factor at the same developmental stage does not perturb CD4 SP and CD8 SP T cell development either, indicating that CSL-dependent signalling by all 4 Notch members is dispensable for TCR-αβ T cell development beyond the β-selection checkpoint (172).

When Notch-1 is inactivated in early DN3 (CD25$^+$CD44$^-$TCR-β^-) cells, before pre-TCR selection, $\alpha\beta$ T-cell development is severely impaired by inhibition of VDJβ rearrangements at the TCR-β chain locus (173). $\gamma\delta$ T-cell development is unaffected in these mice, arguing against a role for Notch-1 signalling in the $\alpha\beta$ versus $\gamma\delta$ T-lineage decision. On the contrary, mice in which the CSL transcription factor is inactivated in the DN3 stage not only show impaired $\alpha\beta$ T-cell development, but also show increased generation of thymic $\gamma\delta$ T cells, suggesting that Notch members other than Notch-1 may be involved in the $\alpha\beta$ versus $\gamma\delta$ lineage decision (172).

Research objectives

A technique to direct hematopoietic stem cells towards the T-cell lineage could be of great therapeutic value to develop strategies for enhancing T-cell development from transplanted donor stem cells after myeloablative therapy. Studies with Notch-1 transgenic and conditional knock-out mice have shown that signalling through the Notch-1 transmembrane receptor is essential for T-cell commitment (156, 158). We showed that overexpression of the active form of Notch-1 (ICN) in human CD34$^+$ hematopoietic stem cells also imposes the T-cell fate (174). However, since constitutive expression of ICN eventually leads to the development of T-cell tumors (175), manipulation of hematopoietic stem cells with ICN for therapeutic applications is not an option.

Recently, activation of physiological Notch signalling by coculturing HSCs on an OP9 stromal cell line engineered to express the Notch ligand Delta-like-1 was also shown to drive T-cell differentiation from murine fetal liver or adult bone marrow HSCs (159). A first research objective of this thesis was to investigate whether this culture system also supports T-cell development from human cord blood and bone marrow hematopoietic stem cells (*Chapter 1*).

From our overexpression studies with ICN we know that Notch-1 signalling is sufficient to drive human progenitors to the T-cell lineage, but whether it is also essential for human T-cell development is not known. Therefore, in a second part of this thesis we addressed this question by inhibiting Notch signalling during hybrid human-mouse fetal thymus organ culture (FTOC) of human cord blood and thymic CD34$^+$ progenitors using different doses of the γ-secretase inhibitor DAPT, which inhibits Notch signalling by preventing cleavage of the intracellular domain of Notch (*Chapter 2*).

The downstream events mediating Notch-1 induced T-cell commitment are not known. Therefore, a third research objective was to investigate whether the Notch-1 target gene HES-1 is responsible for the phenotype obtained with ICN overexpression. To this end, HES-1 was transduced in CD34$^+$ human progenitor cells and their differentiation potential was studied in several *in vitro* and *in vivo* assays (*Chapter 3*).

The identification of genes that are induced or suppressed upon commitment of multipotent stem cells to the lymphoid lineage might lead to the development of strategies to enhance lymphoid development of stem cells. Recently a rare cell population with lymphoid-restricted differentiation potential was identified in umbilical cord blood (54). A single CD34$^+$CD38$^-$CD7$^+$ cell has the capacity to differentiate into all lymphoid lineages, but cannot

differentiate into myeloid cell types such as monocytes, granulocytes or erthrocytes. The final research objective of this thesis was to identify possible candidate genes that regulate the lymphoid commitment step during human hematopoietic differentiation. For that purpose we compared the gene expression between multipotent $CD34^+CD38^-CD7^-$ stem cells and $CD34^+CD38^-CD7^+$ common lymphoid progenitors from cord blood using Affymetrix oligonucleotide microarray technology (*Chapter 4*).

<u>Results</u>

Chapter 1

Human bone marrow CD34+ progenitor cells mature to T cells on OP9-DL1 stromal cell line without thymus microenvironment.

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Human bone marrow CD34$^+$ progenitor cells mature to T cells on OP9-DL1 stromal cell line without thymus microenvironment[☆]

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Abstract

In this paper, we confirm data reported by the group of Zúñiga-Pflücker that human cord blood CD34$^+$38$^-$Lin$^-$ progenitor cells when co-cultured with the murine stromal cell line OP9-DL engineered to express the Notch ligand delta-like-1 mature into T lymphocytes with a phenotypic progression as the one seen in thymus. We show that this is also the case for human T cells starting from CD34$^+$ adolescent bone marrow cells. These findings offer the theoretical possibility to generate ex vivo human T cells and administer them in vivo in patients to overcome their immune deficient window period after transplantation. However, the practical and theoretical problems that this new technology has to overcome before this technique can be applied in clinic are still enormous and discussed.
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Keywords: Progenitor cell; Bone marrow; Thymus

Introduction

The lack of an efficient T cell regeneration after myeloablative therapy followed by stem cell infusion poses a major threat to the patients due to their increased vulnerability to severe infectious diseases. Many immune problems could be alleviated by restoration of the thymic microenvironment. This thymic regeneration may be achieved by various methods. Different approaches are investigated to boost the T cell recovery in the patients. Treatment with IL-7 to enhance T progenitor cell prolifer-

ation is an option, but has the side-effect of promoting homeostatic expansion among peripheral T cells [1,2] and exacerbating graft versus host disease [3] in murine studies. Keratinocyte growth factor (KGF) treatment causes an increased production of intrathymic IL-7, and mice treated with pharmacological doses of KGF display a sustained increase of thymic size [4,5]. Since KGF administration before the conditioning regimes required for successful bone marrow transplantation may protect the thymus micro architecture against myeloablative therapy, KGF treatment is now explored in experimental models [6].

Alternatively, there has been continuous interest to produce on large-scale T lymphocytes with the aim to supply temporally the deficient patient with exogenous T lymphocytes. However, to generate a flexible broad repertoire of the T cell pool, the generation de novo starting from hematopoietic progenitor cells must be achieved. As the generation of human T cells on stromal cells failed, there was a belief that T cells have to mature in a three-dimensional environment. Therefore, attempts

were made to use a tantalum-coated carbon matrix with murine thymic stroma as three-dimensional thymic organoid [7], but with limited success. The most efficient way to produce human T lymphocytes so far has been obtained by fetal thymus organ culture, but with this technique, the number of human T cells generated is still limited and unlikely to allow large scale production. Recently, Schmitt and Zúñiga-Pflücker [8] have shown that efficient T cell generation of murine lymphocytes could be obtained on the stromal cell line OP-9, when the cells were engineered to express the Notch ligand delta like 1. These cells are called OP9-DL1.

This work reiterates that Notch signalling is essential for T cell development. Mammals have four Notch receptors encoded by four different genes (Notch1–4). Notch receptors are single pass transmembrane proteins that are cleaved within the trans-Golgi network during biosynthesis to yield a heterodimeric complex. Extracellular Notch contains epidermal growth factor-like repeats that bind Notch ligands, and LIN12/Notch repeats that likely prevent ligand-independent signaling. There are five Notch ligands (Jagged1, Jagged2, Delta-like1, Delta-like3, and Delta-like4). Notch signaling converts a transmembrane receptor into a nuclear transcriptional coactivator. This multistep process begins with receptor–ligand interactions between adjacent cells. It is still poorly understood how the triggering of the same Notch receptor with different Notch ligands determines a downstream signaling so that cells adopt a different fate. Receptor–ligand interaction initiates two successive cleavages resulting in the release of the cytoplasmic fragment called intracellular notch (ICN). ICN contains several functional domains mediating Notch signal transduction. The first cleavage, mediated by ADAM metalloprotease occurs external to the transmembrane domain. The second cleavage occurs with the transmembrane domain and is mediated by a multiprotein complex with γ-secretase activity whose components include presenelin and nicastrin. The best-characterized function of Notch in the immune system is its role in lymphopoiesis where it is required for T cell commitment from a multipotent progenitor. There is a wealth of information on the function of Notch in the murine hematolymphoid system (for review [9–12]). Data from experiments with human cells are limited. In a review paper, Zúñiga-Pflücker [13] reported that they were able to obtain human T cells from CD34 cord blood cells on the OP9-DL1-engineered cells. Here, we confirm their data and extend them to the production of human T cells starting from CD34 adolescent bone marrow cells. These findings offer the theoretical possibility to generate ex vivo human T cells to overcome the immune deficient window period after transplantation. However, the practical and theoretical problems that this new technology has to overcome before this technique can be applied in clinic are still enormous and are discussed.

Materials and methods

Flow cytometry and cell sorting

Flow cytometry was performed using a FACScalibur (BD Biosciences, Erenbodegem, Belgium) instrument, as previously described [14], FITC-, PE-, biotin-, and APC-conjugated mAbs and streptavidin-APC were purchased from BD Biosciences. For Vβ repertoire analysis PE-conjugated mAbs were purchased from Beckman Coulter (Immunotech, Marseille, France). For analysis, live human cells were gated based on forward- and side-scatter and lack of propidium iodide uptake. The large OP9 or OP9-DL1 cells can be easily discriminated from their forward- and side-scatter profile. Cells were pre purified using anti-CD34 tagged super-paramagnetic Microbeads (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany). Finally progenitor cells were purified using a FACSVantage (BD Biosciences). Sorted cells were > 99.5% pure, as determined by post sort analysis.

OP9-DL1 cells

OP9 cells [15] and OP9-DL1 cells were obtained from Zùñiga-Pflücker and are described in Ref. [8].

Hematopoietic progenitor cell and OP9 cell co-cultures

Hematopoietic progenitor cells were isolated from cord blood or human bone marrow of 12- to 14-year-old children. Cord blood and bone marrow were obtained and used following the guidelines of the medical ethical commission of the University Hospital of Ghent. Mononuclear cells were obtained by centrifugation over Lymphoprep (Nycomed Pharma, Oslo, Norway). Progenitor cells were enriched by anti-CD34 enrichment with super-paramagnetic Microbeads (MACS). With this separation, at least a 90% pure CD34$^+$ progenitor cell population was obtained. Afterwards purified CD34$^+$ cord blood progenitor cells were labeled CD38-PE and CD34-APC and CD3-FITC, CD8-FITC and CD19-FITC and sorted with fluorescence activated cell sorter for CD34$^+$CD38$^-$Lin$^-$ progenitor cells. This yielded a population with a purity of at least 99.5%. Purified CD34$^+$bone marrow progenitor cells were labeled with CD34-APC, CD56-PE, and CD19-FITC and sorted with fluorescence activated cell sorter for CD34$^+$CD19$^-$CD56$^-$ progenitor cells. This yielded a population with a purity of at least 99.5%. Purified progenitors were seeded at 4×10^3 cells/well into 24-well tissue culture plates (Falcon, Becton and Dickinson, Erembodegem, Belgium) containing a confluent monolayer of OP9-GFP cells or OP9-DL1 cells. All co-cultures were performed in complete medium, consisting of α-MEM (Gibco, Invitrogen, Merelbeke, Belgium) supplemented with 20% Fetal Calf Serum (Hyclone, Perbio, Erenbodegem-Aalst, Belgium) in the presence of 5 ng/ml IL-7 and 5 ng/ml Flt3L (R&D, Abingdon, United King-

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dom). Co-cultures were refreshed every 3–4 days by recovering the cells with vigorous pipetting followed by filtering the cells through a cell strainer with a mesh of 70 μm (Falcon) and centrifugation (200G, room temperature, 5 min). The cell pellet was transferred to a fresh confluent monolayer of OP9-DL1 cells in complete medium as described. For analysis, co-cultures were harvested by forceful pipetting at the indicated time points.

Results

Human CD34$^+$ progenitor cells from cord blood mature into T lymphocytes

As shown in Fig. 1, there is a time-dependent progressive phenotypic maturation analogous to the one we have previously described for progenitor cells from fetal liver that mature in vitro fetal thymus organ culture [16,17]. After 18 days of co-culture with OP9-DL1 stromal cells, an important fraction of the human cells expresses CD4 (20–30%). Those cells coexpress CD1 (>80%) and CD7 (>95%) (data not shown). At that time, the number of CD4$^+$CD8$^+$ double positive cells is virtually absent and the expansion of the cells is limited to a 3- to 4-fold (Figs. 1 and 2). From day 18 on, there is a marked increase in cell number, which is accompanied, by a significant increase in both absolute number and frequency of CD4$^+$CD8$^+$ double positive cells. Further analysis on day 35 of culture shows that the CD4$^+$CD8$^+$ T cells display a CD3 phenotype which is characteristic for normal thymocytes, with intermediate and strong expression of CD3. At that time, a significant number of T cells had already expressed the TCR-αβ or TCR-γδ. At
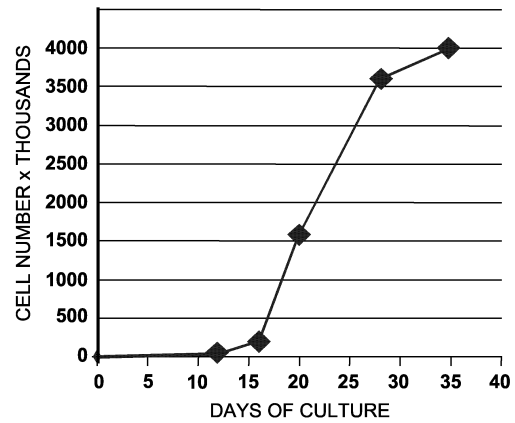

Fig. 2. Cell number of human cells generated on OP9-DL1 cells in function of time. Graph shows number of human cells that are generated on OP9-DL1 stromal cells starting with 4,000 CD34$^+$CD38$^-$Lin$^-$ progenitor cells from cord blood in a well. Note the logarithmic increase between the third and fourth week of co-culture.

later time points, the relative frequency of the TCR-αβ increases and surpasses the frequency of TCR-γδ cells (data not shown). As shown in Fig. 2, the number of human cells increases moderately during the first 2 weeks of co-culture, then the number of cells increases exponentially for the next 2 weeks, finally, the increase in cell number is moderate for longer culture times. At 14–18 days of culture, the sudden increase in cell number and the phenotype of the cells, which consists of cells in transition between immature CD4$^+$CD3$^-$ single positive thymocytes and early CD4$^+$CD8$^+$ double positive CD3^{lo} thymocytes is compatible with the burst in cell growth that is accompanied by thymocytes that express a pre-T cell complex and undergo
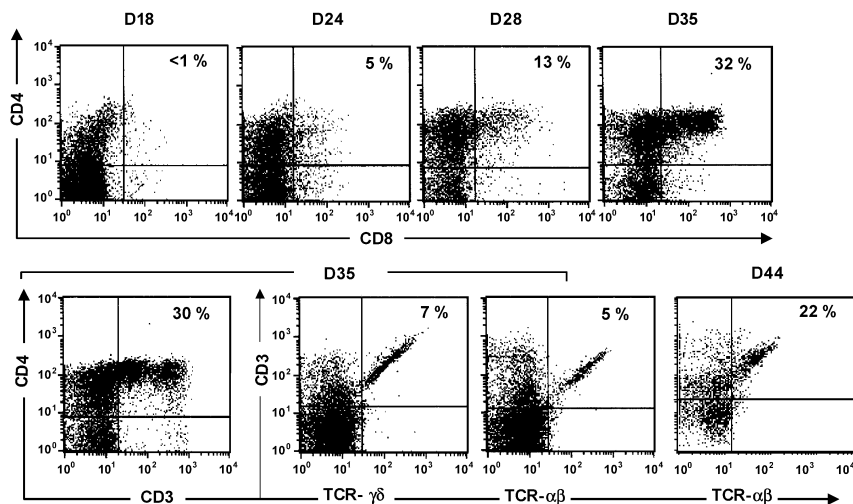

Fig. 1. Differentiation of CD34$^+$CD38$^-$Lin$^-$ progenitor cells from cord blood on OP9-DL1 cells. Flow cytometric analysis for T cells markers from progenitor cells cultured on OP9-DL1 cells for different time points as indicated. Plots are representative for three independent experiments. The figure in the right quadrant indicates the percentage of cells in the right upper quadrant.

positive β-selection. Analysis of Vβ2, Vβ5.1, Vβ7, Vβ8, Vβ13, Vβ17, and Vβ22 showed, respectively 5.6%, 4.7%, 3.0%, 3.4%, 1.7%; 2.3% and 9% on CD4⁺CD3⁺ human cells, which is in line with the distribution reported for normal peripheral T cells [18].

Human CD34⁺ progenitor cells from bone marrow mature into T lymphocytes

As shown in Fig. 3, there is a time-dependent progressive phenotypic maturation with a slightly slower kinetics as compared to the one observed with cord blood progenitor cells. After, respectively, 24, 28, and 35 days of co-culture with OP9-DL1 stromal cells, an important but consistently lower fraction of the human cells coexpresses CD4 and CD8 as compared to the cells generated from cord blood progenitor cells. Further analysis on day 35 of culture, shows that the CD4⁺CD8⁺ T cells display a CD3 phenotype which is characteristic for normal thymocytes, with intermediate and strong expression of CD3. At that time, several T cells had already expressed the TCR-αβ or TCR-γδ (data not shown). At 44 days of co-culture, 38% of the cells have already acquired the CD4⁺CD8⁺ double positive maturation stage. At that time, the relative frequency of the TCR-αβ (11%) surpasses the frequency of TCR-γδ cells.

Discussion

The seminal work of Schmitt and Zùñiga-Pflücker [8,13] showed that it was possible to obtain full maturation starting from mouse bone marrow precursor cells on a stromal OP9-DL1 cell line. These data show unequivocally that the interaction between the Notch receptor and the Delta-like1 ligand is essential for T cell differentiation. The practical implication is that no longer a thymus microenvironment is required to obtain T lymphocytes in vitro. Notwithstanding the theoretical importance of this finding and the research opportunities this observation offers, it also gives the opportunity to produce T cells on a large scale. Therefore, it was of clinical relevance to investigate whether human progenitor cells were also capable to mature to T cells on these engineered OP9-DL1 stromal cell line. One could already postulate that this was feasible, given that human cells can accomplish full T cell maturation in a mouse thymus microenvironment (for review, see Ref. [19]). The group of Zùñiga-Pflücker reported already that is was feasible with human cord blood cells (personal communication and Ref. [13]). In a previous attempt we were not successful to obtain full T cell differentiation when cord blood progenitor cells that have been transduced with an active form of Notch were co-cultured on MS-5 stromal cell line. Only the first steps of differentiation towards the CD4 stage was obtained, but further differentiation beyond the formation or the selection of the pre T cell receptor complex consisting of pre-Tα and TCR-β was not obtained [14]. However, when those cells were intravenously injected in the tail of irradiated NOD-SCID mice, the transduced cells matured into CD4⁺CD8⁺ thymocytes with high expression of CD3 and a high frequency of TCR-αβ cells in the bone marrow [14]. As the thymus of those mice were not reconstituted, we reasoned that probably Notch signalling
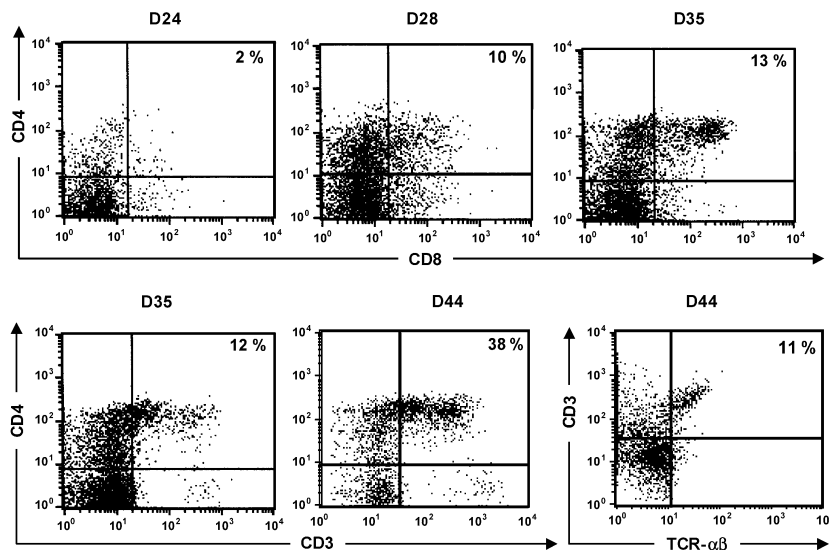

Fig. 3. Differentiation of CD34⁺CD19⁻CD56⁻ progenitor cells from human bone marrow on OP9-DL1 cells. Flow cytometric analysis for T cells markers from progenitor cells cultured on OP9-DL1 cells for different time points as indicated. Plots are representative for two independent experiments. The figure in the right quadrant indicates the percentage of cells in the right upper quadrant.

was sufficient to allow complete human T cell differentiation in the bone marrow microenvironment provided a Notch signal was delivered. Jaleco et al. [20] were also able to demonstrate that another murine stromal cell line S17 engineered to express Delta1 unlike those that expressed Jagged were able to initiate human T cell differentiation in vitro. However, the number of human cells generated were limited and the cells did not reach full T cell maturation. With the stromal cell line OP9-DL1 a high number of T cells (1000- to 4000-fold expansion) and with a phenotype of mature T cells can be obtained. In control experiments on OP9 stromal cells, we were not able to generate human T cells (data not shown), which emphasizes the critical role of Notch signalling to generate T cells (for review, see Refs. [9–12]). Therefore, we may conclude that theoretically the possibility is offered to generate large number of human T cells in vitro. Moreover, this is not only possible with cord blood as a source of progenitor cells but here we show that it is also the case with bone marrow progenitor cells. This is remarkable as it is known that it is very difficult to get consistent T cell generation from bone marrow progenitor cells [21]. However, it is clear that a lot of issues have to be addressed before infusion of in vitro generated T lymphocytes can be put into practice. The preliminary analysis of the Vβ repertoire that is generated is encouraging because it points to a broad repertoire that is similar to the one reported for human peripheral cells [18]. However, a more detailed analysis with Vβ spectratyping and at longer co-culture time is needed and currently under investigation. In this respect, the functional capacity of the human T cells also remain to be addressed. Finally, there is the crucial issue of MHC restriction and auto-immunity. Although it is shown that thymus epithelial cells are dispensable in the generation of T cells, provided the Notch receptor is triggered by Delta-like-1 ligand, we must realize that the stromal cell compartments in the thymus provide the unique combination of interactions, cytokines and chemokines to induce thymocyte precursors to undergo a differentiation program that leads to the generation of functional T cells. In particular, the issue of positive and negative selection has to be carefully addressed, with emphasis on the role of aire protein [22,23] for expression of endogenous proteins for efficient deletion of autoreactive T lymphocytes after interaction with thymus epithelial cells. Nevertheless, in the era of molecular biology, we can envision the use of human stromal cells that have not only been transduced with genes that encode for delta-like-1 ligand, but also for MHC Classes I and II molecules and eventually for aire and other molecules to obtain bona fide T lymphocytes that are applicable for administration in man. Therefore, a careful analysis of the selection processes that now occur with the OP9-DL-1 stromal cell line as such is of importance to understand what is already operating under these conditions. In this respect, the role of human dendritic cells that are generated in those cultures in terms of selection is particularly challenging.

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Chapter 2

Different thresholds of Notch signaling bias human precursor cells toward B-, NK-, monocytic/dendritic-, or T-cell lineage in thymus microenvironment.

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Different thresholds of Notch signaling bias human precursor cells toward B-, NK-, monocytic/dendritic-, or T-cell lineage in thymus microenvironment

Magda De Smedt, Inge Hoebeke, Katia Reynvoet, Georges Leclercq, and Jean Plum

Notch receptors are involved in lineage decisions in multiple developmental scenarios, including hematopoiesis. Here, we treated hybrid human–mouse fetal thymus organ culture with the γ-secretase inhibitor 7 (N-[N-(3,5-difluorophenyl)-L-alanyl]-S-phenyl-glycine t-butyl ester) (DAPT) to establish the role of Notch signaling in human hematopoietic lineage decisions. The effect of inhibition of Notch signaling was studied starting from cord blood CD34+ or thymic CD34+CD1−, CD34+CD1+, or CD4ISP progenitors. Treatment of cord blood CD34+ cells with low DAPT concentrations results in aberrant CD4ISP and CD4/CD8 double-positive (DP) thymocytes, which are negative for intracellular T-cell receptor β (TCRβ). On culture with intermediate and high DAPT concentrations, thymic CD34+CD1− cells still generate aberrant intracellular TCRβ− DP cells that have undergone DJ but not VDJ recombination. Inhibition of Notch signaling shifts differentiation into non–T cells in a thymic microenvironment, depending on the starting progenitor cells: thymic CD34+CD1+ cells do not generate non–T cells, thymic CD34+CD1− cells generate NK cells and monocytic/dendritic cells, and cord blood CD34+Lin− cells generate B, NK, and monocytic/dendritic cells in the presence of DAPT. Our data indicate that Notch signaling is crucial to direct human progenitor cells into the T-cell lineage, whereas it has a negative impact on B, NK, and monocytic/dendritic cell generation in a dose-dependent fashion. (Blood. 2005;106:3498-3506)

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Introduction

T cells develop from pluripotent hematopoietic stem cells (HSCs) through a series of differentiation steps. In humans, differentiating thymocytes can be divided into 4 main subsets based on their expression of CD4 and CD8 coreceptors. The most immature thymocytes are the CD34+ CD4−CD8− double-negative (DN) cells. As a first step toward the expression of a functional T-cell receptor (TCR), CD4+CD3− immature single-positive (CD4ISP) cells start to rearrange the TCRB gene. Subsequently, the TCRβ chain becomes assembled into the pre-TCR complex with the invariant pre-Tα chain. Pre–TCR signaling confers survival and allows development to proceed through a CD4+CD8+TCRlow double-positive (DP) subset of thymocytes. Finally, positive and negative selection results in the maturation of major histocompatibility complex (MHC)–restricted CD4 or CD8 single-positive (SP) T cells.[1,2]

Multiple signal transduction pathways are involved in directing cell fate decisions during T-cell development. These include regulation by Notch proteins, a family of highly conserved transmembrane receptors.[3] Notch is a key player in T-cell development, and its role has been recently reviewed.[4-6] There is good evidence that in mice, reduced signaling through Notch1 causes an early block in T-cell development and results in the expansion of immature B cells.[7-9] However, the role of Notch1 in later stages of T-cell development is contentious. Although earlier data pointed toward a role for Notch1 in the lineage decision between αβ and γδ T cells and between CD4 and CD8 SP thymocytes,[10-12] analysis of 2 different conditional Notch1 knockout mouse strains has failed to confirm these findings.[13,14] This strongly argues against an important role for Notch1 in later stages of thymocyte development, though the possibility remains that other family members compensate for the lack of Notch1.

The role of Notch proteins in lymphocyte development is almost exclusively based on experiments in mice. However, it is widely accepted that differences between the thymuses of mice and humans exist,[15] including the expression of cell-surface markers such as CD25 on early progenitors and the subdivision of the DN stage by CD44 and CD25.[16] The role of Notch in human T-cell differentiation has only been addressed by overexpression of the active form of Notch1 in CD34+ progenitors to evaluate its influence on T-cell differentiation in hybrid human–mouse fetal thymus organ culture (FTOC).[17,18] More recently, it was shown that human T-cell differentiation starting from CD34+ stem cells can be supported by OP-9 stromal cells engineered to express the Notch ligand Delta-like-1.[19,20] In light of these findings, it is important to evaluate the necessity of Notch in human hematopoietic lineage decisions and thymocyte development. It has been observed that presenilin-dependent γ-secretase, which serves to cleave amyloid precursor proteins in neuronal cells, also catalyzes the release of the intracellular domain of Notch proteins.[21,22] Several compounds that inhibit γ-secretase and, consequently, Notch cleavage are

available and can be used to study the role of Notch signaling.[23,24] In this paper, we show that the inhibition of Notch signaling in hybrid human-mouse FTOC impairs the development of human T cells and biases, in a dose-dependent way, development toward B-cell, NK-cell, and monocytic/dendritic-cell differentiation. Thus, our findings show for the first time CD34$^+$ precursor cells as an important site of Notch action in the human thymus.

Materials and methods

Cells and sorting

Pediatric thymuses and cord blood (CB) samples were obtained and were used according to the guidelines of the Medical Ethical Commission of Ghent University Hospital (Belgium). CD34$^+$ CB cells were purified by positive selection with CD34 magnetically activated cell sorter (MACS) beads (Miltenyi Biotec, Bergisch Gladbach, Germany) and stained with CD34–antigen-presenting cells (APCs), CD3–fluorescein isothiocyanate (FITC), CD19-FITC, and CD56–phycoerythrin (PE) (all monoclonal antibodies [mAbs] from BD Immunocytometry Systems [BDIS], Mountain View, CA) to sort CD34$^+$Lin$^-$ cells by flow cytometry (FACSVantage; BDIS). CD34$^+$ thymocytes were purified by positive selection with CD34 MACS beads, stained with CD34-APC and CD1-PE, and sorted for CD1$^-$CD34$^+$ and CD1$^+$CD34$^+$ progenitors. CD4$^+$ISP thymocytes were enriched by negative depletion of CD8$^+$CD3$^+$ thymocytes using DynalBeads (Dynal, Hamburg, Germany) and were labeled with CD4-PE and CD3-FITC, CD8-FITC, HLA-DR-FITC, and CD34-APC to sort CD4$^+$ CD34$^-$CD3$^-$CD8$^-$HLA-DR$^-$ cells. Purity of the cells was always at least 98%.

FTOC and flow cytometry

The mixed human-mouse FTOC was performed as described previously.[25,26] FTOCs were cultured with the γ-secretase inhibitor 7 (N-[N-(3,5-difluorophenyl)-L-alanyl]-S-phenyl-glycine t-butyl ester] (DAPT; Peptides International Inc, Louisville, KY) at various concentrations, as indicated, or with the solvent dimethyl sulfoxide (DMSO). Half the medium was changed weekly. After different time points of FTOC, harvested thymocytes were incubated with anti–mouse FcRγII/III (clone 2.4.G2)[27] and human immunoglobulin G (IgG) (FcBlock; Miltenyi) to avoid nonspecific staining. Cells were stained with anti–mouse CD45-CyChrome (BD PharMingen, San Diego, CA) in combination with one or more of the following anti–human mAbs: CD8β-PE, TCR-αβ-PE, CD79a-PE, CD56-APC (Coulter, Miami, FL), CD34-APC, TCR-γδ-FITC, CD3-APC or -FITC, CD4-APC or -PE, CD14-PE, CD19-FITC or PE, CD20-FITC, CD7-FITC, CD10-PE, CD56-PE, CD69-PE, HLA-DR-APC, or the appropriate control mAb (IgG1 and IgG2a-FITC, APC, or PE) (BDIS). Human viable cells, gated by exclusion of propidium iodide and mouse CD45$^+$ cells, were examined for the expression of the antigens on a FACScalibur using CellQuest Pro software (BDIS). For intracellular staining, cells were fixed and permeabilized using Fix and Perm (Imtec, San Francisco, CA) according to the manufacturer's instructions and were stained with anti–TCRβ1-PE (Ancell, Bayport, MN), CD3-APC (BDIS), or CD79a-PE (Coulter). For DNA analysis, after staining with mAbs for surface antigens, cells underwent gentle fixation with 0.25% paraformaldehyde for 1 hour at 4°C. After washing, the cells were permeabilized with 0.05% Tween 20 for 15 minutes at 37°C, as described.[28] Cells were treated with RNase (Sigma, Bornem, Belgium) and stained with 7-amino-actinomycin D (7-AAD; Becton and Dickinson) for 30 minutes at 25°C. Doublets were excluded by discriminating red fluorescence channel area × width pulses, and DNA content was measured. Apoptotic cells were detected using the annexin V–PE labeling kit from BD PharMingen.

Gene expression analysis by real-time RT-PCR

Total RNA from thymocytes was isolated as described.[29] cDNA synthesis was performed by oligo(dT) priming, and real-time reverse transcription–

polymerase chain reaction (RT-PCR) was performed as described.[29] Hypoxanthine guanine phosphoribosyl transferase (HPRT) mRNA was used for normalization. Human-specific primers were selected using Primer Express software (Applied Biosystems, Foster City, CA) and are shown in Supplemental Table S1, which is available at the *Blood* website (see the Supplemental Materials link at the top of the online article). PCR was performed with an annealing temperature of 60°C. Comparative quantification of the target gene expression in the samples was performed based on cycle threshold (Ct) normalized to HPRT using the ΔΔCt method.[30]

PCR analysis of *TCRB* gene locus recombination

DNA was extracted with the QIAmp DNA minikit (Qiagen, Hilden, Germany), and 50 ng was used for amplification by PCR. PCR conditions were performed as described[31] under the following conditions: 5 minutes at 94°C, 37 cycles of 60 seconds at 94°C, 60 seconds at 63°C (DJ$_\beta$ amplification) or 58°C (VDJ$_\beta$ amplification), followed by 7 minutes at 72°C. Primers for DJ$_\beta$ corresponded to bases 44 to 63 (TBF1) and 3025 to 3006 (TBR1), as previously reported.[32,33] For VDJ$_\beta$ amplification, a mixture of 5′ primers specific for V$_\beta$2-5-8-13 families was used with TBR1. Primers are listed in Table S1 and are shown in Figure 4.

Southern blot analysis

PCR products were run on a 2% agarose gel in 1× Tris-acetate EDTA (ethylenediaminetetraacetic acid) buffer. Specificity of the amplified fragments was validated by their predicted size and Southern blot analysis. Gels were blotted in alkaline buffer (0.4 N NaOH) onto Hybond N$^+$ membranes (Amersham, Little Chalfont, United Kingdom). Membranes were hybridized with a biotinylated TBR3 probe for 16 hours at 55°C, washed, revealed with streptavidin–horseradish peroxidase and the enhanced chemiluminescence (ECL) advanced detection system (Amersham), and visualized using x-ray film (Eastman Kodak, Rochester, NY).

Results

Notch signaling can be blocked by the γ-secretase inhibitor DAPT in hybrid human-mouse FTOC

The efficiency of the γ-secretase inhibitor DAPT to block Notch signaling in hybrid human-mouse FTOC was assessed by its effect on thymocyte development and expression of the Notch target gene *HES1*. Individual thymic lobes obtained from embryonic day 14 to 15 mouse SCID-NOD fetuses were seeded with purified human CB CD34$^+$ progenitor cells, cultured for 28 days in the absence or presence of 10 μM DAPT, and analyzed by flow cytometry (Figure 1). Control FTOC contained a significant number of DP cells, which were absent in DAPT-treated cultures. In contrast, DAPT-treated FTOCs were, to a large degree, composed of CD19$^+$ HLA-DR$^+$ B cells, which were virtually absent in control cultures (Figure 1). This is in agreement with previous findings in mice showing that inactivation of Notch allows B-cell development intrathymically.[7,8] To assess the effect of DAPT on *HES1* expression, we cultured FTOCs that were seeded with CD34$^+$ thymocytes for 10 days with medium alone before a 5-day culture period in the absence or presence of DAPT. Given that in this condition DAPT treatment has only a moderate effect on cellular composition (data not shown), the direct unbiased effect of DAPT on Hes-1 expression could be assessed. *HES1* mRNA levels were reduced by 80% in DAPT-treated cultures (ΔΔC$_t$ = 2.31 ± 0.06, mean ± SEM, for 3 independent experiments).[11,34] FTOCs seeded with CD34$^+$CD1$^-$ thymocytes and cultured for 4 days before a short-term culture of 18 hours with vehicle alone or with 2, 5, or 10 μM DAPT showed a DAPT dose-dependent reduction in *HES1* by 41%, 53%, and 54%, respectively, which was statistically significant (paired t test)
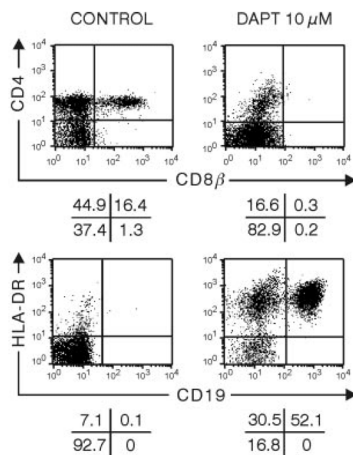
Figure 1. DAPT inhibits Notch signaling in hybrid human–mouse fetal thymus organ culture. Representative flow cytometric analyses of human cells from FTOC seeded with human CD34$^+$ CB progenitor cells and cultured for 28 days in the absence or presence of 10 μM DAPT. Quadrants were set according to isotype controls.

between control and DAPT-treated cultures ($P < .01$) and between 2 μM and 5 μM DAPT-treated cultures ($P < .02$). Our results clearly demonstrate that the γ-secretase inhibitor DAPT is capable of blocking Notch signaling in hybrid human-mouse FTOC.

Inhibition of Notch signaling in FTOC of CB CD34$^+$ progenitor cells arrests T-cell development and shifts differentiation to B, NK, and monocytic-dendritic cells in a dose-dependent way

Because DAPT did not interfere with the entry of the precursor cells into the thymic lobes (data not shown), we performed the experiments by adding DAPT from the start, including the hanging drop. Half the medium was changed weekly to maintain the concentration of active product. Inhibition of Notch with 2 and 10 μM DAPT did not result in a significant change in the absolute human cell numbers generated in FTOC after 28 days. Interestingly, there was a significant decrease in the total cell number when the FTOC was treated with an intermediate dose of 5 μM DAPT (Table 1). This observation must be interpreted

in view of the change in the cellular composition and the cell-specific generation potential.

The frequency and number of CD34$^+$ cells, which represent the most immature cells, were significantly reduced by more than 50% in cultures with 2 and 5 μM of DAPT (Table 1 and Table S2), compatible with the view that Notch supports the maintenance of CD34$^+$ progenitor cells.[35] At a dose of 10 μM, the frequency and total number of CD34$^+$ cells tended to be lower, but were no longer significantly decreased (Table 1 and Table S2). It is possible that the efficient generation of B cells in this condition, which we will present further, is accompanied by an increase in CD34$^+$ B cell–committed progenitors.

In mice, the inhibition of Notch signaling results in the accumulation of B cells.[7] FTOC seeded with CB CD34$^+$Lin$^-$ precursor cells showed a significant dose-dependent increase in frequency and in absolute numbers of B cells after the inhibition of Notch signaling by DAPT (Figure 2A), varying from 12- to 460-fold, depending on the dose of DAPT (Table 1). After 28 days of culture with the highest concentration of DAPT, B cells represented the most predominant population, encompassing more than 50% of all human cells (Figure 1), which were positive for CD10 and CD20 and partly positive for intracellular CD79a (Figure S1A).

In rat FTOC, inhibition of Notch by DAPT results in a dramatic increase in the number of NK cells.[34] Here, DAPT-treated FTOC showed a significant increase in the frequency and number of cells positively staining for CD56, which is expressed on all human NK cells (Figure 2A-B; Table 1), from less than 3% in controls to approximately 10% with the highest concentration of DAPT, corresponding to a 2.5-fold increase in the absolute number of CD56$^+$ cells (Figure 2A; Table 1). At a lower concentration of the inhibitor (5 μM), the frequency of the number of CD56$^+$ cells increased to more than 17%, corresponding to more than a 3.5-fold increase in absolute number of CD56$^+$ cells (Figure 2A; Table 1). Most of the CD56$^+$ cells were negative for CD3, CD4 (Figure 2A), and CD8β surface expression (data not shown) and positive for CD7 (Figure S1B). This corresponds to the phenotype of mature peripheral NK cells and argues that the CD56$^+$ cells in DAPT-treated cultures represent true NK cells.[36] On activation, NK cells are known to up-regulate triggering receptors such as CD69 on their surfaces, endowing them with new recognition capabilities.[37]

Table 1. Influence of γ-secretase inhibition by DAPT on human cellularity and cell number of human subpopulations after 28 days of FTOC seeded with human CD34$^+$Lin$^-$ cord blood progenitor cells

Cell type	Control, no. cells	DAPT treatment		
		2 μM, no. cells	5 μM, no. cells	10 μM, no. cells
Human cells	55 645 ± 8 676	41 591 ± 6 869	24 541 ± 3 191*	32 089 ± 1 755
CD34$^+$	5 618 ± 1 469	1 792 ± 736*	1 411 ± 110*	1 930 ± 188
CD7$^+$	50 646 ± 8 137	27 448 ± 9 725*	8 531 ± 2 563*	4 533 ± 1 060*
CD4ISP	24 828 ± 3 974	18 487 ± 4 598	915 ± 596*	0 ± 0*
DP	8 562 ± 3 238	2 549 ± 951	345 ± 173*	0 ± 0*
IC CD3$^+$	53 119 ± 8 319	35 414 ± 6 671	5 333 ± 1 856*	518 ± 326*
IC TCR-β	14 103 ± 3 490	344 ± 210*	25 ± 25*	0 ± 0*
CD3$^+$	10 952 ± 5 146	1 474 ± 737*	21 ± 21*	0 ± 0*
TCR-αβ	8 449 ± 2 740	291 ± 212*	0 ± 0*	0 ± 0*
TCR-γδ	2 479 ± 313	294 ± 114*	0 ± 0*	0 ± 0*
CD4$^+$HLA-DR$^+$	1 614 ± 266	1 391 ± 496	2 640 ± 502	5 266 ± 790*
CD14$^+$	231 ± 75	241 ± 60	533 ± 107*	1 352 ± 356*
CD56$^+$	1 247 ± 656	2 052 ± 379	4 324 ± 1 134*	3 079 ± 861
CD19$^+$	38 ± 45	454 ± 171*	6 516 ± 2 219*	17 357 ± 3 052*

Results represent mean ± SEM of 4 independent experiments.

*Significant difference ($P < .05$; paired Student t test) compared with the vehicle-treated control cultures.
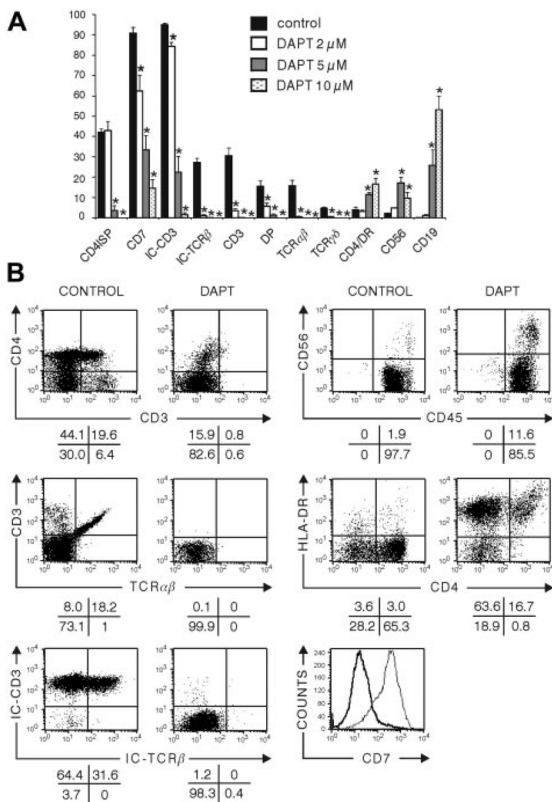
Figure 2. Influence of DAPT inhibition of Notch signaling on differentiation of CD34⁺ CB cells in hybrid human-mouse FTOC. Murine fetal thymic lobes were seeded with human CD34⁺ CB cells and cultured in FTOC for 28 days in the absence of presence of 2, 5, or 10 μM DAPT. (A) Frequencies of subpopulations of human hematopoietic cells at the end of the culture period. Bars represent mean ± SEM of 4 independent experiments. Asterisks indicate statistically significant differences ($P < .05$) compared with vehicle-treated control. (B) Representative dot plots and histogram of flow cytometric analysis of control cultures and cultures treated with 10 μM DAPT. Bold line and thin line in the histogram represent results from the DAPT-treated and control culture, respectively.

Most CD56⁺ cells in DAPT-treated fetal thymuses were negative for CD69 surface expression, suggestive of a nonactivated phenotype (Figure S1B). Collectively, this suggests that in the absence of Notch signaling, NK-cell development from CD34⁺ progenitors is favored. At the highest concentration (10 μM) of DAPT, CD34⁺ progenitor cells are preferentially biased toward B cells, whereas in the presence of intermediate concentrations, the CD34⁺ cells are less efficiently skewed toward B-cell differentiation but are able to generate NK cells. At low concentrations of DAPT, the generation of NK cells is still favored compared with untreated cultures, but not to the same extent as with intermediate DAPT concentrations.

FTOC seeded with human CB CD34⁺ progenitors and cultured in high (10 μM) DAPT concentrations resulted in the emergence of a population with characteristics of monocytic and dendritic cells. These cells coexpressed CD4 and HLA-DR and had relatively high side scatter, and some of them were CD14⁺ (Figure 2B and data not shown). The absolute numbers of CD4⁺HLA-DR⁺ cells and CD14⁺ cells were significantly increased in FTOC treated with high and intermediate concentrations of DAPT and resulted in a more than 3-fold increase (Table 1).

In control FTOCs, CB CD34⁺ progenitors undergo T-cell maturation in the mouse thymic microenvironment. This is shown by the progress of CD34⁺ cells into CD4ISP cells, which are

immature thymocytes that start to rearrange *TCRB*. After 28 days of culture, more than 40% of the human cells achieved this maturation stage (Figure 2A-B). Approximately 15% of the cells already matured to a further stage, with coexpression of CD4⁺CD8 and surface expression of CD3 (Figure 2A-B). The frequency of cells that stain for intracellular TCRβ exceeds that of DP thymocytes, as part of CD4ISP, and CD4⁺CD8αα⁺ cells express intracellular TCRβ.[38] FTOC with human CB CD34⁺ progenitors displayed a complete block of T-cell differentiation at high DAPT concentrations. Although a low number of CD4⁺CD3⁻ cells appeared (Figure 2B), those cells were not CD4ISP immature T-cell precursors; rather, they belonged to the monocytic/dendritic cell lineage according to the coexpression of HLA-DR, the absence of intracellular CD3 (Figure 2A-B), and the different scatter properties (data not shown). At intermediate DAPT concentrations, CD34⁺ CB progenitor cells were able to progress toward T cells, but the number of CD4ISP and DP cells was extremely low. At low DAPT concentrations, the number of CD4ISP and DP cells tended to be lower than for the control, but there was no significant difference. However, some striking differences were observed in the 2 μM DAPT-treated cultures. One was the nearly complete absence of intracellular TCR-β, and the other was a significant reduction in CD7 expression (Table 1).

Inhibition of Notch signaling in CD34⁺CD1⁻ thymic progenitor cells does not lead to the generation of B lymphocytes, allows NK- and monocytic/dendritic-cell maturation, and results in differentiation of abnormal T lymphocytes in FTOC

To have a better view of the effect of Notch inhibition on T-cell maturation, we explored the influence of DAPT on FTOC seeded with CD34⁺ progenitors purified from human thymocytes. Those progenitor cells have already entered the thymus and received Notch signaling[39] and are more efficient generating large numbers of human T cells with faster kinetics in FTOC.

In contrast to FTOCs that were seeded with CB CD34⁺ cells, those seeded with CD34⁺CD1⁻ thymic precursors were unable to generate B cells, even at the highest DAPT concentration (data not shown). This is compatible with the view that CD34⁺ progenitor cells in the thymus have received Notch triggering and possibly other signals that have already irreversibly induced the CD34⁺ precursor cell to a stage wherein the capacity to develop into B cells is lost, but they retained their capacity to develop into NK cells. The absolute number of NK cells was the highest with intermediate concentrations of the inhibitor (Tables 2, 3). Surprisingly, and in contrast to the CB CD34⁺ cultures, there was a significant dose-dependent increase in the frequency and number of DP cells generated in DAPT-treated FTOC. However, those DP cells were abnormal because they lacked surface CD3 and intracellular TCR-β expression (Figure 3A; Tables 2, 3). Furthermore, the inhibition of Notch triggering in CD34⁺ thymic progenitors leads to a reduction of CD7 in a concentration-dependent manner (Figure 3B).

FTOC with increasing DAPT concentrations exhibited a progressive decrease in the number of mature CD3⁺ DP cells, together with an elevation in the number of CD4ISP cells (Tables 2, 3). Importantly, CD7 was markedly down-regulated in those cells (Figure 3B). CD7 is found primarily on T, NK, and pre–B cells. The fact that CD7 expression was unchanged on NK cells argues against unspecific loss of expression or detectability.

Table 2. Influence of γ-secretase inhibition by DAPT on human cellularity of cell subsets in FTOC seeded with human CD34+CD1− thymic progenitor cells after 13 and 19 days of culture

Days after treatment, cell type	Control, no. cells	DAPT treatment	
		5 μM, no. cells	10 μM, no. cells
After 13 days			
Human cells	61 873 ± 10 604*	85 730 ± 25 356	92 214 ± 4 988
CD4ISP	36 481 ± 5 404	38 364 ± 11 521	43 343 ± 2 869
DP	12 618 ± 6 568	52 810 ± 16 178†	62 758 ± 3 316†
IC TCR-β	21 176 ± 8 391	891 ± 340†	623 ± 343†
CD3+	14 467 ± 5 176	723 ± 426†	596 ± 292†
TCR-αβ	9 571 ± 3 505	413 ± 267†	456 ± 271†
TCR-γδ	3 035 ± 893	140 ± 166†	71 ± 62†
After 19 days			
Human cells	208 397 ± 48 574	44 868 ± 7 632†	18 731 ± 7 118†
CD56+	377 ± 72	1 408 ± 343†	1 285 ± 770
CD4ISP	62 544 ± 15 294	7 012 ± 2 060†	1 933 ± 365†
DP	130 330 ± 40 609	30 574 ± 6 641†	16 843 ± 3 806†
IC TCR-β	125 048 ± 30 095	2 291 ± 730†	689 ± 609†
CD3+	109 360 ± 528 583	2 301 ± 448†	1 128 ± 125†
TCR-αβ	89 242 ± 20 756	1 342 ± 263†	630 ± 92†
TCR-γδ	6 096 ± 1 365	214 ± 66†	192 ± 88†

Results represent mean ± SEM of 3 to 5 independent experiments.

*Absolute cell number.

†Significant difference ($P < .05$; paired Student t test) compared with vehicle-treated control cultures.

Inhibition of Notch signaling in differentiating CD34+CD1− thymic progenitor cells in FTOC affects VDJβ, but not DJβ, rearrangement

Southern blot analyses performed on human thymocytes generated in FTOC started with CD34+CD1− thymic progenitor cells. Although both cell populations had a significant number of DP thymocytes and a similar extent of DJβ rearrangement, VDJβ rearrangement of the VDJβ 2-5-8-13 genes was virtually absent at 5 μM DAPT (Figure 4).

DAPT treatment induces thymic CD34+CD1+ cells and CD4ISP cells to develop into aberrant DP cells lacking intracellular TCR-β

To study the impact of Notch signaling on late T-cell–lineage decisions, we grew hybrid FTOC that were colonized with

Table 3. Influence of γ-secretase inhibition by DAPT on frequency of cell subsets in FTOC seeded with human CD34+CD1− thymic progenitor cells after 13 and 19 days of culture

Cell subset	Control	DAPT treatment	
		5 μM	10 μM
After 13 days			
CD4ISP	57.3 ± 1.7	43.0 ± 2.6	35.3 ± 0.8
DP	14.7 ± 6.0	52.6 ± 5.4*	56.7 ± 0.6*
IC TCR-β	26.3 ± 7.1	1.3 ± 0.1*	0.9 ± 0.2*
CD3+	17.9 ± 4.6	1.0 ± 0.2*	0.8 ± 0.2*
TCR-αβ	1.3 ± 0.3	0.5 ± 0.1*	0.3 ± 0.1*
TCR-γδ	4.0 ± 0.5	0.2 ± 0.1*	0.1 ± 0.0*
After 19 days			
CD56+	0.2 ± 0.1	2.7 ± 0.3*	8.3 ± 2.1*
CD4ISP	34.4 ± 6.3	16.6 ± 5.2*	8.7 ± 2.0*
DP	56.3 ± 9.3	68.9 ± 8.8*	71.1 ± 3.6*
IC TCR-β	63.4 ± 7.1	5.3 ± 1.2*	2.1 ± 1.7*
CD3+	50.6 ± 3.5	5.1 ± 0.5*	5.1 ± 0.8*
TCR-αβ	36.8 ± 3.8	2.3 ± 0.6*	2.3 ± 0.5*
TCR-γδ	3.3 ± 0.9	0.4 ± 0.1*	0.9 ± 0.5*

Results represent mean percentage ± SEM of 3 to 5 independent experiments.

*Significant difference ($P < .05$; paired Student t test) compared with vehicle-treated control cultures.
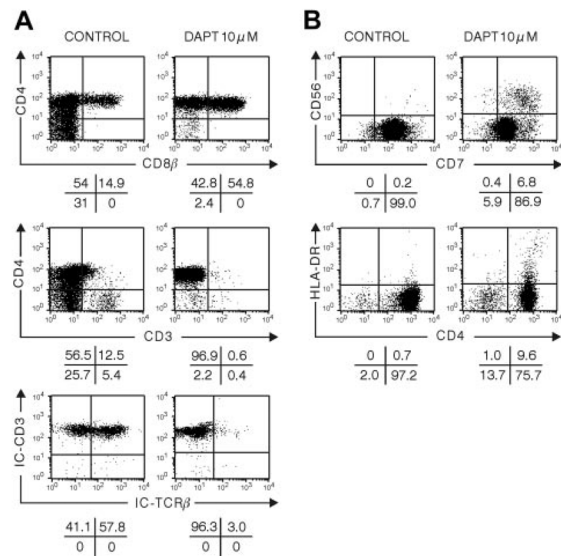
Figure 3. Influence of DAPT treatment on differentiation of human CD34+CD1− thymic progenitor cells in human-mouse hybrid FTOC. Murine fetal thymic lobes were seeded with human CD34+CD1− thymic progenitor cells and cultured for 13 (A) or 19 (B) days in the absence or presence of 10 μM DAPT. Representative dot plots of 4 independent experiments are shown.

thymocytes representing successive stages of differentiation. We studied the development of CD34+CD1+ and CD4ISP human thymocytes cultured for 10 or 20 days in FTOC in the absence or presence of DAPT. At a dose of 10 μM DAPT, few cells were present, precluding detailed phenotypic analysis. Therefore, FTOCs were grown at a lower dose of 5 μM. In control cultures, a frequency of DP cells was obtained that was similar to the one found in vivo and that was higher than the frequency obtained in FTOC seeded with CD34+CD1− thymic or CD34+ CB progenitor cells, which require a longer incubation period to achieve a similar degree of differentiation[40] (compare control treatment in Figure 1 to that in Figures 5 and 6). This shows that FTOC supports human T-cell development irrespective of the maturation degree of the starting cells but that the kinetics depend on the differentiation
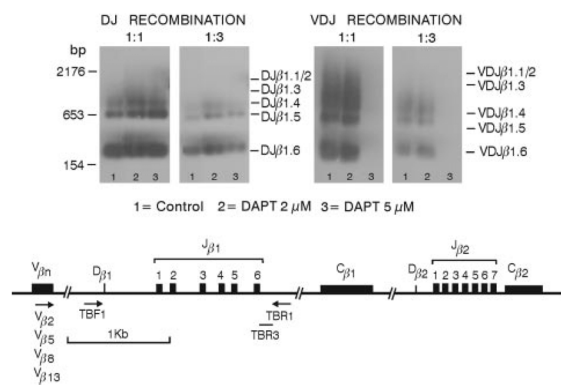
Figure 4. Inhibition of Notch signaling affects VDJβ but not DJβ rearrangements. Southern blots are shown of the products of PCR performed on cell lysates of control or DAPT-treated FTOCs, which were originally seeded with CD34+CD1− human thymic progenitor cells and were cultured for 20 days. DJβ (left) and VDJβ (right) rearrangements are shown for undiluted and 1:3 dilutions of the PCR products. Lanes 1 to 3 represent samples from untreated FTOC or FTOC in the presence of 2 or 5 μM DAPT, respectively.

stage. T-cell maturation, as characterized by TCRαβ expression, occurred normally, and less than 1% of the cells were NK cells (data not shown). Although CD34+CD1+ thymocytes were able to differentiate quickly toward T cells in control conditions, the inhibition of Notch signaling still resulted in a severe block of T-cell development with low numbers of CD3+ cells present after 10 days (Figure 5) and with a strong decrease of cells with intracellular TCR-β expression. Although the frequency of DP thymocytes was not severely changed, the absolute numbers of DP cells were dramatically decreased in the DAPT-treated cultures after 20 days (data not shown). Because no B or NK cells were generated in DAPT-treated cultures (data not shown), these results demonstrate that DAPT directly inhibits T-cell differentiation, not that inhibition of T-cell differentiation would be merely caused by a bias toward B or NK differentiation. When CD4ISP thymocytes were cultured for 10 days in FTOC in the presence of DAPT, aberrant CD4/CD8 DP cells were still obtained that were predominantly negative for surface CD3 and intracellular TCRβ (Figure 6).

Cell-cycle analysis revealed that on DAPT treatment (Table 4), more cells were cycling during the first 10 days of culture. This was also the case when gating on CD4ISP cells. At this point, the number of annexin V+ cells resembling apoptotic cells was comparable, indicating that the emergence of abnormal cells in DAPT-treated FTOC resulted from rapid outgrowth of those cells. After 20 days, however, the DAPT-treated cells stopped cycling and showed a significant increase in the number of apoptotic cells, which resulted in a dramatic decrease in cell number.

Discussion

We demonstrate that Notch signals are required for human T-cell development and that this regulation bears many similarities to murine T-cell development. We used the γ-secretase inhibitor DAPT to interfere with Notch signaling and to assess its role in human T-cell differentiation. Although this approach does not target the human developing cells exclusively but may affect the murine fetal thymic stromal cells and is not entirely specific for the Notch pathway, our results are consistent with the assumption that the observed effects can be ascribed to the inhibition of Notch signaling in human cells. First, there was a decrease in the
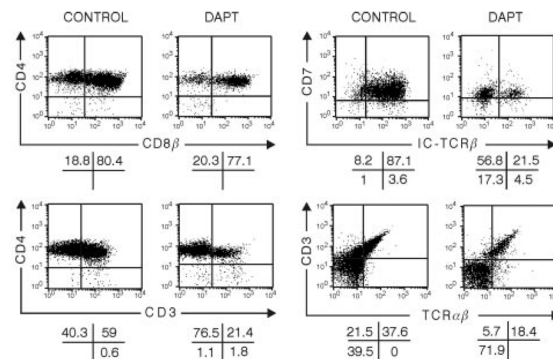

Figure 5. Influence of the inhibition of Notch signaling with DAPT on development in hybrid human-mouse FTOC seeded with human CD34+CD1+ thymic progenitor cells. Murine fetal thymic lobes were seeded with human CD34+CD1+ thymic progenitor cells cultured for 10 days in the absence or presence of 5 μM DAPT. Representative dot plots of the different subpopulations of 3 independent experiments are shown.

Figure 6. Influence of inhibition of Notch signaling with DAPT on development in hybrid human-mouse FTOC seeded with human CD4+ISP thymic progenitor cells. Murine fetal thymic lobes were seeded with human CD4+ISP thymic progenitor cells cultured for 10 days in the absence or presence of 5 μM DAPT. Representative dot plots of the different subpopulations of 3 independent experiments are shown.

downstream target gene HES1 in the human cells. Second, our results are consistent with those of the conditional Cre-lox knockout mice in which only Notch1 in the lymphoid cells was targeted and the stromal cells were intact.[7]

We have now identified the lineage decision of CD34+ precursor cells as an important site of Notch action in human thymocyte development, wherein DAPT arrests T-cell development, similar to findings in mice.[24,41] This is accompanied by a dramatic increase in B-cell number and identifies Notch transmembrane receptors as key players in determining the fate of lymphoid precursor cells in the human thymus.

Notch receptor signaling is important at several stages of hematopoiesis—HSC generation and self-renewal, T-cell commitment, B-cell development, and myeloid differentiation.[42-45] When CD34+Lin− CB cells, a cell population highly enriched for HSCs, were cultured in FTOC, we found a dose-dependent decrease in CD34+ cells with 2 and 5 μM DAPT. This suggests that Notch signaling favors the self-renewal of HSCs. However, because we did not address by detailed phenotypic and functional analyses whether those CD34+ cells were HSCs, we were unable to conclude whether in the thymic microenvironment Notch signaling favors self-renewal and T-cell differentiation or favors only the latter. The observation that FTOC is a time-limited and exhaustive culture argues that HSC self-renewal is not a predominant property of Notch signaling. In contrast, CD34+Lin− CB cells cultured on Delta-like-1–expressing OP-9 stromal cells were maintained during the first week of culture[19] (and M.D.S., unpublished observations, May 2003). It remains to be established whether factors that are differentially expressed in thymus stroma and OP-9-DL1 cells are responsible for HSC self-renewal or whether HSC self-renewal is a matter of different levels of Notch signaling. Furthermore, the increase in the number of dendritic cells and CD14+ cells in DAPT-treated cultures after 10 days of culture (data not shown) suggests that the differentiation of CD34+ cells toward other lineages may contribute to the decrease in CD34+ progenitor cells in DAPT-treated cultures.

Despite the continuous presence of the mouse thymic microenvironment, we observed the differentiation of CD34+ CB progenitor cells in DAPT-treated FTOC into cells with a B-cell phenotype expressing CD19 and HLA-DR. The cells were positive for CD79a and can thus be considered cells in the late pro-B stage. Similar cells are generated when CB CD34+CD38low progenitor cells are
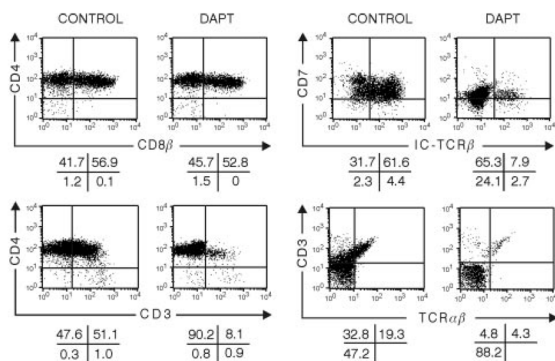
Table 4. Influence of γ-secretase inhibition by DAPT on human cellularity, proliferative status, and annexin V⁺ cells in cell subsets in FTOC seeded with CD34⁺CD1⁻ thymic progenitor cells after different time points of culture

Cell type, treatment	Day 4	Day 7	Day 10	Day 20	Day 26
Cell no.					
Control	16 391 ± 2 911	49 107 ± 315	61 303 ± 9 024	502 068 ± 13 762	828 750
2 μM DAPT	11 389 ± 713	56 108 ± 4 024	108 889 ± 45 035	285 806 ± 43 782	148 353
5 μM DAPT	13 016 ± 2 674	40 626 ± 5 763	123 874 ± 5 633	94 166 ± 75 717	38 500
S/G₂/M cells in total cell population, %					
Control	17 ± 5.9	10 ± 1.4	12 ± 4.2	18 ± 2.4	6.2
2 μM DAPT	22 ± 7.4	18 ± 4.2	20 ± 2.8	4 ± 1.2	1.3
5 μM DAPT	25 ± 1.3	27 ± 0.8	15 ± 0.4	1 ± 0.1	0.5
Annexin V⁺ cells in total cell population, %					
Control	45 ± 12.7	42 ± 5.7	55 ± 3.5	38 ± 6.4	32
2 μM DAPT	49 ± 14.4	37 ± 1.3	38 ± 2.0	32 ± 8.4	36
5 μM DAPT	52 ± 16.5	49 ± 5.4	44 ± 9.1	64 ± 9.3	65
S/G₂/M cells in CD4⁺ subset, %					
Control	ND	8	9 ± 2.3	23 ± 2.1	6.2
2 μM DAPT	ND	20	18 ± 3.1	8 ± 2.6	1.3
5 μM DAPT	ND	27	15 ± 0.6	2 ± 0.7	0.5
Annexin V⁺ cells in CD4⁺ subset, %					
Control	ND	40 ± 1.8	55 ± 3.6	51 ± 10.5	44
2 μM DAPT	ND	36 ± 4.0	38 ± 4.6	41 ± 12.6	48
5 μM DAPT	ND	48 ± 0.1	49 ± 9.2	66 ± 11.9	73
S/G₂/M cells in CD4⁺CD8⁺ subset, %					
Control	ND	ND	ND	12 ± 1.7	3.6
2 μM DAPT	ND	ND	ND	1 ± 0.3	0.5
5 μM DAPT	ND	ND	ND	1 ± 0.3	0.2
Annexin V⁺ cells in CD4⁺CD8⁺ subset, %					
Control	ND	ND	ND	42 ± 12.6	41
2 μM DAPT	ND	ND	ND	43 ± 11.0	51
5 μM DAPT	ND	ND	ND	69 ± 15.8	74

Results represent mean ± SEM of 2 independent experiments, except for figures without SEM shown, when only 1 experiment was performed.
ND indicates not done.

cocultured with the murine stromal cell line MS-5.[46] Taken together, we conclude that Notch signaling represses the B-cell fate in early precursor cells in the thymus.

Importantly, CD34⁺CD1⁻ thymic progenitor cells failed to develop into B lymphocytes under the same conditions. These data suggest that the B-cell–lineage potential of hematopoietic progenitor cells is lost immediately after entry into thymus. One study[47] has shown that murine thymic CD117⁺CD44⁺CD25⁻ (DN1) cells give rise to B lymphocytes when cultured on control OP-9 stromal cells. They observed less than 1% B-cell progenitors in the DN1 thymic cell population. They also observed that low levels of Notch signaling inhibit B-cell potential and that high levels induce T lymphopoiesis. Porritt et al[48] have found that among DN1 prothymocytes, a subset can be characterized with B-lineage potential. This could indicate that cells with B-lineage potential have lost their B-cell potential before seeding the thymus by low levels of Notch signaling and that sustained Notch signaling in the thymus leads to T-lineage commitment. It is possible that FTOC in the presence of DAPT is not sensitive enough to detect a low precursor frequency of B cells or that human thymic CD34⁺CD1⁻ cells have a lower frequency of B-cell precursors than murine DN1 cells. More detailed analysis of thymic CD34⁺ cells and culture on OP-9 stromal cells is required to determine the B-cell–lineage potential of these cells.

Our data indicate that Notch signaling also reduces NK cell differentiation in a thymic microenvironment. A direct effect of altered Notch signaling is compatible with the observation that increasing DAPT concentrations progressively inhibited T-cell maturation in FTOC and, at the same time, led to increased NK cell numbers. These data strongly suggest that the inhibition of Notch signaling is responsible for directing lymphoid progenitors into NK cells. These findings are also consistent with those from experiments in mice[47] and in rats.[34]

It is clear that CD7 expression was influenced by the inhibition of Notch signaling. When CD34⁺Lin⁻ CB cells were cultured in FTOC, both the frequency and the absolute numbers of CD7⁺ cells were significantly lower in DAPT-treated cultures in a dose-dependent way at the end of the culture. Given that after shorter incubation (10 days) the CD34⁺ cells did not express CD7 in the presence of DAPT (data not shown), we concluded that CD7 expression was inhibited from the start. This is compatible with the view that the progression of multipotent CD34⁺ progenitors toward T-/NK-cell progenitors is dependent on Notch signaling. However, CD7 expression was differentially regulated on T and NK cells because CD7 was down-regulated in developing T cells, whereas it was not affected in NK cells. This is best illustrated when thymic CD34⁺CD1⁻ progenitors were cultured in FTOC at low DAPT concentrations. In these conditions, differentiation toward DP thymocytes occurred and was paradoxically enhanced, but CD7 expression level was decreased on T cells and unchanged on NK cells. This precludes the hypothesis that CD7 is directly influenced by Notch signaling and suggests that this occurs in the context of other factors that are present according to the cell type. Even though CD7 is expressed in early T-cell ontogeny, the roles that CD7 plays in T-cell development remain unclear. It has been shown in mice that the number of thymocytes and the induced thymocyte apoptosis is normal in CD7-deficient mice, which indicates that CD7 is not involved in the early stages of T-cell development.[49] It was also demonstrated that NK-cell development and function are not impaired in CD7 disrupted mice.[50]

The function of Notch depends on the cell context and the activity of the Notch pathway itself. It is now known that Notch activity and the quantitative differences in the amount of Notch signaling influence cell fate.[51] Given that, we had to inhibit Notch signaling strongly in CD34+ CB progenitors to arrest T-cell development and to obtain B lymphocytes. A more moderate reduction (2 μM DAPT) allowed the progenitor cells to develop into aberrant CD4ISP and DP cells without intracellular TCR-β. When CD34+CD1+ precursor cells were used, high concentrations of DAPT (10 μM) resulted in cell death whereas a lower concentration sufficed to enhance the development of aberrant DP thymocytes.

Molecular analysis revealed that the lack of *TCRB* chain expression resulted from the absence of VDJ rearrangement, whereas DJ rearrangement still occurred. Similarly, Wolfer et al[14] have shown that thymocytes of mice with a conditional Notch1 deletion differentiate until the DN3 stage, though they lack VDJ rearrangement. Wolfer et al[14] interpreted these findings in view of the potential role of Notch in apoptosis and proposed that defective apoptosis allows the generation and the survival of aberrant cells that normally should have undergone apoptosis. However, because Notch is known to inhibit signaling, it is also possible that the absence of Notch triggering bypasses pre–T-cell complex signaling with the resultant inhibition of VDJ recombination. Inhibition of the VDJ recombination normally occurs in cells after the pre–TCR complex is generated, and it is an important mechanism to mediate allelic exclusion. A similar mechanism has been demonstrated in 2 studies[52,53] showing that transgenesis of an active cytosolic protein kinase D suppressed *TCRB* VDJ rearrangements by premature signaling. It is challenging to consider that Notch signaling acts not only as a T-cell driver but that it modulates signaling intensity to prevent inappropriate premature signaling in developing T cells. This could explain why no full T-cell differentiation toward TCRαβ thymocytes is observed with ICN-transduced human CD34+ progenitor cells in FTOC.[17,18] The Notch signal that is delivered can be too strong and inhibits signaling from the pre-TCR complex.

We conclude that in human lymphoid development, B cells are preferentially generated in the absence of Notch, NK cells are generated in the presence of low amounts of Notch, and T cells require high levels of Notch signals. Notch is also required during later steps of T-cell differentiation to allow the generation of thymocytes with productive rearrangement of the TCRβ chain. The precise molecular mechanisms and their interplay with other signaling cascades remain to be established.

Acknowledgments

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Supplemental Table S1

primer	Sequence 5' to 3'	Reference
Hprt528 sense	CCTTGGTCAGGCAGTATAATCCA	
Hprt630 antisense	TCAAATCCAACAAAGTCTGGCTTA	
Hes633 sense	GGCGGCTAAGGTGTTTGGA	
Hes688 antisense	TTGGGGAATGAGGAAAGCAAACT	
TBF	TGGGAGGGGCTGTTTTTGTA	Ktorza S et al.,1996[36]
TBR	TCCAGGTAAGAAGGGGTGAC	
TBR3probe	CTGACCTCCGTTCTTACACT	
HVB13	CACTGCGGTGTACCCAGGATATGA	
HVB 8	CCATGATGCGGGGACTGGAGTTGC	
HVB 2	GGCCACATACGAGCAAGGCGTCGA	
HVB 5	AGCTCTGAGCTGAATGTGAACGCC	

Supplemental Table S2

	control	DAPT 2 µM	DAPT 5 µM	DAPT 10 µM
cells				
$CD34^+$	$9.7 \pm 1,9$	$\mathbf{4.0 \pm 1.0}$	6.1 ± 1.0	6.0 ± 0.5
$CD7^+$	90.8 ± 2.7	$\mathbf{62.3 \pm 7.8}$	$\mathbf{33.5 \pm 7.0}$	$\mathbf{14.7 \pm 4.1}$
CD4ISP	42.2 ± 1.6	42.9 ± 4.5	$\mathbf{3.7 \pm 2.2}$	$\mathbf{0.0 \pm 0.0}$
DP	14.2 ± 3.7	$\mathbf{5.6 \pm 1.6}$	$\mathbf{1.4 \pm 0.6}$	$\mathbf{0.0 \pm 0.0}$
$cyCD3^+$	95.5 ± 0.9	$\mathbf{84.3 \pm 1.9}$	$\mathbf{22.5 \pm 7.7}$	$\mathbf{1.6 \pm 1.0}$
IC TCR-β ic	27.4 ± 1.9	$\mathbf{1.0 \pm 0.7}$	$\mathbf{0.2 \pm 0.2}$	$\mathbf{0.0 \pm 0.0}$
$CD3^+$	29.1 ± 5.1	$\mathbf{3.6 \pm 0.9}$	$\mathbf{0.1 \pm 0.1}$	$\mathbf{0.0 \pm 0.0}$
TCR-$\alpha\beta$	14.7 ± 3.2	$\mathbf{0.6 \pm 0.3}$	$\mathbf{0.0 \pm 0.0}$	$\mathbf{0.0 \pm 0.0}$
TCR-$\gamma\delta$	4.6 ± 0.5	$\mathbf{0.7 \pm 0.2}$	$\mathbf{0.0 \pm 0.0}$	$\mathbf{0.0 \pm 0.0}$
$CD4^+HLA-Dr^+$	3.1 ± 0.5	3.2 ± 0.7	$\mathbf{10.5 \pm 0.8}$	$\mathbf{16.6 \pm 2.7}$
$CD56^+$	2.2 ± 0.3	4.9 ± 0.3	$\mathbf{17.1 \pm 2.8}$	$\mathbf{9.8 \pm 2.8}$
$CD19^+$	0.1 ± 0.1	1.2 ± 0.5	$\mathbf{25.9 \pm 7.6}$	$\mathbf{53.1 \pm 6.8}$

Results represent mean ± SEM of 4 independent experiments. Figures in bold represent a significant difference ($p<0.05$, paired student T test) as compared to the untreated control cultures.

Supplemental Figure S1
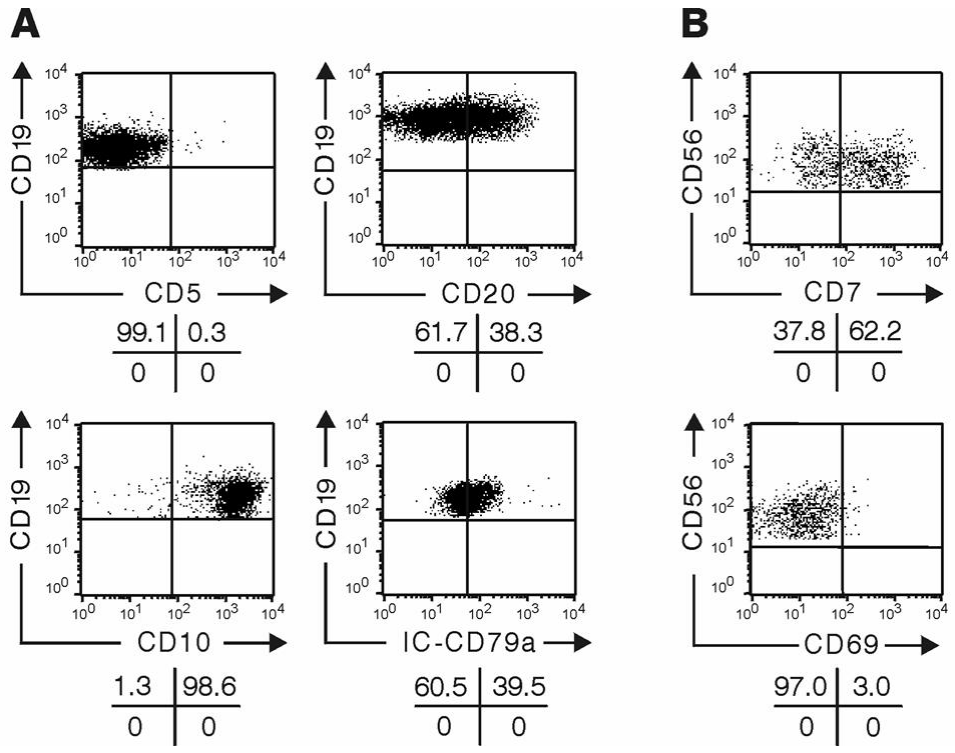
Chapter 3

Overexpression of HES-1 is not sufficient to impose T-cell differentiation on human hematopoietic stem cells.

Hoebeke, I., De Smedt, M., Van de Walle, I., Reynvoet, K., De Smet, G., Plum, J. and Leclercq, G.

Brief report

Overexpression of HES-1 is not sufficient to impose T-cell differentiation on human hematopoietic stem cells

Inge Hoebeke, Magda De Smedt, Inge Van de Walle, Katia Reynvoet, Greet De Smet, Jean Plum, and Georges Leclercq

By retroviral overexpression of the Notch-1 intracellular domain (ICN) in human CD34$^+$ hematopoietic stem cells (HSCs), we have shown previously that Notch-1 signaling promotes the T-cell fate and inhibits the monocyte and B-cell fate in several in vitro and in vivo differentiation assays. Here, we investigated whether the effects of constitutively active Notch-1 can be mimicked by overexpression of its downstream target gene *HES1*. Upon HES-1 retroviral transduction, human CD34$^+$ stem cells had a different outcome in the differentiation assays as compared to ICN-transduced cells. Although HES-1 induced a partial block in B-cell development, it did not inhibit monocyte development and did not promote T/NK-cell–lineage differentiation. On the contrary, a higher percentage of HES-1–transduced stem cells remained CD34$^+$. These experiments indicate that HES-1 alone is not able to substitute for Notch-1 signaling to induce T-cell differentiation of human CD34$^+$ hematopoietic stem cells. (Blood. 2006;107:2879-2881)

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Introduction

Studies with Notch-1–deficient and transgenic mice have shown that signaling through the Notch-1 transmembrane receptor is necessary and sufficient for T-cell commitment.[1-3] In humans, we showed that retroviral overexpression of the intracellular domain of Notch-1 (ICN) in cord-blood CD34$^+$ cells results in inhibition of B-cell development both in vitro and in vivo. Instead, cells are forced to differentiate along the T/NK pathway, resulting in ectopic development of T cells in the bone marrow (BM) of mice injected with ICN-transduced human hematopoietic stem cells (HSCs).[4]

A technique to direct HSCs toward the T-cell lineage could be of great therapeutic value to develop strategies to enhance T-cell development from transplanted donor HSCs after myeloablative therapy. Manipulation of HSCs with ICN is not an option however, since constitutive Notch-1 expression eventually leads to the development of T-cell neoplasms,[5] and mutations in the *NOTCH1* gene have been involved in most cases of human T-cell acute lymphoblastic leukemia (T-ALL).[6] Understanding the cellular events leading to T-cell commitment after ICN overexpression might lead to the identification of an effector molecule that does not cause tumor development when overexpressed in HSCs.

An important target gene of Notch-1 signaling is the basic helix-loop-helix transcription factor *HES1*,[7] which is expressed in thymocytes and thymic stroma and is essential for normal T-cell development.[8,9] HES-1 is up-regulated in hematopoietic precursors after ICN overexpression[10] and after activation of endogenous Notch-1 signaling by binding with the Delta-like-1 ligand.[11,12] Here, we investigated whether HES-1 is the mediator of the effects on human hematopoietic differentiation seen previously with ICN overexpression.

Study design

Production of HES-1 retrovirus

Human HES-1 cDNA was cloned in the retroviral vector pLZRS-IRES-EGFP (LIE)[13] upstream of the internal ribosomal entry site (IRES). ICN-LZRS was prepared by subcloning ICN from the ICN-MSCV construct used before.[4] Infectious retrovirus was produced by transfection of Phoenix-A cells.[14] Expression of HES-1 protein was verified by Western blotting on nuclear extracts of HES-1–transduced HEK-T cells using a polyclonal rabbit anti–HES-1 antibody.

Sorting of CD34$^+$Lin$^-$ cells, retroviral transduction, and differentiation assays

CD34$^+$ cells were isolated from cord-blood mononuclear cells by positive selection with EasySep magnetic beads (StemCell Technologies, Vancouver, BC, Canada), and CD34$^+$ Lin$^-$ cells were sorted after staining with anti–human CD34-APC, CD3-FITC, CD14-FITC, CD19-FITC, or CD19-PE monoclonal antibodies (Becton and Dickinson Immunocytometry Systems, San Jose, CA) and CD56-FITC (Serotec, Oxford, United Kingdom). The purity was always more than 98%. Retroviral transduction and coculture on MS-5 stromal cells were performed as described.[4] Labeling of cells with the indicated antibodies and flow cytometric analysis were performed as described.[4]

Calculation of absolute cell numbers

Absolute cell numbers were calculated from the total viable cell number as counted with the hemocytometer, the frequency of enhanced green fluorescence protein$^+$ (EGFP$^+$) cells and the frequency of the indicated subpopulations as measured by flow cytometry. Because transduction efficiencies were different for LIE, HES-1, and ICN, these numbers were multiplied by a factor to normalize to 1×10^3 EGFP$^+$ cells at the start of all cultures.

Statistical analyses

The paired Student t test was used with a significance level of .05.

Results and discussion

Overexpression of HES-1 reduces B-cell differentiation but does not promote T/NK-cell differentiation

Cord-blood CD34+ cells were transduced with comparable efficiencies by the HES-1 and ICN retrovirus ($17.0\% \pm 5.9\%$ and $19.6\% \pm 7.5\%$ after 1 day, respectively). The transduction efficiency for the control virus LIE was higher, that is, $47.2\% \pm 12.4\%$. As anticipated, B-cell development in MS-5 cocultures was dramatically blocked by ICN overexpression (Figure 1A). In the cultures initiated with HES-1–transduced cells, B-cell differentiation was not completely suppressed but yet significantly reduced (Figure 1A). This reduction was correlated with the level of HES-1 expression and was not caused by a block at an immature B-cell stage (data not shown). These results indicate that the up-regulation of HES-1 in response to Notch-1 signaling might aid to the inhibition of B-cell development but is not the main mediator of this effect.

Whereas overexpression of ICN drives T-cell development, as shown by the massive generation of CD7+cyCD3+ T/NK progenitors under B-cell conditions in MS-5 cocultures, HES-1 overexpression did not mimic this effect (Figure 1A). Furthermore, HES-1, unlike ICN, did not promote NK-cell development in MS-5 cocultures with specific cytokines for NK-cell development (Figure 1B). Therefore, the Notch-1 signal instructing the CD34+ cell to commit to the T/NK lineage is probably not or not solely mediated by HES-1.

Our results are in line with those of Kawamata et al,[15] who also noticed a partial block in B-cell development but no extrathymic T-cell development in the BM of mice transplanted with HES-1– or HES-5–transduced mouse BM Lin− precursors. Also, Maillard et al[16] communicated that overexpression of HES-1 in Notch-1–deficient murine precursors cannot rescue T-cell development in

fetal thymus organ culture (FTOC). Combined with our data, it is clear that overexpression of HES-1 is not sufficient to impose T-cell differentiation on either murine or human HSCs.

HES-1 overexpression has no influence on myeloid differentiation but maintains CD34+ cells

Culturing CD34+ cells on MS-5 in the presence of SCF, FL3, TPO, IL-2, IL-7, and IL-15 (Mix 6) allows their expansion and differentiation toward monocytes. We showed earlier that ICN expression blocks both HSC expansion and differentiation toward monocytes.[4] This was confirmed in the current experiments (Figure 2). HES-1–transduced CD34+ cells, on the other hand, generated comparable frequencies of monocytes as the control (Figure 2A). However, a significantly higher percentage of HES-1–transduced CD34+ cells remained CD34+, and absolute numbers of CD34+ cells were not reduced compared to the control (Figure 2B). The frequency of CD34+ cells also correlated with the level of HES-1 expression (data not shown). Although recent studies indicate that HES-1 maintains stem cells,[17,18] phenotypic analysis of the CD34+ population from our MS-5 cultures did not point to a specific increase of cells with a stem-cell–like phenotype (ie, CD34^{bright}CD133+) (data not shown).

Conclusion

Our experiments indicate that HES-1 is not the sole mediator of T-cell commitment induced by ICN overexpression. Based on the data from Kawamata et al, HES-5 also is not the downstream effector molecule of ICN.[15] Deltex-1, which is also up-regulated after ICN overexpression (Deftos et al[10] and own observation) cannot be the downstream mediator either, since forced expression of Deltex-1 in hematopoietic progenitors results in B-cell development at the expense of T-cell development.[19] More target genes of Notch signaling are being discovered, however. HERP1 and HERP2, which are structurally very similar to HES, are both up-regulated by Notch signaling. They can form heterodimers with
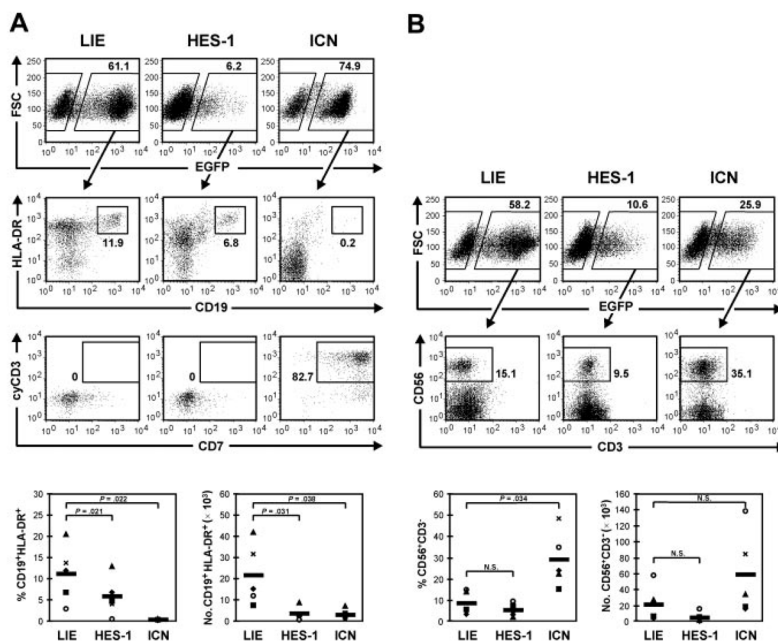
Figure 1. Overexpression of HES-1 reduces B-cell differentiation but does not promote T/NK-cell differentiation. (A) Flow cytometric analysis of MS-5 cocultures with cytokine Mix 2 (SCF and IL-7) for B-cell development. Percentages of B cells (CD19+HLA-DR+) and T/NK progenitor cells (CD7+cyCD3+) in the EGFP+-gated fraction of cultures initiated with LIE-, HES-1–, or ICN-transduced CD34+ cord-blood cells are indicated. Dot plots shown are representative of 5 independent experiments with cells from 5 different donors. (B) Flow cytometric analysis of MS-5 cocultures with cytokine Mix 3 (SCF, IL-2, IL-15) for NK-cell development. Percentage of NK cells (CD56+CD3−) in the EGFP+-gated fraction is indicated. Dot plots shown are representative of 5 independent experiments with cells from 5 different donors. Scatterplots show relative and absolute numbers of B cells and NK cells generated in 5 independent experiments. Each symbol represents an individual experiment. The mean of 5 experiments is represented by thick horizontal lines in each bottom panel. P values obtained with the paired Student t test are shown when significant; N.S. indicates a nonsignificant P value.

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Figure 2. HES-1 overexpression has no influence on myeloid differentiation but maintains CD34+ cells. Flow cytometric analysis of MS-5 cocultures with cytokine Mix 6 (SCF, FL3, TPO, IL-2, IL-7, and IL-15). Percentages of CD4+CD14+ monocytes (A) and CD45+CD34+ cells (B) in the EGFP+-gated fraction of cultures initiated with LIE-, HES-1–, or ICN-transduced CD34+ cord-blood cells are indicated. Dot plots shown are representative of 5 or 6 independent experiments with cells from 5 to 6 different donors. Scatterplots show relative and absolute numbers of monocytes and CD34+ cells generated in 5 to 6 independent experiments. Each symbol represents an individual experiment. The thick horizontal lines in each bottom panel represent the mean. *P* values obtained with the paired Student *t* test are shown when significant; N.S. indicates a nonsignificant *P* value.
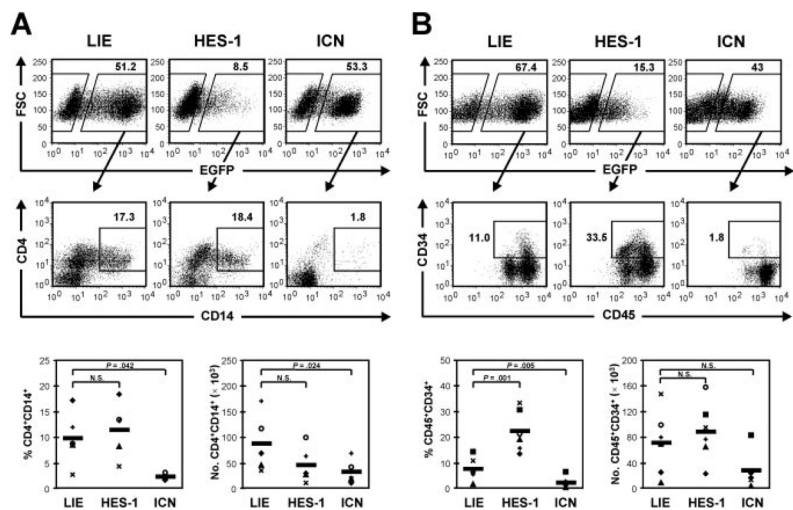
HES proteins that bind with greater affinity to the promoter of downstream genes and have a stronger repression activity than the respective homodimers.[20] This might explain why overexpression of HES-1 alone has little effect. Possibly one or more other transcription factors that are up-regulated by ICN overexpression must be overexpressed together with HES-1. HERP1 *(Hey1)* and HERP2 *(Hey2)* knock-out mice have been established recently,[21] so it will be interesting to find out whether T-cell development is influenced in these mice. Another possible scenario is that ICN drives cells to the T/NK pathway by up-regulating T/NK-committed genes. For example, the pre–TCR- α chain *(PTCRA)* gene has been shown to be a direct target gene of Notch-1 in T cells.[10,22] Furthermore, several studies point to the existence of CBF1, suppressor of hairless Lag-1 (CSL)–independent Notch signaling pathways.[23] Although the CSL pathway is active in

ICN-transduced cord blood CD34+ cells, as shown by the up-regulation of HES-1, it is possible that the effects of ICN on differentiation are mediated by CSL-independent mechanisms.

Acknowledgments

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ERRATUM

Page 2881 of the article, line 6 "HERP1 (*Hey1*) and HERP2 (*Hey2*)" should be "HERP1 (*Hey2*) and HERP2 (*Hey1*)".

Chapter 4

Molecular characterization of the CD34$^+$CD38$^-$CD7$^+$ common lymphoid progenitor from human cord blood.

Hoebeke, I., De Smedt M., Stolz, F., Pike-Overzet, K., Staal, F.J.T., Plum, J. and Leclercq, G.

Molecular characterization of the CD34$^+$CD38$^-$CD7$^+$ common lymphoid progenitor from human cord blood

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Abstract

Hematopoietic stem cells in the BM give rise to all types of blood cells. According to the classic model of hematopoiesis, the differentiation paths leading to the myeloid and lymphoid lineages segregate early. This model is supported by the isolation of cell populations with lymphoid- or myeloid-restricted differentiation potential from murine and human BM. A 'common lymphoid progenitor' has also been isolated from the CD34$^+$CD38$^-$ fraction of human umbilical cord blood based on its expression of the CD7 cell surface marker. In the present study, we confirm its lymphoid-restricted differentiation potential and show in addition that this population has strong T-cell differentiation potential. In an attempt to unravel the molecular mechanisms underlying lymphoid commitment, we performed Affymetrix oligonucleotide array analyses on sorted CD34$^+$CD38$^-$CD7$^+$ and CD34$^+$CD38$^-$CD7$^-$ cells. Our analysis revealed the differential expression of many transcription factors, RNA binding molecules, signal transduction molecules, cell cycle genes and enzymes. Of the genes with reported expression in hematopoietic tissues, we found that lymphoid-affiliated genes were mainly upregulated in the CD7$^+$ population while myeloid-specific genes were found to be downregulated in the CD7$^+$ cells, supporting the hypothesis that lineage commitment is accompanied by the shutdown of inappropriate gene expression and the upregulation of lineage-specific genes. In addition, our analysis identified several highly expressed genes that have not been described in hematopoiesis before and thus are interesting candidates for future research.

Introduction

All blood cells ultimately derive from a rare population of hematopoietic stem cells in the BM that are multipotent and have the ability to self-renew. According to the classic model of hematopoiesis, all lymphoid cells (T, B and NK cells) develop through a common precursor stage, the so-called 'common lymphoid progenitor' (CLP), and accordingly, cells from the myeloid lineages share a 'common myeloid progenitor' (CMP). This model was supported by the prospective isolation of cell populations with CLP and CMP function from the murine BM (1, 2). Recent evidence however indicated that BM CLPs are not physiological T-cell progenitors, as early thymic progenitors (ETPs) do not have the CLP phenotype (3) and CLPs are not present in the peripheral blood (4). Instead, the thymus is most likely seeded by a multipotent progenitor. During fetal hematopoiesis, B and T cells do not share a direct common progenitor either, as CLPs were not found in the fetal liver (5). Instead, fetal B and T cells would develop through B/myeloid and T/myeloid intermediates.

The first report of a human CLP came from Galy et al. who showed that a subpopulation of adult and fetal BM Lin⁻CD34⁺ cells expressing the early B- and T-cell marker CD10 is not capable of generating monocytic, granulocytic, erythroid or megakaryocytic cells, but can differentiate into dendritic cells, B, T and NK cells (6). These Lin⁻CD34⁺CD10⁺ cells homogenously expressed CD38. According to Ishii et al. expression of the chemokine receptor CXCR4 on BM CD34⁺ cells would be sufficient to restrict their differentiation potential to the lymphoid lineage (7). A human CMP was recently also identified in the Lin⁻CD34⁺CD38⁺ fraction of BM and cord blood. These CMPs are CD45RA⁻ and express low levels of IL-3Rα (8).

In cord blood, expression of CD10 on CD34⁺ cells does not discriminate progenitor cells with lymphoid-restricted potential from multipotent cells (9). However, Hao et al. detected in the most primitive CD34⁺CD38⁻ cord blood fraction a subpopulation expressing CD7, an antigen that was previously identified on early human T-lymphoid progenitors, and they showed that single CD34⁺CD38⁻CD7⁺ cord blood cells can generate B cells, NK cells and dendritic cells, but are devoid of myeloid or erythroid differentiation potential. T-cell potential was not addressed by these investigators (9).

In a recent study, Haddad et al. compared the differentiation potential of cord blood CD34⁺CD45RA^hiLin⁻CD10⁺ cells, which correspond to the BM CLP, with that of cord blood CD34⁺CD45RA^hiCD7⁺ cells, which comprise the CD34⁺CD38⁻CD7⁺ CLP, as these uniformly express CD45RA. The authors showed that the differentiation potential of CD34⁺CD45RA^hiCD7⁺ cells is skewed toward the T/NK lineages, while

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CD34$^+$CD45RA^{hi}Lin$^-$CD10$^+$ cells predominantly possess B-cell differentiation potential. Additionally, both populations retain some degree of myeloid differentiation capacity. Gene expression data from microarray analyses supported their conclusions.

In the present study, we confirm that the CD34$^+$CD38$^-$CD7$^+$ cord blood population is lymphoid-committed and we show that it also has strong T-lymphoid differentiation potential in Fetal Thymus Organ Culture (FTOC). Next, we investigated the differential gene expression between CD34$^+$CD38$^-$CD7$^-$ multipotent cells and CD34$^+$CD38$^-$CD7$^+$ common lymphoid progenitor cells using Affymetrix GeneChip technology.

Materials and methods

Cell sorting

Within 12 hours after collection of human umbilical cord blood samples, mononuclear cells were isolated over a Lymphoprep density-gradient (Axis-Shield PoC AS, Oslo, Norway) and CD34$^+$ cells were isolated by positive selection with MACS magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were labelled with anti-CD34-allophycocyanin (APC), anti-CD38-phycoerythrin (PE) and anti-CD7-fluorescein isothiocyanate (FITC) monoclonal antibodies (BD Biosciences, San Jose, CA) and CD34$^+$CD38$^-$CD7$^+$ and CD34$^+$CD38$^-$CD7$^-$ cells were sorted with a FACSVantage Cell sorter (Becton and Dickinson Immunocytometry Systems (BDIS), San Jose, CA). The purity of the sorted cells was checked on a FACSCalibur (BDIS) and was always > 95%. Sorted cells were either directly used in MS-5 co-cultures or FTOC, or either stored in 200 µl TRIZOL (Invitrogen, Carlsbad, CA) at −70°C for later RNA isolation and use in microarray experiments or Real-Time PCR.

Co-culture on MS-5 stromal cells

The differentiation of stem cells to most lymphoid (except T cells) and myeloid cell types can be accomplished *in vitro* by culturing them in the presence of the appropriate human recombinant cytokines on a feeder layer of the murine stromal cell line MS-5 (10). Four days before their use in co-culture experiments, MS-5 cells (kindly provided by L. Coulombel, Institut Gustave Roussy, Villejuif, France) were seeded in 96-well plates at a density of 5 x 10^3 cells per well. Co-cultures were initiated by incubating human sorted cells in 200 µl IMDM medium (Invitrogen) supplemented with 5% human AB serum (Valley Biomedical, Winchester, VA), 5% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamin (all from Invitrogen) and the following cytokines: SCF (50 ng/ml), FL (50 ng/ml), TPO (10 ng/ml), IL-2 (5 ng/ml), IL-7 (20 ng/ml) and IL-15 (10 ng/ml) (Mix 6) (all cytokines from

R&D Systems, Abingdon, UK). After 20 days of culture at 37°C and 7% CO_2, the progeny of the cells was counted with a Bürker hemocytometer excluding dead cells with trypan blue, and their phenotype was determined by flow cytometry.

Fetal Thymus Organ Culture (FTOC)

Thymic lobes were isolated from fetal day 15 NOD-SCID mice obtained from our own pathogen-free breeding facility. Each lobe was placed in a well of a Terasaki-plate and 25 µl complete IMDM medium containing 1000 human cells was added. The plates were inverted and incubated at 37°C with 7% CO_2 for 72 h. After this 'hanging drop' culture, during which the precursor cells migrate into the thymic lobes, the lobes were transferred to a Nuclepore polycarbonate membrane (Whatman, Brentford, UK) on a Gelfoam sponge (Pharmacia & Upjohn, Kalamazoo, MI) soaked in complete IMDM medium supplemented with 10% human AB serum and cultured for 32 days at 37°C with 7% CO_2. After the first 14 days, half of the medium was replaced with fresh medium. Thymocytes were harvested by mechanical disruption of the thymic structure and viable cells were counted by trypan blue exclusion. Then cells were stained with appropriate antibodies and analysed by flow cytometry.

Flow cytometry

Before labelling with antibodies, cells were pre-incubated 15 min with anti-mouse FcRγII/III (clone 2.4.G2, a kind gift of Dr. J. Unkeless, Mount Sinai School of Medicine, New York, NY) and human IgG (Miltenyi Biotec) to block murine and human Fc receptors respectively. Cells were incubated with appropriate amounts of combinations of the following mouse anti-human monoclonal antibodies: CD19-PE, CD34-APC, CD4-APC, CD33-FITC, CD14-FITC (all from BD Biosciences), CD56-APC and CD8β-PE (both from Immunotech, Beckman Coulter, Fullerton, CA). Cell populations containing mouse leukocytes (from FTOC) were simultaneously stained with anti-mouse CD45-CyChrome (BD Pharmingen, San Diego, CA). After 45 min, cells were washed with ice-cold PBS + 1% BSA + 0.1% NaN_3, propidium iodide (4 µg/ml) was added and cells were analysed on a FACSCalibur. Propidium iodide positive and mouse CD45 positive cells, representing dead cells and mouse leukocytes respectively, were excluded from analysis, which was performed with CellQuest software (BDIS).

RNA isolation and amplification

The TRIZOL lysates of different sorts, corresponding to a total of 100,000 sorted cells, were pooled and total RNA was extracted and purified on an RNeasy column (Qiagen, Venlo, The Netherlands) according to the instructions of the manufacturers. The RNA was concentrated to 10 µl with Microcon YM-50 columns (Millipore, Billerica, MA) and subjected to Degenerative Oligonucleotide Primer (DOP) mediated amplification. The detailed protocol, which was developed in our lab, can be found in the online Supplementary Methods section. Briefly, mRNA was first reverse transcribed using a T7-promoter oligo(dT) primer. After RNase H treatment, second strand synthesis was initiated using the 22-nt DOP-primer. In vitro transcription of the cDNA with T7 RNA-polymerase was done to generate cRNA, which was used in a second round of amplification using random hexamers for synthesis of the first strand, and the T7-promoter oligo(dT) primer for synthesis of the second strand. cDNA was transcribed and biotin-labelled using the ENZO BioArray HighYield RNA Transcript Labeling Kit (ENZO, Farmingdale, NY) according to the manufacturer's instructions. Biotinylated cRNA was purified on an RNeasy column and its quality was determined on the Agilent 2100 BioAnalyzer (Agilent, Palo Alto, CA). RNA isolation and amplification was done twice for both $CD34^+CD38^-CD7^+$ and $CD34^+CD38^-CD7^-$ cells (biological duplicates).

Microarray analysis

Biotinylated cRNA was fragmented and hybridized to Affymetrix GeneChip arrays according to the guidelines of the manufacturer. In a first experiment, cRNA was hybridized to Affymetrix HG-U133A arrays, while in a second experiment it was hybridized to Affymetrix HG-U133 Plus 2.0 arrays. Statistical analysis of the microarray data was performed as described before (11). Briefly, after background removal and quantile normalization by Robust Multi-chip Average (RMA) analysis (12), the raw perfect match (PM) probe intensity levels were used in a per probe set two-way analysis of variance (ANOVA) (with factors 'probe' and 'cell population') to generate an average expression level for the 2 biological repeats and a p-value for the significance of the difference between the $CD34^+CD38^-CD7^+$ and $CD34^+CD38^-CD7^-$ cell populations. The p-values were adjusted for multiple testing using Sidak step-down adjustment and differences with adjusted p-values < 0.05 were considered significant.

Bioinformatics

The differentially expressed genes were categorized according to Gene Ontology (GO) terms using the Affymetrix NetAffx center (http://www.affymetrix.com/analysis/index.affx) and the freely available programs Onto-Express (13) and Ease (http://david.niaid.nih.gov/david/ease.htm). Further information on the genes was gained by manually searching OMIM (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM) and GeneCard databases (http://www.genecards.org/), which both have links to PubMed literature references. Expression pattern information was derived from GeneNote, a database of human genes and their expression profiles in healthy tissues based on microarray experiments performed on the Affymetrix HG-U95 set (http://genecards.weizmann.ac.il/genenote/), and SymAtlas (http://symatlas.gnf.org/SymAtlas/). Mapping of the genes on pathways and networks was done using the commercial package Ingenuity Pathways Analysis (Ingenuity, Redwood City, CA). MatchMiner (http://discover.nci.nih.gov/matchminer/index.jsp) was used to find common genes in the lists of differentially expressed genes of our study and those of Dik et al. (11) and Van Zelm et al. (14).

Real-Time PCR analysis

Total RNA was extracted from sorted cells using TRIZOL and was DNAse treated on an RNeasy column according to the manufacturer's guidelines. The RNA was concentrated with Microcon YM-50 columns and oligo(dT)-primed reverse transcription was performed with SuperScript^{TM} II (Invitrogen). Real-Time PCR analysis with Sybr Green I (Eurogentec, Seraing, Belgium) was performed with an ABI PRISM 7000 (Applied Biosystems, Foster City, CA) using the standard temperature protocol (40 cycles of 10 min 95°C, 15 sec 95°C, 60 sec 60°C). Reaction mixtures contained 300 nM of forward and reverse primers and 0.04% BSA (Sigma, St. Louis, MO). Primers were designed using Primer Express 2.0 software (Applied Biosystems) and sequences can be found in Supplementary Table 1. Expression levels were normalized to the expression of the reference gene *HPRT* using the $\Delta\Delta C_T$ method (15).

Results

CD34$^+$CD38$^-$CD7$^+$ cord blood cells generate B and NK cells but no myeloid cells in co-cultures on MS-5 stromal cells

To confirm that CD34$^+$CD38$^-$CD7$^+$ cord blood cells are committed to the lymphoid lineage, we stringently sorted both CD34$^+$CD38$^-$CD7$^+$ (hereafter called CD7$^+$) and CD34$^+$CD38$^-$CD7$^-$ (hereafter called CD7$^-$) populations (see Figure 1) and determined their differentiation potential in MS-5 co-cultures with a mix of 6 cytokines (SCF, FL3, TPO, IL-2, IL-7 and IL-15) permitting both lymphoid and myeloid differentiation.
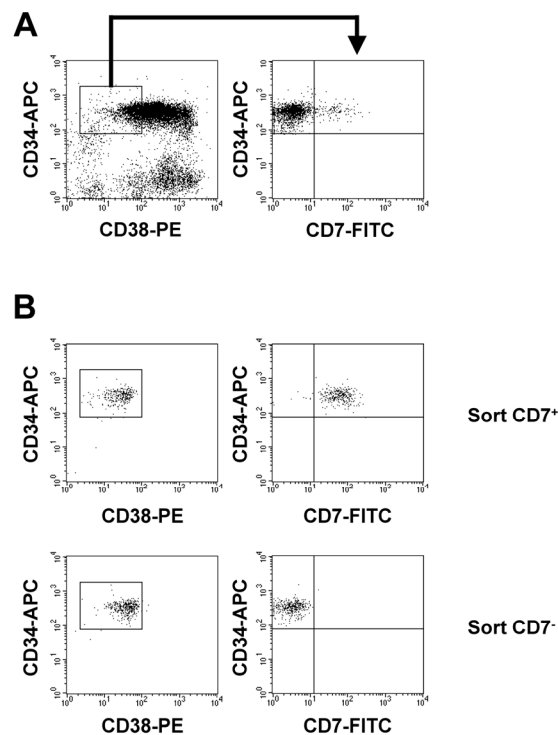
Figure 1. Isolation of CD34$^+$CD38$^-$CD7$^+$ and CD34$^+$CD38$^-$CD7$^-$ cells from human umbilical cord blood.
(A) Indication of sorting strategy. Cells in the upper left and upper right quadrants of the dot plot showing CD7 expression on electronically gated CD34$^+$CD38$^-$ cells were sorted. (B) Reanalysis of sorted cells with FACSCalibur showed purity of > 95%.

In initial experiments, we noticed that the CD7$^-$ cells proliferated much faster than the CD7$^+$ cells. Therefore, even the slightest contamination of the CD7$^+$ population with CD7$^-$ cells would obscure the real differentiation potential of the CD7$^+$ cells. For this reason the sorted cells were plated at 10 cells per well, which ensures that most wells only contain CD7$^+$ cells. Figure 2 gives an overview of a typical experiment consisting of 50 wells with CD7$^+$ cells and 30 wells with CD7$^-$ cells. After 20 days of culture, cells were counted and stained with antibodies for CD19, CD56 and CD33 to identify B cells, NK cells and myeloid cells respectively. On cells from the 20 wells with the highest cell number, a second staining was

performed with antibodies against the hematopoietic stem cell marker CD34 and the monocyte marker CD14. Five wells initiated with CD7$^+$ cells did not contain enough cells for reliable FACS analysis and were excluded from analysis. Cytokine mix 6 is optimized to support both expansion of CD34$^+$ stem cells and differentiation towards monocytes (16). Indeed, CD7$^-$ cells proliferated well and most cells differentiated into the myeloid lineage (CD33$^+$), while also a low number of cells remained CD34$^+$. On the contrary, the wells initiated with CD7$^+$ cells contained only B and NK cells, and almost no CD34$^+$ cells or myeloid cells. Absolute cell numbers were 13-fold reduced compared to wells initiated with CD7$^-$ cells. Remarkably, the proportion of B and NK cells in the different wells initiated with CD7$^+$ cells was highly variable, with most wells being enriched in either of the two cell types, and only five wells containing similar numbers of B and NK cells. This explains the large standard deviation on the average frequencies of B and NK cells.
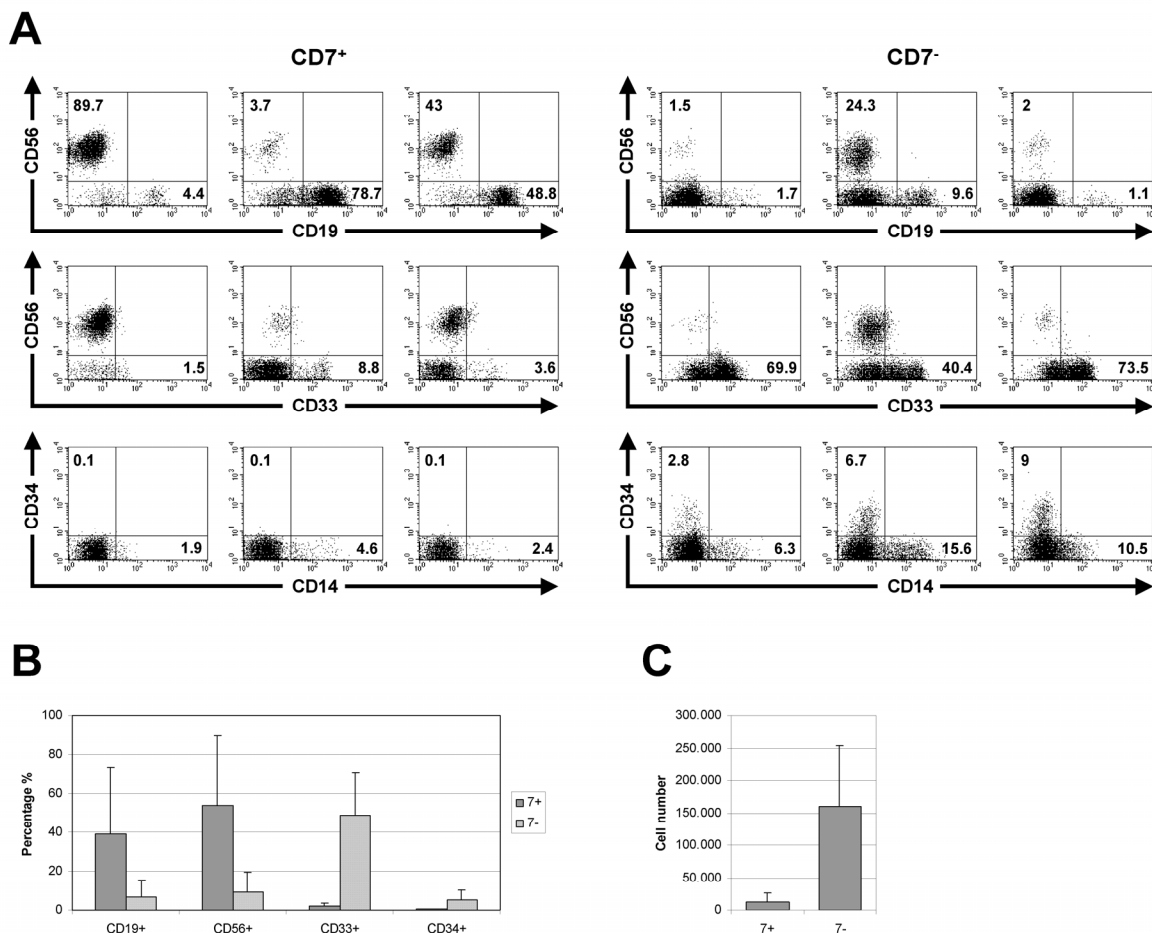
Figure 2. CD34$^+$CD38$^-$CD7$^+$ cord blood cells have lymphoid-restricted differentiation potential. (A) Flow cytometric analysis of MS-5 co-cultures with cytokine mix 6 (SCF, FL3, TPO, IL-2, IL-7 and IL-15) after 20 days. Dot plots for 3 wells of 50 wells initiated with 10 CD7$^+$ cells and 3 wells of 30 wells initiated with 10 CD7$^-$ cells are shown. (B) Mean frequencies of B cells (CD19$^+$), NK cells (CD56$^+$), myeloid cells (CD33$^+$) and HSCs (CD34$^+$) obtained with CD7$^+$ cells and with CD7$^-$ cells. Error bars represent the standard deviation. (C) Average absolute cell number obtained from 10 cells after 20 days of MS-5 culture with cytokine mix 6.

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CD34⁺CD38⁻CD7⁺ cord blood cells efficiently generate T cells in FTOC

The co-culture system on MS-5 stromal cells does not support T-cell differentiation. The capacity of the CD7$^+$ and CD7$^-$ cells to generate T cells was compared in Fetal Thymus Organ Cultures (FTOC). Three independent experiments were initiated with 1000 cells per thymic lobe. Thymocytes were harvested after 32 days of organ culture and analysed for the expression of CD4 and CD8β (see Figure 3). At that time, cultures initiated with CD7$^+$ cells clearly had generated a higher percentage of double positive (DP) thymocytes than those initiated with CD7$^-$ cells. Also from the absolute cell number it is clear that CD7$^+$ cells performed much better in FTOC than CD7$^-$ cells. CD7$^+$ cells generated about 10 times more cells than the CD7$^-$ cells (150,197 ± 5,668 versus 14,549 ± 17,886). These data indicate that, as expected from a common lymphoid progenitor, CD7$^+$ cells generate T cells with a much faster kinetics than the more immature CD7$^-$ cells.
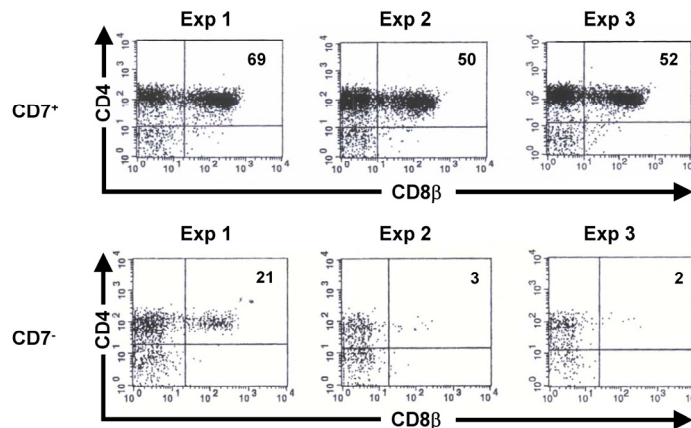
Figure 3. CD34$^+$CD38$^-$CD7$^+$ cord blood cells have strong T-cell generation capacity. Flow cytometric analysis of FTOC after 32 days of culture. Percentages of CD4$^+$CD8$^+$ double positive thymocytes obtained in 3 independent experiments are indicated in the upper right quadrant.

RNA amplification and microarray analysis

Because of the rare nature of the CD34$^+$CD38$^-$CD7$^+$ cord blood population, it was necessary to perform RNA amplification to obtain a sufficient amount of RNA to hybridize on the GeneChips. For each of two separate experiments, total RNA was isolated from a total of 100,000 sorted CD34$^+$CD38$^-$CD7$^+$ and CD34$^+$CD38$^-$CD7$^-$ cells pooled from 3 to 8 separate sorts, in each of which 1 to 3 cord blood units were pooled, which guaranteed a normalization of interindividual sources of variation.

In the first microarray experiment, cRNA was hybridized to Affymetrix HG-U133A arrays, which are comprised of more than 22,000 probesets representing 18,400 transcripts, including 14,500 well-characterized human genes. In the second experiment cRNA was

hybridized to Affymetrix HG-U133 Plus 2.0 arrays, which are comprised of more than 54,000 probesets representing 47,000 transcripts, including 38,500 well-characterized human genes. The expression values of the 22,215 common probesets in both experiments were used to calculate a correlation coefficient between the two biological repeats. As shown in Figure 4, the correlation between the biological repeats was very high (correlation coefficients of 0.92 and 0.96 for the CD7$^-$ and the CD7$^+$ arrays, respectively), which allowed us to use the average expression values of the two repeats for further analysis. Of the 22,215 probesets that were present on both GeneChips, 201 were significantly differentially expressed (adjusted p < 0.05). Of these, 110 probesets representing 101 genes were upregulated in the CD7$^+$ population, while 91 probesets representing 89 genes were downregulated in the CD7$^+$ population.
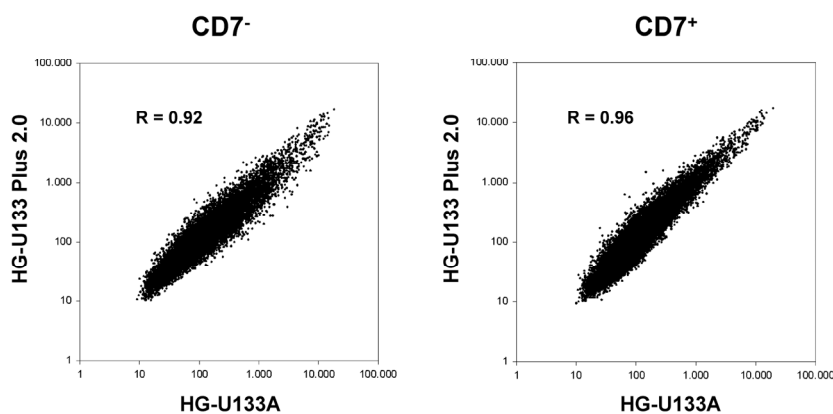
Figure 4. Correlation between the microarray experiments. Expression levels for each of the 22,215 common probesets after hybridization on the HG-U133A chip (first experiment) and the HG-U133 Plus 2.0 chip (second experiment) are shown. The correlation coefficient R is indicated.

Validation of microarray data

The mRNA expression of the markers used for cell sorting, namely CD34, CD38 and CD7, as measured by the microarrays, correlated well with their cell surface expression on the sorted populations. To further validate the microarray data, the expression of 11 transcripts was confirmed by Real-Time PCR on unamplified RNA from freshly sorted cells. Except for *RGS2*, fold changes obtained by Real-Time PCR analysis correlated well with those obtained by the microarray analysis (see Figure 5).

A.

B.

$y = 0,9795x + 0,5729$
$R^2 = 0,9795$
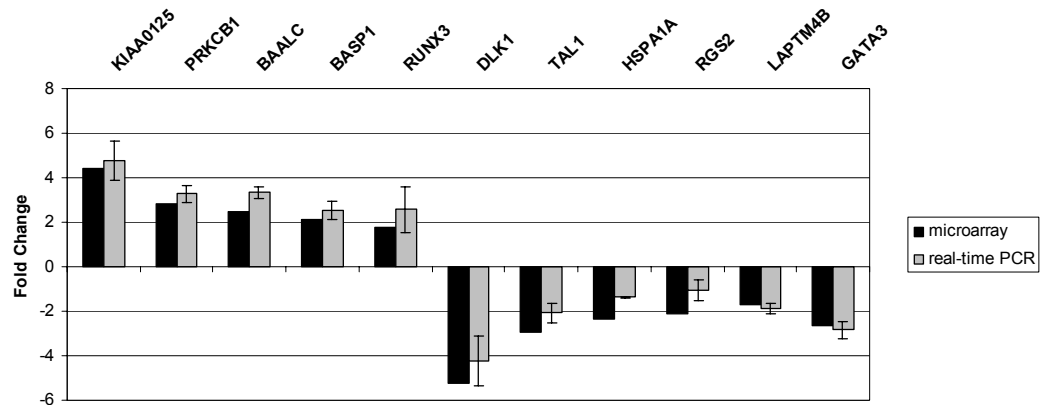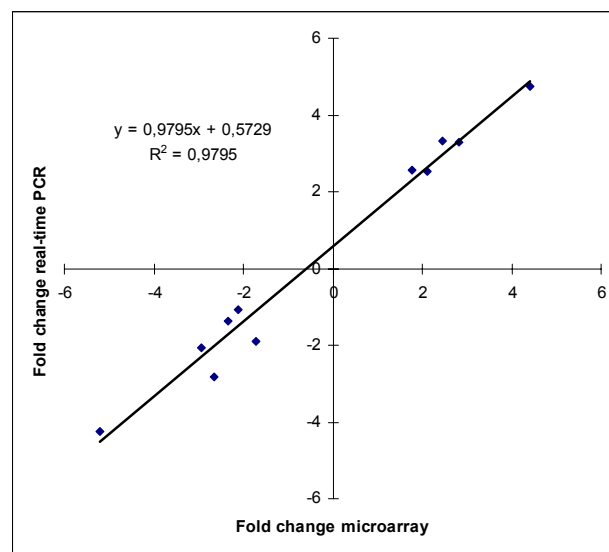
Figure 5. Validation of microarray data by Real-Time PCR. (A) The fold change (expression in CD7$^+$ cells relative to expression in CD7$^-$ cells) of a selection of differentially expressed genes as determined by microarray analysis and Real-Time PCR analysis. Bars represent the SD on the mean of duplicate PCR reactions, except for *RGS2* and *PRKCB1*, where they represent the SD on the mean of biological repeats. mRNA levels were normalized to *HPRT* mRNA expression. (B) Correlation between the fold changes determined by microarray analysis (on X-axis) and Real-Time PCR analysis (on Y-axis).

Results from the microarray analysis

Using annotation software such as Onto-Express, Ease and the NetAffx Analysis Center from Affymetrix, the significantly differentially expressed genes were grouped into functional categories (see Tables 1 and 2). Figure 6 shows the Gene Ontology (GO) terms of the categories Molecular Function, Biological Process and Cellular Component that have at least three genes annotated to them. Gene categories that are significantly overrepresented in the list of upregulated genes include transcription factors, RNA binding molecules, components of the ubiquitin-protein ligase complex, splice factors, transporters and signal

transduction molecules. The list of downregulated genes is significantly enriched for structural components of the ribosome, components of the cytoskeleton, signal transduction molecules and molecules involved in protein biosynthesis and cell proliferation.

Upregulated genes

GO: Molecular Function

Corrected P-value	Probesets Nr.	%	
0.012	11	9.9%	DNA binding
0.179	11	9.9%	ATP binding
0.143	9	8.1%	zinc ion binding
0.010	7	6.3%	RNA binding
0.310	6	5.4%	protein binding
0.052	6	5.4%	transferase activity
0.002	6	5.4%	ubiquitin-protein ligase activity
0.199	6	5.4%	transcription factor activity
0.881	6	5.4%	receptor activity
0.133	4	3.6%	calcium ion binding
1.0	3	2.7%	GTP binding
0.256	3	2.7%	hydrolase activity
0.112	3	2.7%	nucleic acid binding
0.414	3	2.7%	molecular_function_unknown

GO: Biological Process

Corrected P-value	Probesets Nr.	%	
0.180	11	9.9%	regulation of transcription, DNA-dependent
3.2E-4	6	5.4%	protein ubiquitination
0.160	5	4.5%	transport
0.155	5	4.5%	development
0.002	5	4.5%	regulation of transcription from Pol II promoter
0.096	4	3.6%	protein amino acid phosphorylation
0.005	4	3.6%	small GTPase mediated signal transduction
0.371	4	3.6%	intracellular signaling cascade
0.469	4	3.6%	apoptosis
0.035	3	2.7%	intracellular protein transport
0.020	3	2.7%	nuclear mRNA splicing, via spliceosome
0.0	3	2.7%	negative regulation of transcription

GO: Cellular Component

Corrected P-value	Probesets Nr.	%	
0.007	29	26.1%	nucleus
1.0	9	8.1%	integral to membrane
0.941	6	5.4%	cytoplasm
0.858	6	5.4%	membrane
6.7E-4	6	5.4%	ubiquitin ligase complex
1.0	4	3.6%	cytoskeleton
0.510	4	3.6%	plasma membrane
0.177	3	2.7%	membrane fraction

Downregulated genes

GO: Molecular Function

Corrected P-value	Probesets Nr.	%	
0.185	9	9.7%	protein binding
0.175	5	5.4%	calcium ion binding
0.427	5	5.4%	signal transducer activity
2.0E-4	5	5.4%	structural constituent of ribosome
0.177	5	5.4%	transcription factor activity
0.006	4	4.3%	actin binding
0.176	4	4.3%	DNA binding
0.005	4	4.3%	electron transporter activity
0.236	4	4.3%	RNA binding
0.259	4	4.3%	zinc ion binding
2.5E-4	3	3.2%	GTPase activator activity
0.712	3	3.2%	molecular_function unknown
0.671	3	3.2%	oxidoreductase activity

GO: Biological Process

Corrected P-value	Probesets Nr.	%	
0.057	11	11.8%	regulation of transcription, DNA-dependent
0.246	8	8.6%	signal transduction
0.059	5	5.4%	development
0.003	5	5.4%	cell proliferation
0.027	5	5.4%	intracellular signaling cascade
0.002	5	5.4%	protein biosynthesis
0.033	4	4.3%	cell-cell signaling
0.549	4	4.3%	immune response
0.001	3	3.2%	perception of sound
0.309	3	3.2%	cell growth and/or maintenance
0.161	3	3.2%	regulation of cell cycle
1.0	3	3.2%	apoptosis
0.121	3	3.2%	metabolism

GO: Cellular Component

Corrected P-value	Probesets Nr.	%	
0.905	17	18.3%	nucleus
1.0	8	8.6%	integral to plasma membrane
0.732	8	8.6%	integral to membrane
0.0	5	5.4%	ribosome
0.425	4	4.3%	cytoplasm
0.191	4	4.3%	extracellular space
0.438	4	4.3%	intracellular
0.192	4	4.3%	plasma membrane
0.208	4	4.3%	membrane
0.801	4	4.3%	mitochondrion
0.754	3	3.2%	soluble fraction
0.649	3	3.2%	cellular_component unknown
0.004	3	3.2%	actin cytoskeleton

Figure 6. Functional profile of significantly upregulated and downregulated genes constructed by Onto-Express. Gene Ontology (GO) terms in the categories Molecular Function, Biological Process and Cellular Component which have at least 3 genes annotated to them are shown. The number of probesets and percentage of probesets annotated to each GO term are indicated. Note that a probeset can be annotated to more than one GO term. GO terms that are significantly overrepresented in the lists of up- and downregulated genes (Bonferroni-corrected P-value < 0.05) are shown in red.

Table 1.   Genes significantly upregulated in CD7$^+$ cells, categorized according to function and ranked according to fold change (FC). Listed gene names are approved by the HUGO Gene Nomenclature Committee. Alternative gene names are given between brackets. Genes reported to be expressed in hematopoietic tissue are underlined. References are available as online supplementary material.

Gene Name	Description	Ref.	Probeset	FC
Transcription				
IRF8	interferon consensus sequence binding protein 1 (interferon regulatory factor 8). Expressed exclusively in cells of the immune system. Drives myeloid progenitor cells toward macrophages, while inhibiting granulocytic differentiation. Essential for differentiation of CD8α^+ dendritic cells.	(1-3)	204057_at	4.47
SETBP1 (SEB)	SET binding protein 1		205933_at	2.93
MEF2A	MADS box transcription enhancer factor 2, polypeptide A (myocyte enhancer factor 2A). Required for the transcriptional activation of IL-2 in T cells.	(4)	212535_at	2.87
LRRFIP1 (GCF2)	leucine rich repeat (in FLII) interacting protein 1. Highly expressed in peripheral blood leukocytes. Transcriptional repressor of TNF-α.	(5, 6)	201862_s_at	2.72
RERE	arginine-glutamic acid dipeptide (RE) repeats. Transcriptional repressor. Overexpression leads to cell death.	(7)	200940_s_at	2.43
TARBP1	TAR (HIV) RNA-binding protein 1		202813_at	2.33
SMARCA4 (BRG1)	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4. Essential for T-cell development. Silenced in several tumor cell lines. Tumor suppressor activity by cooperating and complexing with the retinoblastoma tumor suppressor protein (pRB), which inhibits cell cylce progression by repressing transcription of specific growth-related genes.	(8-12)	214728_x_at	2.33
HLX1 (HB24)	H2.0-like homeo box 1 (Drosophila). Expressed throughout the myeloid/macrophage lineage and at early stages of B-cell development.	(13)	214438_at	2.29
SATB1	special AT-rich sequence binding protein 1 (binds to nuclear matrix/scaffold-associating DNA's). Regulates the expression of numerous genes during thymocyte differentiation by recruiting chromatin remodelling factors.	(14-17)	203408_s_at	2.24
TRIM33 (TIF1G)	tripartite motif-containing 33. Mutation of the zebrafish ortholog disrupts both embryonic and adult hematopoiesis. Also has ubiquitin protein ligase activity.	(18)	210266_s_at	2.21
NCOR2 (SMRT)	nuclear receptor co-repressor 2		207760_s_at	2.10
TCF4 (E2-2)	transcription factor 4. Required for both B- and T-cell development.	(19)	212387_at	2.04
BCL6	B-cell CLL/lymphoma 6 (zinc finger protein 51). Transcriptional repressor essential for the differentiation of germinal center B cells.	(20)	203140_at	2.02
RUNX3	runt-related transcription factor 3. Best known for silencing the expression of CD4 during the development of CD8 SP thymocytes. Also required for normal development of primitive and definitive hematopoietic cells.	(21, 22)	204198_s_at	1.78
CHD3 (Mi-2a)	chromodomain helicase DNA binding protein 3. Highly expressed in thymus (GeneNote).		208806_at	1.69
RNA binding				
SFPQ (PSF)	splicing factor proline/glutamine rich (polypyrimidine tract-binding protein-associated)		221768_at	3.18
METTL3	methyltransferase like 3. Associated with pre-mRNA splicing components and involved in posttranscriptional modification by methylating adenosine residues of some mRNAs.		213653_at	2.76
EIF4A1(DDX2A)	Eukaryotic translation initiation factor 4A, isoform 1		214805_at	2.55
SF3B1	splicing factor 3b, subunit 1, 155kD		201071_x_at	2.44
RNPC2 (HCC1)	RNA-binding region (RNP1, RRM) containing 2. Splicing factor.		208720_s_at	2.20
HNRPA1	heterogeneous nuclear ribonucleoprotein A1. Involved in splice site selection and responsible for the transport of mRNA from the nucleus to the cytoplasm.		214280_x_at	1.90
DDX21	DEAD (Asp-Glu-Ala-Asp) box polypeptide 21. RNA helicase involved in ribosomal RNA synthesis.		208152_s_at	1.86

Cell cycle

Gene	Description	Ref	Probe	Value
MN1	meningioma (disrupted in balanced translocation) 1. Functions as a transcriptional co-activator. Found in chromosomal translocation with TEL/ETV6 in AML. Ectopic expression of MN1-TEL fusion protein perturbs both myeloid and lymphoid cell growth. MN1 is expressed in HSCs, CMPs and GMPs. Enforced expression in osteoblastic cells results in profound decrease in cell proliferation by slowing S-phase entry.	(23-26)	205330_at	3.06
ATR	ataxia telangiectasia and Rad3 related. Exerts cell cycle delay following UV light-induced DNA damage by phosphorylating cell cycle checkpoint control proteins such as Chk1, Chk2 and p53.	(27)	209902_at	2.50
CCNL1	cyclin L1. Involved in regulation of RNA polymerase II transcription by phosphorylation of the C-terminal domain of RNA polymerase II. Also member of the pre-mRNA processing machinery.	(28)	220046_s_at	2.50
MACF1	microtubule-actin crosslinking factor 1. Involved in cell cycle arrest.		208634_s_at	2.40
NF1	Neurofibromin 1 (neurofibromatosis, von Recklinghausen disease, Watson disease). Tumor suppressor capable of inhibition of cell growth in culture. Negative regulator of Ras-signalling by promoting conversion of active Ras-GTP to inactive Ras-GDP. NF1-deficiency causes juvenile myelomonocytic leukemia. NF1$^{-/-}$ murine FL cells are hypersensitive to GM-CSF and generate a JMML-like phenotype. NF1$^{-/-}$ HSCs demonstrate growth advantage of differentiated myeloid and lymphoid cells.	(29-32)	216115_at	2.17
MCM3AP (GANP)	MCM3 minichromosome maintenance deficient 3 (S. cerevisiae) associated protein. Upregulated in germinal center B cells. Inhibits initiation of DNA replication by acetylating MCM.	(33-35)	212269_s_at	2.04
KNTC1 (ROD)	kinetochore associated 1. Essential component of the mitotic checkpoint that prevents cells from prematurely exiting mitosis.	(36)	206316_s_at	1.97
RBBP6 (RBQ-1)	retinoblastoma binding protein 6. Preferentially bound by underphosphorylated pRB, which is present in resting cells.	(37-39)	205178_s_at	1.94
POLS (TRF4)	polymerase (DNA directed) sigma. Involved in DNA repair.	(40)	202466_at	1.74

Apoptosis

Gene	Description	Ref	Probe	Value
NALP1	NACHT, leucine rich repeat and PYD (pyrin domain) containing 1. Activator of caspases. Highly expressed in peripheral blood leukocytes. Also central role in the processing of pro-IL-1-β.	(41)	210113_s_at	2.03

Receptor activity

Gene	Description	Ref	Probe	Value
CCR9	CC chemokine receptor 9A (CCR9) mRNA, alternatively spliced, complete cds. Receptor for the thymus-expressed chemokine TECK/CCL25. Regulates the coordinated migration of thymocytes through the thymus. Expression of CCR9 is tightly regulated during T-cell development, and forced premature expression of CCR9 in DN thymocytes partially blocks further development.	(42-50)	207445_s_at	3.78
EMR2	egf-like module containing, mucin-like, hormone receptor-like 2. Highly expressed on blood monocytes, macrophages and myeloid DC, but not on resting or activated lymphocytes.	(51)	207610_s_at	3.02
ITGA4 (CD49D)	integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor). Highly expressed by CD34$^+$ HSCs. Mediates adhesive interactions with the extracellular matrix of the BM stroma that are essential for hematopoiesis and homing of HSCs to the BM.	(52)	213416_at	2.60
P2RY14 (GPR105)	purinergic receptor P2Y, G-protein coupled, 14. Receptor for UDP-conjugated sugars. Expressed by the most primitive, quiescent stem cell population of human bone marrow.	(53)	206637_at	2.47
PLXND1	plexin D1. Receptor for semaphorins.		38671_at	1.89

Signal transduction

Gene	Description	Ref	Probe	Value
THRAP2	Thyroid hormone receptor associated protein 2		216109_at	3.94
RAB31	RAB31, member RAS oncogene family. Small GTP-ase of RAB family.		217763_s_at	3.51
RPS6KA2 (RSK3)	ribosomal protein S6 kinase, 90kDa, polypeptide 2		212912_at	3.33
PRKCB1	protein kinase C, beta 1. Pre-B cell receptor signalling molecule. Essential for B-cell development.	(54)	207957_s_at	2.83
RAB14	RAB14, member RAS oncogene family. Small GTP-ase of RAB family.		200927_s_at	2.67

BTK	Bruton agammaglobulinemia tyrosine kinase. Pre-B cell receptor signalling molecule. Essential for B-cell development.	(55, 56)	205504_at	2.66
TRAF5	TNF receptor-associated factor 5. Highly expressed in spleen and thymus (GeneNote). Activates NF-κB and functions downstream of the lymphotoxin-β receptor. Implicated in apoptosis.	(57)	204352_at	2.35
TRAF4	TNF receptor-associated factor 4. Expressed in primary T cells and Jurkat. Implicated in apoptosis.	(58, 59)	202871_at	2.28
EVL	Enah/Vasp-like. Involved in signal transduction leading to dynamic changes in the cytoskeleton. Highly expressed in thymus and spleen.	(60)	217838_s_at	2.24
NUDT3	Nudix (nucleoside diphosphate linked moiety X)-type motif 3		212605_s_at	2.15
TRAF3IP3 (T3JAM)	TRAF3 interacting protein 3. Highly expressed in lymphoid tissues (GeneNote).		213888_s_at	2.02
TNS3 (TENS1)	Tensin 3. Role in EGF-signalling pathway.		217853_at	1.86
DOCK1 (DOCK180)	dedicator of cytokinesis 1. Interacts with RAC1. Also involved in cytoskeletal rearrangements required for phagocytosis of apoptotic cells and cell motility.		203187_at	1.83
HA-1	minor histocompatibility antigen HA-1		212873_at	1.73
TNFAIP3 (A20)	tumor necrosis factor, alpha-induced protein 3. Inhibits TNF-induced NFκB activation. Has de-ubiquitinating activity and ubiquitin ligase activity. Highly expressed in lymphoid organs.	(61)	202643_s_at	1.60

Transport

SCN3A	sodium channel, voltage-gated, type III, alpha polypeptide		210432_s_at	6.18
ABCA1	ATP-binding cassette, sub-family A (ABC1), member 1 (ABCA1), mRNA. Cholesterol transporter.		203504_s_at	3.88
OSBPL3 (ORP3)	oxysterol binding protein-like 3 (ORP3). Highly expressed in most primitive CD34$^+$CD38$^-$ cells and downregulated upon proliferation and differentiation.	(62)	209626_s_at	3.74
SLC2A5 (GLUT5)	solute carrier family 2 (facilitated glucose transporter), member 5 (SLC2A5), mRNA.		204430_s_at	3.68
SLC38A1 (ATA1)	solute carrier family 38, member 1. Aminoacid transporter.		218237_s_at	3.09
SLC24A3	solute carrier family 24 (sodium/potassium/calcium exchanger), member 3		219090_at	2.34
SLC33A1	solute carrier family 33 (acetyl-CoA transporter), member 1		203164_at	2.34
TMEM41B	transmembrane protein 41B. Sugar porter activity.		212622_at	2.30
SLC35E2	solute carrier family 35, member E2		217122_s_at	2.13
TPR	translocated promoter region (to activated MET oncogene). Implicated in the import of proteins into the nucleus.		201730_s_at	2.00
ITSN2	intersectin 2. Involved in clathrin-mediated endocytosis.		209907_s_at	1.97
KNS2	kinesin 2. Transport of organelles and chromosomes along microtubuli during cell division.		216187_x_at	1.66

Enzymatic activity

TRIB2	tribbles homolog 2 (Drosophila). Inhibitor of cell division in *Drosophila*. Highest expression in peripheral blood leukocytes. Controls MAPK activity.	(63, 64)	202478_at	5.18
UBR2	ubiquitin protein ligase E3 component n-recognin 2		212760_at	2.60
ADAM28 (MDCL)	a disintegrin and metalloproteinase domain 28. Expressed on the surface of human lymphocytes.	(65)	205997_at	2.51
STK32B	serine/threonine kinase 32B		219686_at	2.37
LOC23117 DKFZp547E087 LOC348162 LOC440354 LOC613037	KIAA0220-like protein hypothetical gene LOC283846 hypothetical protein 348162 PI-3-kinase-related kinase SMG-1 pseudogene similar to the PI-3-kinase-related kinase SMG-1 family pseudogene 2		211996_s_at	2.28
DPEP2	dipeptidase 2		219452_at	2.02
ADA	adenosine deaminase. Mutations in this gene lead to human SCID disease, characterized by a deficiency of both B and T cells. Treatment of T-ALL patients with ADA-inhibitors leads to a complete conversion from T-lymphoblastic to promyelocytic leukemia.	(66, 67)	204639_at	1.89
USP34	ubiquitin specific protease 34		212066_s_at	1.70

Enzyme regulator activity

PSCD4 (cytohesin 4)	pleckstrin homology, Sec7 and coiled-coil domains 4. Guanine nucleotide-exchange factor for ADP-ribosylation factors. Expressed abundantly in leukocytes, but not in other tissues.	(68)	219183_s_at	3.16
ARHGAP25	Rho GTPase activating protein 25. Highly expressed in lymphoid tissues (GeneNote).		38149_at	3.10
ITIH4 (IHRP)	inter-alpha (globulin) inhibitor H4 (plasma Kallikrein-sensitive glycoprotein). Acute phase protein.		37201_at	1.83

Cytoskeleton

PALLD (KIAA0992)	palladin. Component of actin-containing microfilaments.	(69)	200897_s_at	3.73
PNN	pinin, desmosome associated protein. Also involved in mRNA processing. Potential tumor suppressor.	(70)	212036_s_at	3.14
BASP1	brain abundant, membrane attached signal protein 1. Highly expressed in lymphoid tissues.	(71)	202391_at	2.10
TBCD	tubulin-specific chaperone d. Involved in the folding of actin and tubulin.		211052_s_at	2.03

Miscellaneous

SPON1 (f-spondin)	spondin 1, (f-spondin) extracellular matrix protein		209436_at	3.19
TNFAIP2 (B94)	tumor necrosis factor, alpha-induced protein 2. Expressed in lymphoid tissues and peripheral blood monocytes.	(72)	202510_s_at	3.08
IGHM	immunoglobulin heavy constant mu. Essential for B-cell development.	(73)	209374_s_at	2.94
LPIN1	lipin 1. Antiproliferative effect on pro-B cells when ectopically expressed.	(74)	212276_at	2.60
NACA	nascent-polypeptide-associated complex alpha polypeptide. Required for the intracellular translocation of newly synthesized polypeptides. May also act as a transcriptional co-activator. Expressed in cord blood CD34$^+$ progenitor cells, maintained during in vitro erythroid differentiation but suppressed during megakaryocyte and granulocyte differentiation.	(75)	222018_at	1.96

Not annotated

KIAA0125	KIAA0125 gene product (KIAA0125), mRNA. Localizes to Ig heavy chain locus. Highest expression in BM, spleen and thymus (GeneNote).		206478_at	4.40
LSR68 (C14orf43)	lipopolysaccharide specific response-68 protein (LSR68), mRNA		220494_s_at	4.09
SH3TC1	SH3 domain and tetratricopeptide repeats 1. Highly expressed in thymus (SymAtlas).		219256_s_at	4.07
KIAA0087	KIAA0087 gene product		207161_at	3.41
JMJD1A	jumonji domain containing 1A		212689_s_at	3.31
-	MRNA full length insert cDNA clone EUROIMAGE 362430		215679_at	2.70
FLJ13197	hypothetical protein FLJ13197. Expressed in some subtypes of ALL.	(76)	219871_at	2.64
BAALC	brain and acute leukemia, cytoplasmic. Mostly expressed in neuroectoderm-derived tissues, but also in some cases of AML and ALL. Highly expressed by CD34$^+$ cells from BM and blood, downregulated upon in vitro differentiation.	(77, 78)	218899_s_at	2.46
FLJ10707	hypothetical protein FLJ10707		221806_s_at	2.40
SUV420H1	suppressor of variegation 4-20 homolog 1 (Drosophila)		218242_s_at	2.30
LOC388388	Hypothetical LOC388388		210230_at	2.13
COBL	cordon-bleu homolog (mouse). Involved in neural tube formation.		213050_at	2.10
FLJ22635	hypothetical protein FLJ22635. Highly expressed in lymphoid tissues (GeneNote).		219359_at	2.10
LOC339287	hypothetical protein LOC339287		212708_at	2.09
C11orf21 (SMS3)	chromosome 11 open reading frame 21. Highly expressed in lymphoid tissues (GeneNote).		220560_at	2.06
IQSEC1	IQ motif and Sec7 domain 1		203906_at	1.91
-	CDNA FLJ39679 fis, clone SMINT2010068		214996_at	1.90

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Table 2. Genes significantly downregulated in CD7$^+$ cells, categorized according to function and ranked according to fold change (FC). Listed gene names are approved by the HUGO Gene Nomenclature Committee. Alternative gene names are given between brackets. Genes reported to be expressed in hematopoietic tissue are underlined. References are available as online supplementary material.

Gene Name	Description	Ref.	Probeset	FC
Transcription				
NFIB	Nuclear factor I/B		213032_at	5.39
MLLT3 (AF9)	myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 3. Downregulation of MLLT3 leads to a reduced expression of the myeloid-specific transcription factor HOXA10.	(79, 80)	204918_s_at	4.86
KLF2 (LKLF)	Kruppel-like factor 2 (lung). Essential for primitive erythropoiesis. Also highly expressed in naïve CD4$^+$ and CD8$^+$ T cells and responsible for their quiescent phenotype by downregulating expression of the c-myc proto-oncogene.	(81, 82)	219371_s_at	3.77
EVI1	ecotropic viral integration site 1. Transcriptional repressor whose expression is restricted to a transient stage of myeloid differentiation. Involved in megakaryocyte differentiation. Overexpressed in some myeloid leukemia.	(83-85)	221884_at	3.64
CEBPB (NF-IL6)	CCAAT/enhancer binding protein (C/EBP), beta. Highly expressed in myeloid cells. Activates transcription of IL-6 and several myeloid genes. Ectopic expression in multipotent hematopoietic progenitors leads to differentiation along the myeloid lineage, while dominant-negative versions inhibit myeloid differentiation.	(86-89)	212501_at	3.45
MAFF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian). Interacts with the upstream promoter region of the oxytocin receptor gene.	(90)	36711_at	3.10
ELF1	E74-like factor 1 (ets domain transcription factor). Involved in the regulation of many T- and B-cellspecific genes. Also expressed in several myeloid cell types and involved in the regulation of SCL and LMO2 expression.	(91-96)	212420_at	3.01
TAL1 (alias SCL)	T-cell acute lymphocytic leukemia 1 (TAL1). Pivotal for the generation of all hematopoietic lineages. Expression is maintained during differentiation along erythroid, mast and megakaryocytic lineages, but is repressed after commitment along other hematopoietic lineages.	(97, 98)	206283_s_at	2.95
ETV5 (ERM)	ets variant gene 5 (ets-related molecule). Lymphoid-specific transcription factor that is upregulated in activated Th1 cells and overexpressed in B-cell lymphomas.	(99, 100)	203349_s_at	2.61
IFI16	interferon, gamma-inducible protein 16. Transcriptional repressor constitutively expressed in BM CD34$^+$ cells. Expression is maintained upon differentiation along the monocytic lineage, but is strongly downregulated upon differentiation to the granulocytic and erythroid lineages. Also constitutively expressed in lymphoid cells. Implicated in cell cycle regulation.	(101-104)	208965_s_at	1.91
HMGB3	high-mobility group box 3. Highly expressed in murine BM erythroid cells, HSCs, and most CLP and CMP cells. Long-term repopulating activity is entirely contained in the subpopulation of HSCs that express HMGB3. Enforced expression of HMGB3 inhibits both myeloid and B-cell differentiation.	(105)	203744_at	1.68
RNA binding				
LSM5	LSM5 homolog, U6 small nuclear RNA associated (S. cerevisiae). Involved in pre-mRNA splicing.		211747_s_at	2.56
KHDRBS3 (T-STAR) (SLM2)	KH domain containing, RNA binding, signal transduction associated 3. Involved in pre-mRNA splicing. Primarily expressed in the testes, skeletal muscle, heart and brain. Generally acts as a growth suppressor.	(106-108)	209781_s_at	1.96
Cell cycle				
NDN	necdin homolog (mouse). Neuron-specific growth suppressor. Functions also as a transcription factor.	(109)	209550_at	3.00
GADD45A (DDIT)	growth arrest and DNA-damage-inducible, alpha. Induced by ionising radiation and alkylating agents. Inhibits entry of cells into S phase and stimulates DNA repair.	(110)	203725_at	2.67
CKS2 (CKSHS2)	CDC28 protein kinase 2. Interacts with cyclin-dependent kinases (CDKs) that regulate mitosis. Highly expressed in BM and thymus (GeneNote).	(111, 112)	204170_s_at	2.10

Receptor activity

CXCR4	chemokine (C-X-C motif), receptor 4. Broadly expressed on hematopoietic cells. Mediates migration to its ligand SDF-1. CXCR4/SDF-1 interaction is essential for homing of HSCs to the BM and is important for both fetal and adult hematopoiesis.	(113-118)	217028_at	2.14
NPR3 (ANPRC)	natriuretic peptide receptor C/guanylate cyclase C (atrionatriuretic peptide receptor C). Important in the maintenance of blood pressure and extracellular fluid volume.		219789_at	2.08
LEPR (OBR)	leptin receptor. Receptor for the adipocyte-derived hormone leptin and involved in regulation of body weight by the hypothalamus. Also expressed on HSC populations and mature leukocytes, predominantly on monocytes/macrophages. Leptin signalling increases SCF-induced proliferation of primitive HPCs and has a role in immune response and inflammation.	(119-126)	202377_at	2.03
KIAA1049	KIAA1049 protein		213311_s_at	1.96

Receptor binding

TNFSF10 (TRAIL) (Apo-2L)	tumor necrosis factor (ligand) superfamily, member 10. Membrane-bound cytokine that induces rapid apoptosis of tumor cell lines. Positive regulator of myeloid differentiation.	(127, 128)	202687_s_at	2.65
ICAM4	intercellular adhesion molecule 4, Landsteiner-Wiener blood group. Erythroid-specific surface marker, suggested role in erythroid differentiation.	(129)	207194_s_at	2.57
OXT	oxytocin, prepro- (neurophysin I). Neuropeptide produced by thymic epithelial cells. Its receptor is expressed by all thymocyte subsets. Oxytocin signalling would be involved in the control of T-cell proliferation and survival.	(130, 131)	207576_x_at	2.29
ICAM2	intercellular adhesion molecule 2. Expressed at high levels on vascular endothelial cells and at low levels on most leukocytes and platelets. Mediates leukocyte adhesion by binding the integrin receptor LFA-1. This interaction protects ICAM2-expressing cells from apoptosis.	(132-134)	213620_s_at	2.21
IL1B	interleukin 1, beta. Secreted primarily by activated monocytes/macrophages. Expression is regulated by PU.1 and CEBPB.	(135)	39402_at	2.06

Signal transduction

TAX1BP3 (TIP-1)	Tax1 (human T-cell leukemia virus type I) binding protein 3. Might be involved in Wnt/β-catenin signalling.		209154_at	2.78
GRK5	G protein-coupled receptor kinase 5		204396_s_at	2.60
TNS1	Tensin. Actin binding protein. May be involved in linking signal transduction pathways to the cytoskeleton.		221748_s_at	2.56
NUDT4 (DIPP2)	nudix (nucleoside diphosphate linked moiety X)-type motif 4		206302_s_at	2.55
PIP5K1B (STM7)	phosphatidylinositol-4-phosphate 5-kinase, type I, beta. Required for actin organization.		205632_s_at	2.48
RAB38	RAB38, member RAS oncogene family		219412_at	2.43
PTPN11 (SHP-2)	protein tyrosine phosphatase, non-receptor type 11 (Noonan syndrome 1). Acts upstream of the Ras oncogene. Indispensable for both myeloid and lymphoid hematopoietic cell development. Dominant active mutations cause Noonan syndrome and are common in juvenile myelomonocytic leukemia (JMML) and other childhood hematopoietic malignancies. Also highly expressed in most adult leukemia cells.	(136-143)	209896_s_at	2.34
PSEN2 (PS2)	presenilin 2 (Alzheimer disease 4). Critical component of γ-secretase complex responsible for the proteolytic cleavage of Notch after ligand binding. Loss of PSEN2 results in an increased production of granulocytes without affecting other hematopoietic lineages.	(144, 145)	211373_s_at	2.33
ARHGEF12 (LARG)	Rho guanine exchange factor (GEF) 12. Fusion partner of the MLL gene in a case of myeloid leukemia. Highly expressed in HSC fractions and immature erythroid cells. Involved in the regulation of the actin cytoskeleton.	(146, 147)	201334_s_at	2.30
RGS2 (GOS8)	regulator of G-protein signalling 2, 24kD. Upregulated during granulocytic differentiation of several myeloid cell lines.	(148)	202388_at	2.12
S100A6 (calcyclin)	S100 calcium-binding protein A6 (calcyclin). Specifically expressed during G1 phase of cell cycle. Expressed in granulocytes, but not in lymphocytes. Overexpressed in cases of AML.	(149-152)	217728_at	2.11
RALBP1 (RLIP76)	ralA binding protein 1. Interacts with the GTP-bound form of ralA, ralB, cdc42 and rac1.		202844_s_at	2.07
BST2	bone marrow stromal cell antigen 2. First identified on BM stromal cells, but also expressed on myeloid and lymphoid hematopoietic cells. Its promoter is activated by the erythroid-specific transcription factor GATA1.	(153, 154)	201641_at	1.83

Transport

ATP1B1	ATPase, Na+K+ transporting, beta 1 polypeptide		201242_s_at	5.79
MAL	mal, T-cell differentiation protein. In endoplasmatic reticulum membrane of T cells. Expressed only in intermediate and late stages of T-cell differentiation.	(155)	204777_s_at	2.72
TFR2	transferrin receptor 2. Highly expressed in the liver and by platelets and the erythromegakaryocytic cell line K562. Major role in cellular iron uptake by internalising the carrier protein transferrin.	(156, 157)	210215_at	2.62
TIMM13	translocase of inner mitochondrial membrane 13 homolog (yeast). Mediates import and insertion of hydrophobic membrane proteins into the mitochondrial inner membrane.		218188_s_at	2.40
SLC39A8	solute carrier family 39 (zinc transporter), member 8		209267_s_at	2.37

Protein biosynthesis

RPL36A	ribosomal protein L36a		201406_at	3.50
HSPB1 (HSP27)	heat shock 27kD protein 1. Associates with α and β tubulin. May functionas a molecular chaperone and in signal transduction pathways.		201841_s_at	2.75
BZW2	basic leucine zipper and W2 domains 2. Translation initiation factor activity.		217809_at	2.41
RPS27L	ribosomal protein S27-like		218007_s_at	2.30
RPL35	ribosomal protein L35		200002_at	2.11
RPS27 (MPS-1)	ribosomal protein S27 (metallopanstimulin 1)		200741_s_at	2.06
RPS6	ribosomal protein S6		200081_s_at	1.90

Enzymatic activity

PRDX2 (NKEFB)	peroxiredoxin 2 (Natural killer cell-enhancing factor B). Major cytosolic factor of red blood cells that enhances NK-cell activity in vitro. May play an important role in the differentiation of erythroid cells. Role in eliminating peroxides generated during metabolism.	(158)	39729_at	3.56
SUCLG2	succinate-CoA ligase, GDP-forming, beta subunit		212459_x_at	3.44
ALDH6A1 (MMSDH)	Aldehyde dehydrogenase 6 family, member A1 (methylmalonate semialdehyde dehydrogenase). Oxidoreductase activity. Mitochondrial enzyme.		221589_s_at	2.19
SHMT2	serine hydroxymethyltransferase 2 (mitochondrial). Interconversion of serine and glycine.		214096_s_at	2.16
AKR7A2 (AFAR)	aldo-keto reductase family 7, member A2 (aflatoxin aldehyde reductase). Detoxification of aldehydes and ketones.		214259_s_at	2.16
FLJ22222	hypothetical protein FLJ22222. Oxidoreductase activity.		53071_s_at	2.10
PHYH	phytanoyl-CoA hydroxylase (Refsum disease). Role in lipid metabolism.		203335_at	1.99
UROD	uroporphyrinogen decarboxylase. Involved in haem biosynthetic pathway; highly expressed in erythroid cells.	(159)	208970_s_at	1.92
CTDSPL (SCP3)	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase-like. Negatively regulates RNA polymerase II activity.	(160)	201906_s_at	1.72
COX7A2L	cytochrome c oxidase subunit VIIa polypeptide 2 like.		201256_at	1.67
CAT	Catalase. Serves to protect cells from the toxic effects of hydrogen peroxide by promoting its conversion to water and molecular oxygen.		201432_at	1.64

Enzyme regulator activity

SERPINE2 (nexin)	serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2		212190_at	3.27

Cytoskeleton

KIAA1102	KIAA1102 protein. Actin binding protein.		212328_at	4.52
PLS3 (T-plastin)	plastin 3 (T isoform). Actin binding protein. Highly expressed in actively dividing cells.		201215_at	3.21
MARCKS	myristoylated alanine-rich protein kinase C substrate. Actin crosslinking protein. Involved in leukocyte motility.		201670_s_at	2.39
ACTR2 (ARP2)	ARP2 actin-related protein 2 homolog (yeast). Implicated in the control of actin polymerisation.		200727_s_at	2.23
ACTN1	actinin, alpha 1. Actin binding protein.		208636_at	2.13

Miscellaneous

|				
DLK1 (Pref-1)	delta-like 1 homolog (Drosophila). Transmembrane protein homologous to the Notch ligands. Inhibits Notch activation. Expressed on thymic epithelial cells and fetal stromal cells that maintain repopulation activity of HSCs in vitro. Expression increases during megakaryocytic differentiation of CD34$^+$ HSCs and after ectopic expression of the myeloid cell nuclear differentiation antigen.	(161-165)	209560_s_at	5.21
CFH	complement factor H. Serum glycoprotein that controls the function of the alternative complement pathway.		213800_at	3.52
TMEM45A	transmembrane protein 45A		219410_at	3.25
TJP2	tight junction protein 2 (zona occludens 2). Involved in the organization of epithelial and endothelial intercellular junctions.		202085_at	2.70
KLHL7 (KLHL6)	kelch-like 7 (Drosophila)		220239_at	2.63
CRYGD	crystallin, gamma D. Dominant structural component of vertebrate eye lens.		207532_at	2.59
TRA1 (GP96)	tumor rejection antigen (gp96) 1. Molecular chaperone that functions in the processing and transport of secreted proteins.		200598_s_at	2.41
CFHL1; CFH	complement factor H-related protein 1; complement factor H. Secreted plasma protein synthesized primarily by hepatocytes.	(166)	215388_s_at	2.36
HSPA1A (HSP70-1)	heat shock 70kD protein 1A. Likely involved in regulation of cell growth. Mediates the folding of newly translated polypeptides.	(167)	200799_at	2.35
FHL2 (DRAL)	four and a half LIM domains 2. Transcriptional co-activator which translocates to the nucleus upon activation of the Rho GTPase signalling pathway. Enhances the transcriptional activation of Wnt-responsive genes by β-catenin. Associates with PS2 in vitro.	(168-170)	202949_s_at	2.27
TMEM14A	transmembrane protein 14A		218477_at	2.14
C1QBP	complement component 1, q subcomponent binding protein. First component of pathway of complement activation.		214214_s_at	2.04
LAPTM4B	lysosomal associated protein transmembrane 4 beta. Overexpressed in several cancers, thus most likely involved in cell proliferation.	(171-173)	214039_s_at	1.72

Not annotated

BEX1	brain expressed, X-linked 1. Nuclear protein. Expressed in acute myeloid leukemias.	(174)	218332_at	6.12
PRG1 (Serglycin)	proteoglycan 1, secretory granule. Peptide core of the proteoglycan in the secretory granules of promyelocytic leukemic HL-60 cells.	(175)	201859_at	3.76
FLJ22746	hypothetical protein FLJ22746		220637_at	3.39
C11orf10	chromosome 11 open reading frame 10		218213_s_at	2.20
DREV1	DORA reverse strand protein 1. Highly expressed in CD33$^+$ and CD14$^+$ cells (SymAtlas). Coded by the opposite strand of the DORA gene such that DORA is in an intron of the DREV1 gene. DORA is expressed uniquely in cells of the immune system, particularly in macrophages.	(176)	217868_s_at	2.00
C20orf67 (PCIF1)	chromosome 20 open reading frame 67. Phosphorylated CTD-interacting factor 1. Interacts with the phosphorilated C-terminal domain of the RNA polymerase II largest subunit. May play a role in mRNA synthesis.	(177)	222044_at	1.91
PMAIP1 (APR)	phorbol-12-myristate-13-acetate-induced protein 1. Essential mediator of p53-dependent apoptosis. Highly expressed in adult T-cell leukemia cell line.		204285_s_at	1.84
GABARAPL1	GABA(A) receptor-associated protein like 1. Estrogen-regulated protein. Promotes tubulin assembly and microtubule bundling.		211458_s_at	1.81
TXNIP (VDUP1)	thioredoxin interacting protein. Regulator of the cellular redox status, possesses tumor suppressive activity and would act as a transcriptional repressor. Would be critical for the development and function of NK cells.	(178-180)	201008_s_at	1.79

The differentially expressed genes were also imported into Ingenuity Pathways Analysis software to identify functional relationships between genes based on known interactions in the literature (see Table 3 and Figure 7A). Interestingly, the biological function 'Hematological system development and function' is associated with three of the four highest ranked biological networks. Canonical signalling pathways associated with the differentially expressed genes are shown in Figure 7B.

Table 3. Biological networks generated by Ingenuity Pathway Analysis. Genes in bold are differentially expressed in the microarray analysis. Only networks with a significance score of 3 or higher ($P \le 0.001$) are shown. The first network is shown in Figure 7.

| Network	Genes in network	Score	Top Functions
1	**ADA**, **BTK**, **C1QBP**, **CAT**, **CEBPB**, **CXCR4**, **EVI1**, **FHL2**, **GADD45A**, H2-D1, **HSPA1A**, **HSPB1**, **IFI16**, IFITM3, **IL1B**, **IRF8**, LR8, **MAL**, MIA, PHLPB, **PMAIP1**, **PRKCB1**, **PTPN11**, **RGS2**, **RPS6**, **RPS6KA2**, **S100A6**, **SERPINE2**, **SMARCA4**, **TCF4**, **TNFAIP2**, **TNFAIP3**, **TNFSF10**, **TRA1**, **TRAF4**	52	Cellular Growth and Proliferation, Cell Death, Hematological System Development and Function
2	CASP10, CDK2, CDKN2B, **CKS2**, CYCS, **DDX21**, FTH1, GADD45G, GSK3A, GTF2I, **HSPB1**, **KLF2**, LGALS3, **LRRFIP1**, **MARCKS**, MYC, **NALP1**, PDCD8, **PNN**, PPIA, PPP1R8, PRDX1, **PRDX2**, **PSEN2**, PTEN, **RPL35**, **RPL36A**, **RPS27**, **SF3B1**, SF3B3, **SHMT2**, TF, **TFR2**, TFRC, TNFRSF8	20	Cellular Function and Maintenance, Small Molecule Biochemistry, Cell Death
3	**ABCA1**, APP, ARG1, **ARHGEF12**, **BZW2**, CD59, **DOCK1**, **EVL**, F13A1, FLOT1, FPRL1, G0S2, G6PD, IL13, ITGAE, KIF5A, KIF5B, KIF5C, **KNS2**, **MN1**, MUC5AC, NHLH1, **PLS3**, PTK2, RELA, **RUNX3**, **SATB1**, **SLC2A5**, **SPON1**, **TAL1**, **TAX1BP3**, TGFB1, **TNFAIP2**, **TNS**, USF2	20	Hematological System Development and Function, Tissue Morphology, Organismal Survival
4	ADCYAP1, AKAP12, ANK2, **BASP1**, **BCL6**, **BST2**, CABP1, CCL6, **CTDSPL**, CYBB, FPRL1, HMGN2, HOXA9, **ICAM4**, **ITGA4**, ITGB7, ITPR1, KLF6, MADCAM1, **MCM3AP**, **NCOR2**, ORM1, **RAB31**, **RALBP1**, RB1, **RBBP6**, RPSA, **SCN3A**, SERPINB9, SKIIP, SLC1A3, SLC8A1, TNF, **TPR**, **UBR2**	17	Cellular Movement, Hematological System Development and Function, Immune Response
5	ARF1, ARF5, **ATP1B1**, **ATR**, BCL3, BLM, **CHD3**, CHEK1, CHEK2, EEF1E1, **IFI16**, **IGHM**, ING1, **IQSEC1**, **KIAA0992**, **KNTC1**, MSN, NBS1, **NDN**, **PHYH**, **PIP5K1B**, PLK1, **PSCD4**, **RAB14**, RAD17, RDX, RPA1, RPA2, RXRB, SMARCB1, **THRAP2**, THRB, TP53, VIL2, WRN	17	Cell Cycle, DNA Replication, Recombination, and Repair, Cancer
6	**CKS2**, **DLK1**, E2F4, EGFR, **ELF1**, EXOSC5, GADD45B, **HMGB3**, KITLG, KLF6, LEP, **LSM5**, **MACF1**, MAPK8, **NFIB**, PLSCR1, **PRG1**, RIPK4, **SERPINE2**, **SFPQ**, SFTPC, SKI, SMAD4, SMURF2, SNRP70, TGFB1, TNFRSF17, TOP1, TPM1, **TRA1**, **TRAF5**, **TRIM33**, **TXNIP**, USF2, WAP	17	Cancer, Cellular Growth and Proliferation, Gastrointestinal Disease
7	**ACTN1**, **ACTR2**, ACTR3, **ADAM28**, ANP32A, ARHGAP5, CDC42, CDH5, CDK5R2, **COX7A2L**, **CRYGD**, DAF, EGF, **EIF4A1**, **ETV5**, F2, G6PD, HGFAC, HNRPL, **ITSN2**, KHDRBS1, **KHDRBS3**, **MEF2A**, MKNK1, **NACA**, **NF1**, NR4A3, PDCD4, RASGRP3, SET, **SETBP1**, SIM1, THBD, VEGF, WAS	15	Cell-To-Cell Signaling and Interaction, Cellular Assembly and Organization, Cell Morphology
8	AGT, AMBP, C3, **CCR9**, CD209, **CFH**, CLDN1, EDNRA, ERG, **GRK5**, **HLX1**, **HNRPA1**, HSD11B1, **ICAM2**, IFIT1, IGL@, IL4, IL15, ITIH1, ITIH2, ITIH3, **ITIH4**, ITIH5, JUN, LSP1, **MAFF**, MST1, MYH7, NFE2, **NPR3**, **OXT**, **RNPC2**, TBX21, **TJP2**, **TNS**	15	Molecular Transport, Cellular Movement, Cell-To-Cell Signaling and Interaction

A.
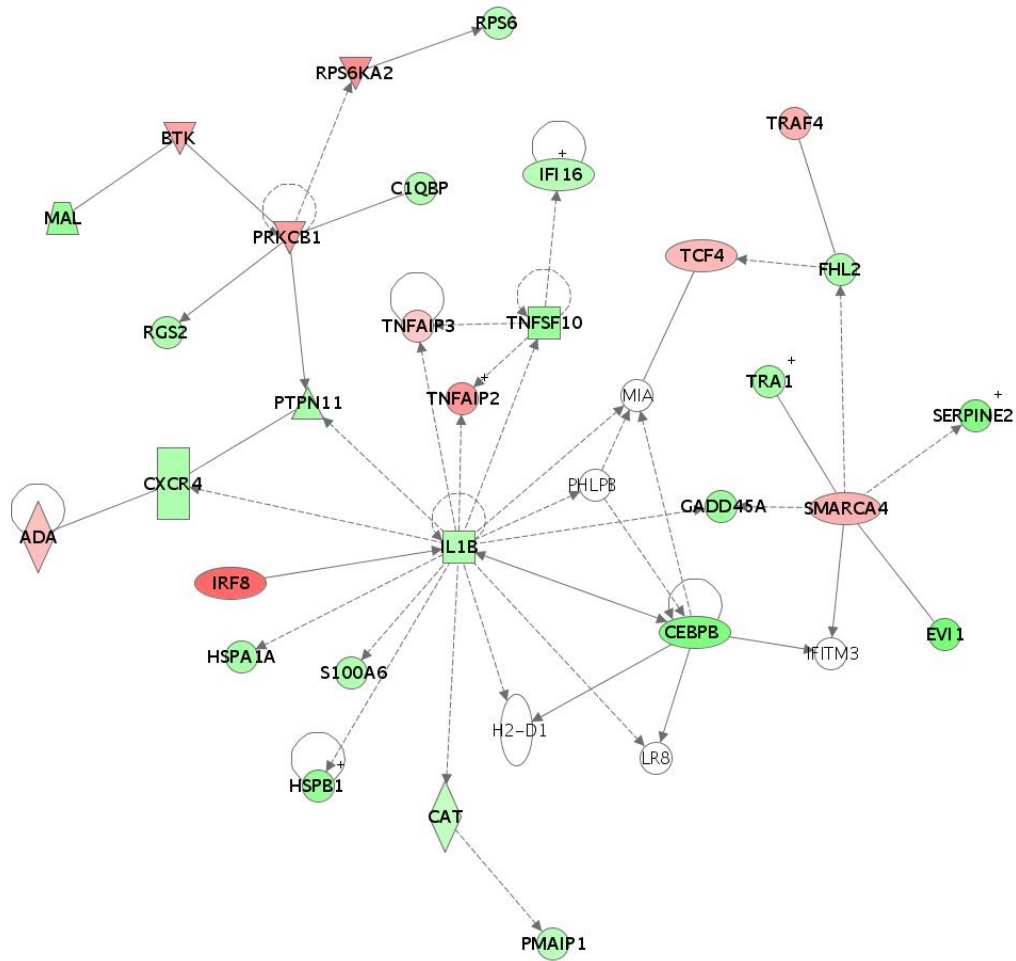
B.
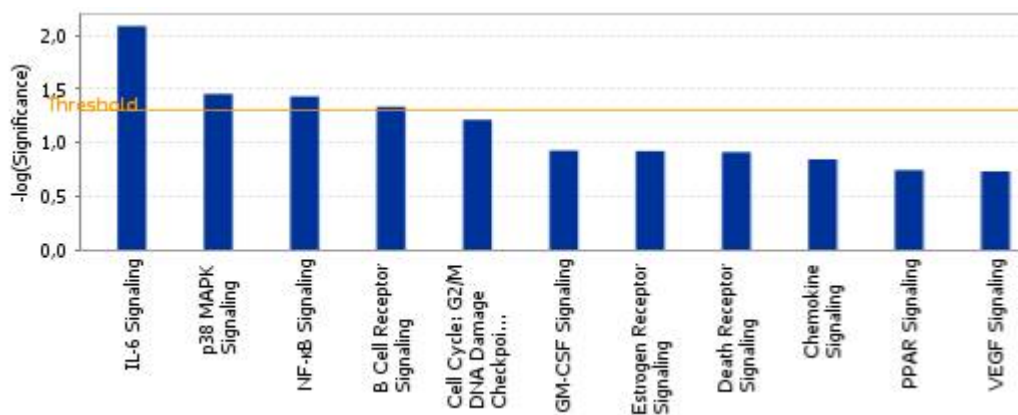
Figure 7. Data analysis using Ingenuity Pathways Analysis. (A) Biological network containing 30 differentially expressed genes (Network 1 from table 3). (B) Canonical signaling pathways associated with the differentially expressed genes. The probability that the association between genes and a pathway is explained by chance alone is indicated on the Y-axis.

Next, we did an extensive literature survey for the differentially expressed genes. The gathered gene information is added to tables 1 and 2. We found that about one third of both upregulated and downregulated genes has been reported to be expressed in hematopoietic tissue. These genes are underlined in tables 1 and 2. Remarkably, more than 75% of the differentially expressed transcription factors have been involved in hematopoiesis. In addition, many differentially expressed genes are involved in cell proliferation, gene expression regulation, cytoskeleton regulation and protein degradation. In the following paragraphs, we discuss these genes in more detail.

Genes involved in hematopoiesis

Several genes that were significantly upregulated in the CD7$^+$ population play an essential role in lymphoid development. ***IGHM***, ***BTK*** and ***PRKCB1*** are essential for B-cell development, ***SMARCA4*** and ***SATB1*** are essential for T-cell development and ***TCF4*** (alias ***E2-2***) is required for both B- and T-cell development. Mutations in ***ADA*** lead to human SCID disease, characterized by a deficiency of both B and T cells. ***BCL6*** is essential for the differentiation of germinal center B cells. Interestingly, *BCL6* was recently shown to be a direct target of transcriptional activation by **IRF8** (alias **ICSBP1**) (17), which is also highly upregulated in the CD7$^+$ population. **MCM3AP**, a protein that is associated with MCM3 of the DNA replication complex, is also specifically upregulated in germinal center B cells (18, 19). The fact that LYN-deficient mice have impaired development of germinal centers in spleen and have decreased expression of MCM3AP (20), creates a link between MCM3AP and LYN, which is non-significantly upregulated in the CD7$^+$ population.

Other genes with a known function in lymphoid cells include the chemokine receptor ***CCR9*** and the trancription factors ***MEF2A*** and ***RUNX3***. Interestingly, RUNX3 was recently shown to transactivate the promoter of ***ITGA4*** (alias ***CD49D***) (21), which is also significantly upregulated in the CD7$^+$ population. ITGA4 mediates adhesive interactions of HSCs with the extracellular matrix of the BM stroma, which are essential for normal hematopoiesis and homing of HSCs to the BM (22).

The list of lymphoid-affiliated genes is greatly expanded when also the genes that are non-significantly upregulated in CD7$^+$ cells are considered. These include amongst others the transcription factors *ZNF1A1* (*Ikaros*), *KLF6* and *NFATC3*, the membrane proteins *CD10* (*CALLA*), *LY86* (Lymphocyte Antigen 86) and *LRMP* (Lymphoid Restricted Membrane Protein), the signalling molecules *Lyn*, *SYK* and *ZAP70*, the lymphoid-specific helicase *HELLS* and the early B-cell marker *IGJ* (immunoglobulin J chain). Also *EZH2*, *FUS*,

DCLRE1C, *IGF2R* and *SMCY* are involved in lymphoid development according to Ingenuity software. The expression of these genes might be truly differential, because also *GATA3* and *CD7*, although both differentially expressed as determined by Real-Time PCR, did not pass the stringent significance test of the microarray analysis. The multiple comparison correction of the p-value to reduce the chance of generating false positives also increases the chance of not detecting truly differentially expressed genes.

In addition, many significantly upregulated genes are specifically expressed in lymphoid tissues, suggesting a possible role in lymphoid development. These include ***TRAF4***, ***TNFAIP3***, ***EVL***, ***FLJ13197***, ***BASP1*** and ***ADAM28***. Interestingly, ADAM28 is a ligand for the leukocyte integrin ITGA4 (alias CD49D) (23), which is also significantly upregulated in the CD7$^+$ population (see higher). EVL binds the SH3 domain of LYN (24), which is also upregulated, although not significantly. ***TRAF3IP3***, ***CHD3***, ***ARHGAP25***, ***FLJ22635*** and ***c11orf21*** are also highly expressed in lymphoid tissues according to GeneNote and GeneAtlas.

Several other significantly upregulated genes are expressed in the hematopoietic system, namely ***TRIB2***, ***TNFAIP2***, ***PSCD4***, ***P2RY14***, ***HLX1***, ***EMR2***, ***LRRFIP1***, ***TRIM33***, ***TRAF5*** and ***NALP1***. **SETBP1** binds SET (25), which is disrupted by a translocation in acute undifferentiated leukemia (AUL), and which is also upregulated in CD7$^+$, albeit not significantly. ***BAALC*** is highly expressed by CD34$^+$ BM progenitor cell subsets and is downregulated upon *in vitro* differentiation (26). The oxysterol binding protein ***OSBPL3*** (alias **ORP3**) is highly expressed in the most primitive CD34$^+$CD38$^-$ fraction and is downregulated upon proliferation and differentiation (27). The physiological function of OSBPL3 remains to be elucidated, but since oxysterols are potent inhibitors of cell growth and induce apoptosis in CD34$^+$ cells (28), oxysterols and their binding proteins might have a regulatory role in HSC proliferation and differentiation. Oxysterols are hydroxylated derivatives of cholesterol, and interestingly, a cholesterol transporter, ***ABCA1***, is significantly upregulated in the CD7$^+$ population, supporting the hypothesis that cholesterol and its derivatives play a role in hematopoiesis.

Amongst the significantly downregulated genes a considerable number of genes are affiliated to myeloid differentiation. For instance, the transcription factors ***TAL1*** (alias ***SCL***), ***CEBPB*** (alias ***NF-IL6***), ***EVI1*** and ***IFI16*** are well known for regulating myeloid development. Since IFI16 is also constitutively expressed by lymphoid cells (29, 30), it might also play a role in lymphoid development. It seems plausible that IFI16 expression must be downregulated in the CLP for lymphoid development to take place, but is upregulated again

later in development. **TNFSF10** (alias **TRAIL**) and **RGS2** are also believed to be involved in myeloid differentiation. Expression of the pro-inflammatory cytokine **IL1B** is regulated by the Ets domain transcription factor PU.1/Spi-1, which is constitutively expressed in monocytic cells (31). The *IL1B* promoter also contains a binding site for CEBPB that would be important for maximal transcriptional activity (31). The lower expression of *IL1B* in the CD7$^+$ population therefore could be the direct result of the 3.4-fold decrease in *CEBPB* expression.

Other myeloid cell-specific genes include **PRG1**, **UROD**, **PRDX2** and **ICAM4**. Also **DLK1** (alias **Pref-1**) might be involved in myeloid development, as *DLK1* is specifically upregulated after ectopic expression of the myeloid cell nuclear differentiation antigen (*MNDA*) in K562 cells (32). In addition, levels of *DLK1* mRNA would increase markedly during megakaryocytic differentiation of CD34$^+$ HSCs (33). DLK1 is a transmembrane protein homologous to the Delta/Serrate ligands of Notch. Although it lacks the characteristic DSL motif necessary for Notch binding, DLK1 interacts with Notch and inhibits Notch activation, as shown by the decrease in HES-1 expression (34). Therefore, it is also possible that the downregulation of *DLK1* in the CD7$^+$ population correlates with increased Notch-signalling. However, *HES-1* or other downstream target genes of Notch are not differentially expressed between CD7$^+$ and CD7$^-$ cells. DLK1 is also expressed on the surface of fetal stromal cell lines that maintain repopulation activity of hematopoietic precursors *in vitro* (35). DLK1, either added in soluble form or expressed on stromal cells, promotes the formation of 'cobblestone areas' of proliferation, which contain both primitive high-proliferative progenitors and stem cells with repopulation capacity (35). DLK1 is also expressed on the surface of thymic epithelial cells (36). **DREV1** is coded by the opposite strand of the *DORA* gene in such way that *DORA* is embedded in an intron of the *DREV1* gene (37). Interestingly, DORA is expressed uniquely in cells of the immune system, particularly in macrophages.

In addition, several downregulated genes are expressed in myeloid leukemias, suggesting a possible role in myeloid development. These include **MLLT3**, **BEX1**, **ARHGEF12**, **PTPN11** (alias **SHP2**), **MN1** and **S100A6** (alias **Calcyclin**).

Several other significantly downregulated genes are expressed in the hematopoietic system, some of them also in the lymphoid lineage. As mentioned before for IFI16, it is possible that the expression of these genes needs to be downregulated in the progenitor stage for lymphoid differentiation to take place, and their expression might be upregulated in later stages of lymphoid development. For instance, **HMGB3** is highly expressed in murine BM erythroid cells, in HSCs, and in most CLP and CMP cells. Enforced expression of *HMGB3* in

BM cells inhibits both myeloid and B-cell differentiation, suggesting that endogenous *HMGB3* expression levels must drop to allow myeloid and B-cell differentiation (38). This category of genes also includes the transcription factors **KLF2**, **ETV5** and **ELF-1** and the T-cell differentiation protein **MAL**. Also the T-cell specific transcription factor **GATA3** belongs to this category. *GATA3* is essential for T-cell differentiation (39) and is expressed in HSC, CLP and pro-T and pre-T cells, but not in B-cell progenitors or myeloid progenitors (2). *GATA3* is clearly downregulated in the CD7$^+$ population according to our Real-Time PCR data (see Figure 5), although the downregulation detected by the microarray was not statistically significant. The neuropeptide **OXT** (oxytocin) is normally produced by thymic epithelial cells (40), while its corresponding receptor is expressed on thymocytes (41). Oxytocin signalling would be involved in the control of T-cell proliferation and survival (41). Interestingly, the transcription factor **MAFF**, which is also strongly downregulated in the CD7$^+$ population, was shown to bind the promoter of the oxytocin receptor gene (42).

Several of the downregulated genes might be associated with HSC function, and their downregulation might therefore be correlated with the onset of differentiation. For instance, long-term repopulating activity of HSCs was shown to be contained to the HSC fraction expressing **HMGB3** (38), and consequently, LT-HSCs were recently shown to express higher levels of *HMGB3* (43). Also **NDN** and **MLLT3** showed higher expression in LT-HSCs (43). Elimination of reactive oxygen species by catalase (**CAT**) was recently shown to be required for HSC self-renewal (44) and accordingly, *CAT* was upregulated in LT-HSC (43). The downregulation of other transcripts implicated in oxidoreductase activity (**PRDX2**, **ALDH6A1**, **AKR7A2** and **FLJ22222**) in the CD7$^+$ population might also correlate with loss of self-renewal.

Also expressed in the hematopoietic system are **ICAM2**, **BST2**, **TXNIP**, **CXCR4** and **LEPR**. Interestingly, leptin binding leads to recruitment and phosphorylation of the SH2 domain-containing tyrosine phosphatase **PTPN11** (alias **SHP-2**) (45), which is also downregulated in the CD7$^+$ population (see above). LEPR and PTPN11 therefore might be components of a single signalling pathway in HSCs that is attenuated upon lymphoid commitment. **PSEN2**, a critical component of the γ-secretase complex that is responsible for the proteolytic cleavage of the Notch receptor after ligand binding (46), is also downregulated in the CD7$^+$ population. Interestingly, PSEN2 was shown to interact with the transcriptional co-activator **FHL2** (47), which is also downregulated.

Genes involved in proliferation and apoptosis

The list of upregulated genes contains many genes known to act as negative regulators of cell proliferation, for instance *LPIN1* (*Lipin1*), *MCM3AP*, *ATR*, *POLS*, *SMARCA4*, *MACF1*, *KNTC1*, *RBBP6*, *TRIB2* and *NF1*. Murine Nf1$^{-/-}$ fetal liver cells are hypersensitive to GM-CSF (48) and generate a JMML-like phenotype in recipient mice (49). Therefore, upregulation of *NF1* in CLP could function to make these cells less sensitive to GM-CSF.

Many downregulated genes are positive regulators of cell proliferation. These include *LEPR*, *IFI16*, *S100A6* (alias *Calcyclin*), *CKS2*, *LAPTM4B*, *HSPA1A* and several ribosomal proteins such as *RPL35*, *RPS27*, *RPS27L*, *RPS6* and *RPL36A*, the latter whose overexpression was shown to increase cell proliferation (50). A decrease in the expression of mRNAs that encode ribosomal proteins accompanies shut-off of cell division (51). However, some downregulated genes are negative regulators of cell proliferation, for instance *NDN*, *KHDRBS3* (alias *T-STAR*), *TXNIP* and *GADD45A.*

In addition, several genes involved in apoptosis were differentially expressed: *NALP1*, *RERE*, *TRAF4* and *TRAF5* were upregulated, while *PMAIP1* was downregulated. Overall, this expression pattern of positive and negative regulators of proliferation tends to point to an intrinsic lower proliferative capacity of the CD7$^+$ population compared to the CD7$^-$ population.

Genes involved in transcription/translation regulation

In addition to transcription factors, also many other proteins involved in gene expression were differentially expressed. The list of significantly upregulated genes contains for instance many genes involved in splicing, such as *CCNL1*, *SFPQ*, *SF3B1*, *RNPC2*, *PNN* and *METTL3*, which is also involved in posttranscriptional modification by methylating adenosine residues of some mRNAs. *HNRPA1* is involved in splice site selection and is responsible for the transport of mRNA from the nucleus to the cytoplasm. *EIF4A1* is a translation initiation factor, and *NACA* is required for the intracellular translocation of newly synthesized polypeptides. *TPR* is implicated in the import of proteins into the nucleus. *SLC38A1* is an amino acid transporter. The RNA helicase *DDX21* is involved in ribosomal RNA synthesis. Several other splicing factors and proteins involved in protein biosynthesis are present in the list of non-significantly upregulated genes.

These types of genes are also found amongst the significantly downregulated genes. *C20orf67* may play a role in mRNA synthesis (52), while *LSM5* and *KHDRBS3* (alias *T-STAR*) are involved in pre-mRNA splicing. *BZW2* has translation initiation factor activity

and ***HSPA1A*** mediates the folding of newly translated polypeptides. ***CTDSPL*** (alias ***SCP3***), belongs to a family of 3 closely related small CTD phosphatases that dephosphorylate Ser residue 5 in the C-terminal domain (CTD) of the largest subunit of RNA polymerase II and thus negatively regulate RNA polymerase II activity (53). SCP1 is expressed in all tissues but the brain and functions to silence the expression of neuronal genes in non-neuronal tissues. Upon neuronal differentiation of murine ES cells, the expression of SCP1 is downregulated (54). It would be interesting to find out whether SCP3 fulfills an analogous function in suppressing lymphoid-specific genes in non-lymphoid cells.

The differential expression of factors of the transcription/translation machinery might be an important mechanism for regulating lymphoid-specific gene/protein expression in the CD7$^+$ CLP cells. There is evidence that alternative splicing of mRNA, which permits the generation of several proteins from one gene by alternative exon usage, can be modulated in a cell type- or developmental stage-specific way (55). Tissue-specific splicing can be the result of concentration differences of ubiquitously expressed splicing factors, but tissue type- or developmental stage-specific splicing factors have also been described (56).

Genes involved in regulation of the cytoskeleton

Many differentially expressed genes are involved in the regulation of the cytoskeleton. The list of upregulated genes contains for instance ***MACF1***, ***Palladin***, ***TBCD*** and ***DOCK1***. More cytoskeleton-related genes are significantly downregulated: ***PLS3***, ***KIAA1102***, ***TNS1***, ***ACTN1***, ***PIP5K1B***, ***ARHGEF12***, ***MARCKS***, ***ACTR2***, ***HSPB1*** and ***GABARAPL1***. Although little is known about the cytoskeleton of hematopoietic stem and progenitor cells (57), it might be involved in the regulation of differentiation in several ways. The cytoskeleton is a scaffold for various signal transduction pathways (58). In addition, alterations in its structure can lead to the relocation and clustering of certain cytoskeleton-linked surface molecules. Moreover, the cytoskeletal organization can regulate gene expression by controlling the nuclear import of certain transcription factors (59).

Genes involved in protein degradation

A number of genes coding for proteins involved in the ubiquitin-proteasome system of proteolysis, were significantly upregulated: ***TNFAIP3*** (alias ***A20***), ***UBR2***, ***TRIM33*** and ***USP34***. Other proteolytic enzymes that were upregulated are ***ADAM28*** and ***DPEP2***. The list of non-significantly upregulated genes contains much more genes involved in ubiquitination. Proteins degraded by the ubiquitin-proteasome pathway include cyclins and other regulators

of the cell cycle, and transcription factors (60). Protein degradation is thus a mechanism to regulate the concentration of many regulatory proteins.

Genes not expressed in hematopoietic tissue

The list of downregulated genes also contains genes that are normally expressed in non-hematopoietic tissue, for instance **CRYGD**, **CFHL1** and **KHDRBS3** (alias **T-STAR**). These genes were weakly expressed in the CD7$^-$ population, and shut down in the CD7$^+$ population. The expression of these genes in the CD7$^-$ population is in line with the reported 'promiscuous' gene expression in stem cells (61).

Discussion

The CD7$^+$ subpopulation of CD34$^+$CD38$^-$ human cord blood cells was identified by Hao et al. as a primitive common lymphoid progenitor population with the ability to generate B, NK and dendritic cells, but with no potential for myeloid or erythroid differentiation (9). In this paper, we confirmed the lymphoid-restricted differentiation potential of this cell population in a co-culture assay using MS-5 stromal cells. In addition, we showed that this cell population has strong T-cell differentiation potential in hybrid human-mouse FTOC. Therefore, CD34$^+$CD38$^-$CD7$^+$ cord blood cells have full lymphoid differentiation potential and are true CLPs.

Gene expression profiling of CD34$^+$CD38$^-$CD7$^+$ cells and their CD7$^-$ counterparts using Affymetrix oligonucleotide microarrays revealed the differential expression of many transcription factors, cell cycle genes, signal transduction molecules and proteins involved in gene expression and cytoskeleton regulation. Many genes involved in negative regulation of the cell cycle were significantly upregulated in the CD7$^+$ population, while positive regulators were mostly downregulated. Whether CD7$^+$ cells intrinsically have a lower proliferative capacity remains to be determined, as the cytokines used in the MS-5 cocultures support myeloid differentiation, and thus it is not surprising that a lymphoid-restricted progenitor does not perform well in those culture conditions.

Many upregulated genes are lymphoid-affiliated, whereas many downregulated genes are related to the myeloid lineage. This expression pattern is in agreement with gene expression studies on murine hematopoietic stem and progenitor cells, which showed that CLPs express markers of B, T and NK cells but no myeloid markers, and conversely, CMPs express granulocytic/monocytic and megakaryocytic/erythroid markers but no lymphoid markers (61, 62). The low-level 'promiscuous' expression of lineage-specific genes before

commitment to a particular lineage, is referred to as 'lineage priming' (63). Upon commitment to a particular lineage, cells upregulate the appropriate lineage-specific genes, and suppress the inappropriate genes of the alternative lineages. Surprisingly, murine HSCs express many myeloid-affiliated genes but almost no lymphoid-affiliated genes, while their immediate progeny, multipotent progenitors (MPP), exhibit both myeloid and lymphoid promiscuity (61, 62). Moreover, murine HSCs express a lot of genes affiliated to non-hematopoietic tissues (62), which may explain their reported capacity to differentiate into non-hematopoietic cell types (64). Consistent with these data, our list of significantly downregulated genes also contained a number of non-hematopoietic genes.

Our study is the first to describe a global gene expression profile of a human common lymphoid progenitor. In their recent study, Haddad et al. determined the gene expression in the related cord blood population CD34$^+$CD45RA^{hi}CD7$^+$, which probably comprises CD34$^+$CD38$^-$CD7$^+$ cells, because the latter also express CD45RA (9). The Haddad CD34$^+$CD45RA^{hi}CD7$^+$ population displayed strong NK and T cell potential, but also substantial myelo/erythroid potential (65). This is not surprising because most CD34$^+$CD45RA^{hi}CD7$^+$ cells are CD38$^+$ and, as mentioned in the paper by Hao et al. and as we noticed ourselves (data not shown), even low expression of CD38 on CD34$^+$CD7$^+$ cells is sufficient to confer these cells myeloid differentiation potential. CD38 is a marker expressed on more mature progenitors of all lineages. So therefore, the CD34$^+$CD45RA^{hi}CD7$^+$ population is presumably heterogeneous and contains both lymphoid- and myeloid-committed progenitors, which can also express low levels of the CD7 antigen. Accordingly, the Haddad CD7$^+$ population expressed increased levels of the T-cell receptor γ chain (cDNAs *TRG*, *TRGC2* and *TRGV9*), terminal deoxynucleotidyl transferase (*DNTT*), and strikingly many genes that are specifically expressed in myeloid cells: calgranulin A (*S100A8*), the macrophage colony stimulating factor receptor CD115 (*CSF1R*), the myeloid-specific transcription factor CEBP/D (*CEBPD*), and the neutrophil granule proteins lysozyme (*LYZ*), myeloperoxidase (*MPO*), elastase 2 (*ELA2*), azurocidin (*AZU1*), cathepsin G (*CTSG*) and proteinase 3 (*PRTN3*). None of these genes was expressed by our CD34$^+$CD38$^-$CD7$^+$ or CD7$^-$ populations, indicating they are immature and not contaminated with T-cell committed or myeloid cells.

Haddad et al. recently identified an identical CD34^{hi}CD45RA^{hi}CD7$^+$ population in human fetal BM and showed that the most immature CD34^{hi}CD1a$^-$ fetal thymocytes share their surface phenotype, as well as their mRNA expression of *MPO* and germline *TRGV9*, which suggests that the CD34^{hi}CD45RA^{hi}CD7$^+$ fetal BM cells colonize the fetal thymus (66).

Using CFSE labelling, the authors showed that these cells indeed efficiently enter murine thymic lobes in a hanging-drop culture, in contrast to their CD34^{hi}CD45RA^{int}CD7^{-} counterparts. However, the possibility exists that it is actually the CD34^{+}CD38^{-}CD7^{+} subpopulation that are the true thymic immigrants. A direct comparison in FTOC using equal numbers of both cell populations would clarify this issue. In addition to their T-cell differentiation potential, CD34^{hi}CD1a^{-} fetal thymocytes would possess strong NK-cell potential, as well as B- and dendritic cell potential, but would lack granulocyte/macrophage potential (66). Analogously, Weerkamp et al. recently showed that the most immature thymocyte subset (CD34^{+}CD1a^{-}) from the adult human thymus has differentiation potential for B, NK and dendritic cells as well as myeloid and erythroid lineage potential (67), leading to the hypothesis that the human thymus is seeded by a multipotent stem cell-like progenitor instead of a common lymphoid progenitor. Still, the multilineage differentiation capacity of both fetal and adult early thymocytes remains to be shown at the single cell level, thus the possibility exists that, analogously to the corresponding murine DN1 thymocyte stage (CD44^{+}CD25^{-}) (68), the CD34^{+}CD1a^{-} thymic subset is heterogeneous and contains multiple progenitors with different differentiation potential. It also remains elusive whether the DN1 subsets derive from the same thymus-seeding cell or have different ancestors. Therefore, it might be possible that T-committed progenitors in the human thymus derive from CD34^{+}CD38^{-}CD7^{+} thymic immigrants, while the other thymic cell types might derive from other progenitors in the CD34^{hi}CD45RA^{hi}CD7^{+} cord blood population.

Interestingly though, multiple differentially expressed genes in the Haddad study were also differentially expressed in our study. For instance, *IRF8* (*ICSBP1*), *CCR9*, *RAB31*, *TRAF4*, *SCN3A*, *SLC2A5*, *TRIB2*, *KIAA0992* (*Palladin*) and *SPON1* are upregulated in the CD7^{+} population of both studies, and in our study they are amongst the most highly upregulated genes with a fold change of more than 3-fold. *GRK5*, *NPR3*, *TJP2*, *BEX1*, *PSEN2*, *DLK1*, *TNFSF10* (*TRAIL*), *CFH*, *CFHL1*, *HSPB1*, *EVI1*, *MLLT3*, *TAL1*, *LAPTM4B* and *FLJ22746* are downregulated in the CD7^{+} population of both studies. *BEX1* showed the strongest downregulation in our study (6-fold), and *NPR3* and *LAPTM4B* showed high average expression levels. The expression of these genes in hematopoietic tissue has not been documented before, so therefore it would be worthwhile to study these genes in more detail, as they might represent novel stem cell related or myeloid cell related genes.

Supporting a role in hematopoietic differentiation of the differentially expressed genes from our study is the observation that 146 of the 190 significantly differentially expressed genes are also differentially expressed between one or more consecutive differentiation stages

of early human T- and B-cell development (11, 14) (See supplementary table 2). Of those, 80 are differentially expressed during T- and B-cell development, 38 only during T-cell development and 28 only during B-cell development. For many of those genes the differential expression (up- or downregulation) between CD7$^+$ and CD7$^-$ is the same as between cord blood CD34$^+$Lin$^-$ and the most immature T- or B-cell stage (CD34$^+$CD38$^-$CD1$^-$ and pro-B respectively).

In conclusion, our molecular characterization of the human cord blood CD34$^+$CD38$^-$CD7$^+$ common lymphoid progenitor generated plenty of interesting genes for further study. Overexpression and RNA interference of selected genes will learn whether their upregulation or downregulation is critical for the developmental transition of multipotent stem cells to the common lymphoid progenitor stage.

Acknowledgments

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Supplementary Methods

First cycle of amplification

RT-synthesis I (RT-I)

Total RNA derived from 100,000 cells was reverse-transcribed in a total volume of 20 µl, which included 10x first strand buffer, 2 µl of 25 mM MgCl$_2$, 2 µl of 0.1 M DTT (both from Invitrogen), 500 ng PAGE-purified oligo-d(T)$_{24}$-T7 primer [5'-GGC CAG TGA ATT GTA ATA CGA CTC ACT ATA GGG AGGCGG 24dT-V-3'] (Microsynth, Balgach, Switzerland), 1 µl dNTPs (10 mM) (Fermentas, St.Leon-Rot, Germany), 1 µl RNase inhibitor (RnaseOUT, 40 U/ml, Invitrogen) and 400 U SuperScript II Reverse Transcriptase (Invitrogen). The mixture of RNA and primer, in a volume of 10 µl, was heated to 70°C for 10 min and chilled on ice before adding buffer, deoxynucleotides, DTT, RNase inhibitor and reverse transcriptase. The reactions were incubated at 42°C for 60 min and chilled on ice. Finally, 1 U RNase H (Invitrogen) was added, followed by an incubation at 37°C for 20 min, a deactivation step at 65°C for 20 min and purification by Microcon YM-50 columns (Millipore). 1 µl of complementary DNA (cDNA) thus generated was used to check the integrity of the starting RNA and the generated cDNA by real-time PCR for the housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT).

Second strand synthesis I (SSS-I)

For each reaction the cDNA generated from RT-I (20 µl) was used and 2 µl (10 µM) HPLC-purified degenerative oligonucleotide primer (DOP) [5' CCG ACT CGA GNN NNN NAT GTG G-3'; N = A,C,G,T] (Hoffmann-La Roche Ltd. Basel. Switzerland) was added. Second strand synthesis was performed in a total volume of 100 µl, which included 10x AmpliTaq Gold Buffer, 6 µl of 25 mM MgCl$_2$ (both from Applied Biosystems, San Jose, CA), 2 µl of 10 mM dNTPs (Fermentas), 2.5 U AmpliTaqGold Polymerase (Applied Biosystems) and HPLC-purified water (Ambion, Cambridgeshire, UK). All cDNA samples were subjected to second strand synthesis under the following conditions using a Master Gradient Thermocycler (Eppendorf, Hamburg, Germany): 95°C for 10 min, 30°C for 10 min, 30 min 30°C to 70°C (3.5°C/2 min 30 s), 15 min 70°C, 4°C for a maximum of 20 min. Finally, a purification step using Microcon YM-50 columns (Millipore) was performed.

Linear T7-RNA in vitro transcription (IVT-I)

The column purified dsDNA in a volume of 8 µl was supplemented with 4 µl of 5x transcription buffer, 1.5 µl of 100 mM GTP, ATP, CTP and UTP, and 2 µl (5000 U) of T7-Polymerase (all from the Ribomax Large Scale RNA production Systems – SP6 and T7 kit, Promega, Leiden, The Netherlands) and linearly transcribed in vitro during 4 h at 37°C. RNA recovery and removal of template DNA was achieved by DNase treatment (RQ1 RNase free Dnase, Promega) and RNeasy purification (Qiagen).

Second cycle of amplification

RT synthesis II (RT-II)

The amplified RNA samples were reverse transcribed into cDNA using 500 ng of random hexamers with 10x first strand buffer, 2 µl of 25 mM $MgCl_2$, 2 µl 0.1 M DTT (all from Invitrogen), 1 µl RNase inhibitor (Invitrogen) and 1 µl of 10 mM dNTPs. The mixture of RNA and primer was heated to 70°C for 10 min and chilled on ice before adding buffer, deoxynucleotides, DTT, RNase inhibitor and 400 U (2 µl) SuperScript II Reverse Transcriptase (Invitrogen). Thereafter, synthesis was continued using an Eppendorf Master Gradient cycler at 25°C for 10 min, 37°C for 60 min, 70°C for 10 min, 4°C for 20 min. 1 µl of cDNA of RT-II was removed to check the integrity by real-time PCR for HPRT. Finally, 1 µl of RNase H (Invitrogen) was added and the cDNA sample was incubated at 37°C for 30 min.

Second strand synthesis II (SSS-II)

The reaction mixture of RT-II (20 µl) was supplemented with 500 ng (1 µl) PAGE-purified oligo-d(T)$_{24}$-T7 primer [5'-GGC CAG TGA ATT GTA ATA CGA CTC ACT ATA GGG AGGCGG 24dT-V-3'] (Microsynth), 10 µl 10x AmpliTaq Gold Buffer, 6 µl of 25 mM $MgCl_2$ (both from Applied Biosystems), 2 µl of 10 mM dNTPs (Fermentas), 2.5 U AmpliTaqGold Polymerase (Applied Biosystems) and HPLC purified water (Ambion) to obtain a total volume of 100 µl. The reaction was subjected to 95°C for 10 min, 37°C for 10 min, 42°C for 10 min, 21 min from 42°C to 70°C (4°C/3 min), 70°C for 10 min and 4°C for a maximum of 20 min. Finally, a purification step using Microcon YM-50 columns (Millipore) was performed.

Linear T7-RNA in vitro transcription II (IVT-II) and labelling

The column-purified dsDNA was transcribed and biotin-labelled using the ENZO BioArray HighYield RNA Transcript Labelling Kit (ENZO, Farmingdale, NY) according to the

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manufacturer's instructions. cRNA recovery and removal of template DNA was achieved by DNase treatment (Promega) and RNeasy purification (Qiagen). Purified amplified RNA was quantified either by absorbance at 260 nm or by using a capillary electrophoresis system (Agilent, Palo Alto, CA).

Supplementary Table 1. Primer sequences

KIAA0125-FW	GTTTGAATTTCTGCACGCTGTT
KIAA0125-RV	CACTCATACACAAAATACCCAGCAT
PRKCB1-FW	TCCAAACTTCCAGAAACTCATCAA
PRKCB1-RV	TGAAGCATTTTGGTATCAGACACA
BAALC-FW	AGAAGGAAATGCAGGGCACAT
BAALC-RV	TGGTTTAACTTCTGGTTGCTGTCT
BASP1-FW	AGCTTTCAGACAGAGCCCACTTA
BASP1-RV	TCTGGAGAGGAAGAATGGAGGAT
RUNX3-FW	CCCTAGGTGGTCTCATAATTCCA
RUNX3-RV	ATCCCTCACCTCAATGCCTTCT
DLK1-FW	GGCACTGTGGGTATCGTCTTC
DLK-RV	AGGTCCTCCCCGCTGTTG
TAL1-FW	ACCCAAACATATGCACATTCACTT
TAL1-RV	ACGCACCCTTGATGACCAAA
HSPA1A-FW	GCCGAGAAGGACGAGTTTGA
HSPA1A-RV	ACCCTGGTACAGTCCGCTGAT
RGS2-FW	ATGGTCCGTGTTTGCATTGTTA
RGS2-RV	CTGCAGTTTTCAACACCATAGCA
LAPTM4B-FW	CTTGTATGCGCTTTTTACCTTGAC
LAPTM4B-RV	CAGGAGAGTTGCTGACTTTGTAACA
GATA3-FW	TGGGCTCTACTACAAGCTTCACAATAT
GATA3-RV	TTGCTAGACATTTTTCGGTTTCTG
HPRT-FW	AGATGGTCAAGGTCGCAAGC
HPRT-RV	GTCAAGGGCATATCCTACAACAAAC

Supplementary Table 2. Significantly differentially expressed genes in human CD34$^+$CD38$^-$CD7$^+$ CLP that are also significantly differentially expressed between 1 or more consecutive differentiation stages of early human T- and B-cell development (Dik et al., 2005 and Van Zelm et al., 2005).

B and T cell development		only T cell development	only B cell development
C11orf21	TRAF5	SLC35E2	PIP5K1B
ATP1B1	RAB14	RERE	CFH
CEBPB	TENS1	TRAF3IP3	KNTC1
PRKCB1	RAB31	ICAM4	CHD3
LRRFIP1	GRK5	MCM3AP	SCN3A
TAX1BP3	SH3TC1	HSPA1A	C1QBP
EIF4A1	ITSN2	TNFSF10	SLC2A5
SPON1	BAALC	DPEP2	JMJD1A
SLC39A8	SMARCA4	TPR	HNRPA1
ITGA4	RUNX3	CCR9	TMEM14A
SLC38A1	KLHL7	ETV5	THRAP2
ACTN1	MAFF	C20orf67	GADD45A
LEPR	BCL6	NUDT4	AKR7A2
SETBP1	BASP1	PSCD4	BZW2
PRDX2	DLK1	MAL	METTL3
TNFAIP3	HLX1	ALDH6A1	POLS
ACTR2	MACF1	FHL2	USP34
P2RY14	SUCLG2	ICAM2	PMAIP1
TRIB2	BTK	TFR2	LPIN1
TAL1	NPR3	SLC33A1	SUV420H1
SF3B1	EMR2	RALBP1	UBR2
HSPB1	CKS2	EVL	MARCKS
GABARAPL1	MLLT3	PLXND1	TJP2
CAT	ADA	RPS6KA2	SHMT2
IQSEC1	OSBPL3	TMEM41B	FLJ22746
SATB1	NALP1	OXT	FLJ13197
IFI16	TNS	KNS2	KIAA0220
ADAM28	NUDT3	DDX21	LOC339287
STK32B	BST2	SERPINE2	
ELF1	IL1B	TRAF4	
TXNIP	RGS2	HMGB3	
EVI1	ATR	ARHGAP25	
KIAA0125	FLJ22635	TRA1	
IGHM	FLJ22222	PSEN2	
TCF4	LOC284262	KIAA0087	
BEX1	KIAA1049	HA-1	
NACA		LOC388388	
CXCR4		EUROIMAGE 362430	
KLF2			
PRG1			
LAPTM4B			
IRF8			
TARBP1			
TBCD			

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General discussion

Bone marrow transplantation or transplantation of hematopoietic stem cells is used to correct hereditary blood diseases and auto-immune diseases and to restore the hematopoietic system when it is destroyed after chemotherapy or radiation treatment for cancer. The bone marrow may be taken from the patient prior to chemotherapy or radiation treatment (autograft), or it may be taken from a donor (allograft). While the myeloid compartment is restored 2 to 4 weeks after transplantation, lymphoid differentiation usually takes much longer. Especially restoration of the T-cell compartment lags behind, creating a period with low T-cell numbers during which the patient is extremely vulnerable to infections. This lag phase could be considerably shortened by adding lymphoid or T-cell committed progenitors to the transplant. Therefore, identification of the molecular regulatory mechanisms involved in the lymphoid and T-lineage decisions might lead to the development of strategies to direct multipotent stem cells to differentiation along the T-cell lineage. Such *in vitro* manipulated stem cells then would differentiate much faster to mature T cells after transplantation.

Numerous murine studies have identified signalling through the Notch-1 transmembrane receptor as a master regulatory mechanism of T-cell commitment. Constitutive Notch-1 signalling, either by retroviral expression of the active intracellular domain of Notch-1 (ICN) or by co-culturing progenitor cells on OP9 stromal cells expressing the Notch ligand Delta-like-1 (OP9-DL1), directs stem cells towards the T-cell fate. In human, Notch-1 signalling has not been studied that extensively yet. Our lab showed earlier that, analogous to the mouse, retroviral transduction of human CD34^{+} hematopoietic precursor cells with ICN blocks B cell development and instead drives them towards the T cell lineage (174). In chapter 1 of this thesis, we show that human CD34^{+} cord blood and adult bone marrow progenitors can efficiently differentiate into mature T cells on the OP9-DL1 cell line, indicating that the 3-dimensional thymic structure is not required for human T-cell development as long as Notch signalling by Delta-like-1 is provided. However, other signals delivered by the OP9 stromal cells are also important, as S17 stromal cells engineered to express Delta-like-1 do not support full T-cell differentiation (159, 176). Our observation that human progenitors transduced with ICN and cultured on MS-5 stromal cells do not differentiate further than immature T/NK progenitors expressing CD7 and intracellular CD3, suggests that MS-5 cells do not support full T-cell differentiation either (174).

The OP9-DL1 culture system offers a number of advantages over the mixed human/mouse fetal thymic organ culture (FTOC) for studying T-cell development *in vitro*. First, no animals need to be sacrificed. Second, low numbers of cells can be efficiently grown in these cultures, making it possible to study the T-cell potential of rare cell populations or even single cells. Moreover, the OP9 coculture system will allow to study the contribution of individual components of the Notch signalling pathway separately. Also, Notch-signalling can be easily turned on and off by transferring developing progenitors from OP9 cells expressing Notch ligands to unmanipulated OP9 cells and back. This will allow to study the role of different Notch ligands during specific stages of thymocyte development.

In addition, the OP9-DL1 culture system might have important therapeutic applications. For instance, *in vitro* cultured T cells could be used to transplant T cell immunodeficient patients. The culture system might also be adapted to culture T cells directed against certain pathogens or against epitopes specifically present on tumor cells. Our demonstration that adult bone marrow progenitors efficiently develop into T cells on the OP9-DL1 cell line is important in this regard, as it enables the use of a patient's own stem cells to differentiate *in vitro* to T cells, avoiding problems with incomplete HLA matching in the case of an allogeneic transplant. However, some important issues need to be addressed before this could be put into practice. First, as it was shown that bone marrow $CD34^+$ cells generate fewer T cells in FTOC with increasing age (177), it remains to be determined whether bone marrow progenitors from older patients differentiate as efficiently to T-cells on OP9-DL1 as the bone marrow progenitors from young children we used in our experiments. Second, although functionally mature murine T cells develop *in vitro* on OP9-DL1, the functionality of *in vitro* developed human T cells still needs to be shown. In addition, positive and negative selection need to be carefully addressed. In theory, positive selection could be achieved *in vitro* by transducing the OP9-DL1 cells with the patient-matched MHC-I and MHC-II molecules. Preventing the development of autoreactive T cells is much more difficult to achieve, as negative selection in the thymus is mediated by the AIRE-induced presentation of a battery of peripheral-tissue antigens by thymic epithelial cells to the developing T cells (178). Therefore the best strategy to restore a deficient T cell compartment and prevent auto-immunity would be to culture stem cells *in vitro* until they are T-committed and then transplant T-committed progenitors that continue their differentiation in the patient's thymus where they undergo positive and negative selection.

Studies with conditional Notch-1 knock-out mice have shown that Notch-1 signalling is essential for T-cell development in the mouse. Creating a similar knock-out phenotype in

humans is of course impossible, therefore, as described in chapter 2, we used an alternative strategy to address whether Notch signalling is essential for human T-cell development. Inhibition of physiological Notch signalling during hybrid human-mouse fetal thymus organ culture (FTOC) by adding the γ-secretase inhibitor DAPT to the culture medium was shown to disrupt T-cell differentiation of human progenitors and to direct them along the B, NK or monocytic-dendritic cell lineages, depending on the degree of Notch inhibition and the differentiation stage of the human progenitors. These experiments indicate that Notch signalling is essential for proper T-cell development of human hematopoietic progenitors. Although the phenotype induced by DAPT recapitulates the phenotype displayed by Notch-1 knock-out mice, it remains uncertain whether Notch-1 is the critical factor for human T cell development, since γ-secretase is involved in the proteolytic cleavage of all 4 Notch receptors and DAPT therefore inhibits signalling through all Notch receptors. Moreover, it is arguable that the effect of the γ-secretase inhibitor is not restricted to inhibition of Notch signalling in the Notch-expressing human hematopoietic cells. Indeed, DAPT could also inhibit proteolytic processing of other important signalling molecules on the surface of hematopoietic cells or thymic epithelial cells. In addition, γ-secretase inhibitors might interfere with the endocytosis of the ligand-bound extracellullar Notch-domain by the signalling cell, which was shown to be important for Noch-signalling in the receiving cell (179). These uncertainties can be ruled out by the use of dominant-negative Mastermind-like (MAML), which does not interfere with Notch receptor activation but inhibits Notch signalling by sequestering ICN and preventing it from activating the CSL transcription factor (146). Still, dominant-negative MAML also inhibits Notch signalling by all 4 Notch receptors. Therefore, to get a definite answer to the question whether signalling through Notch-1 is specifically responsible for directing the T-cell fate of human progenitors, silencing of Notch-1 expression, e.g. by RNA interference, will have to be performed.

Our studies with DAPT indicate that the strength of Notch signalling determines the outcome of differentiation. At intermediate DAPT doses, leaving some residual Notch-signalling, CD34$^+$ cord blood progenitors preferentially developed into NK cells, while at high DAPT doses, inhibiting all Notch-signalling, they mostly developed into B cells. Low doses of inhibitor were sufficient to disturb normal T cell development. These results correlate well with those of Schmitt et al. who studied the differentiation of murine fetal liver-derived hematopoietic progenitor cells on OP9-DL1 stroma in the presence of different concentrations of γ-secretase inhibitor (68). The data of both studies indicate that inhibition of

B lymphopoiesis is already achieved by low levels of Notch signalling, while the induction of T cell differentiation requires high levels of Notch signalling. Quantitative differences in the amount of Notch signalling affecting hematopoietic precursor cell fate outcome was also observed by the group of Bernstein when culturing human precursors with different densities of immobilized extracellular Delta-like-1 protein (180). While lower densities maximally enhanced the generation of CD34$^+$ cells *in vitro* and enhanced their *in vivo* repopulation activity, higher densities of the ligand increased apoptosis of CD34$^+$ precursors and repopulating cells and stimulated lymphoid maturation. These data are important to take into account when developing methods for expanding hematopoietic stem cells *in vitro*, which is an important research goal for improving engraftment of adult patients with umbilical cord blood hematopoietic stem cells. Differences in the strength of Notch signalling could also be the explanation for the observed differential effects on cell fate outcome by activation of Notch signalling by Delta or Jagged ligands (176, 181). Alternatively, Delta and Jagged might transmit qualitatively distinct Notch signals, leading to the activation of distinct downstream target genes. The observations that Notch receptors and ligands are differentially expressed during distinct stages of T cell maturation and in distinct thymic cell compartments (182), and that thymocytes migrate to distinct regions within the thymic microenvironment as they mature (62), suggest that distinct ligand-receptor interactions are important during intrathymic T cell development. Moreover, the strength and duration of Notch signalling can be modulated by a number of molecules that affect ligand binding or modulate intracellular signals. The culture system on OP9-DL1 stromal cells will be a very useful tool to study the role of individual components of the Notch signalling pathway in T cell development.

Because long-term constitutive expression of Notch-1 eventually leads to the development of T-cell tumors (175), manipulation of stem cells with ICN for driving them to the T cell lineage is not an option. Therefore the identification of the downstream effector of Notch-1-induced T-cell commitment could be very useful. The basic helix-loop-helix transcriptional repressor HES-1 was the first target gene of Notch signalling to be identified and studies with HES-1 knock-out mice have shown that its expression is essential during early T-cell development. HES-1 therefore seemed to be a good candidate for mediating the effects of ICN overexpression. However, as described in chapter 3, retroviral expression of HES-1 in CD34$^+$ hematopoietic precursor cells did not recapitulate the phenotype we obtained with ICN overexpression. Although it partly reduced B cell development, HES-1 did not block myeloid differentiation and could not drive progenitors to the T cell lineage. Despite the fact that overexpression of HES-1 shows that it is not sufficient to induce T-cell commitment,

studies with HES-1 knock-out mice have shown that at least in murine fetal T-cell development HES-1 is necessary for the expansion of early T lymphocytes (183, 184). It remains to be investigated whether HES-1 fulfils the same function in adult thymopoiesis. Conditional knock-out mice and/or RNA-interference constructs should be useful tools to solve this question.

We investigated whether the lower B cell numbers obtained by HES-1 overexpression were the result of a block at an immature B-cell stage, and found that this was not the case (unpublished data). Early B-cell progenitors ($CD34^+CD19^+/CD22^+$) were not overrepresented in the MS-5 cultures initiated with HES-1-transduced stem cells, and we found that HES-1-overexpressing cells that are committed to the B-cell lineage go through every stage of normal B-cell development. Interestingly, Zweidler-McKay et al showed recently that Notch signalling induces growth inhibition and apoptosis in a wide variety of malignant murine and human B-cell lines (185). Also, overexpression of HES-1 is sufficient to induce B-cell growth arrest and apoptosis according to these authors. Therefore, the possibility exists that the reduced B-cell development that we observed with ICN/HES-1 overexpression in multipotent hematopoietic progenitors is the result of HES-1-induced growth arrest and/or apoptosis of developing pre-B cells. We did not investigate this hypothesis in our study. The mechanism by which HES-1 induces cell growth arrest and apoptosis in B-cell lines was not revealed in the study of Zweidler-McKay et al. However, in a study conducted by Huang et al., HES-1 was identified as a regulator of p53 activity. According to these authors, HES-1 overexpression can induce apoptosis by activating p53 (186). Furthermore, it has been shown that HES-1 mRNA and protein both oscillate in C2C12 myoblasts in a 2-hour cycle after activation of Notch signalling by exposure to X63 myeloma cells expressing the mouse Delta-1 ligand (187). This oscillatory expression is regulated by a negative feedback loop, i.e. HES-1 negatively regulates its own expression by directly binding to its own promoter (188). The role of this 'ultradian clock' is unknown, but one cannot rule out that it also has a function in hematopoiesis and that disturbing the oscillation by constitutive HES-1 expression inhibits differentiation. Data supporting the theory that the HES-1 expression level has to be critically balanced for differentiation to occur, comes from Ross et al., who showed recently that overexpression of HES-1 and siRNA-mediated reduction of HES-1 expression both inhibit adipocyte development (189).

In addition to reducing B cell differentiation, we found that HES-1 overexpression maintained $CD34^+$ cells. These results are in accordance with reports that showed that retroviral transduction of HES-1 in murine and human hematopoietic stem cells preserves

their stem cell phenotype and enhances their *in vivo* reconstitution capacity (190, 191). HES-1 overexpression was also shown to delay the differentiation of 32D mouse myeloid progenitor cells (192) and to inhibit erythroid/megakaryocytic differentiation from murine Lin⁻Sca-1⁺ hematopoietic progenitors (193). Therefore, it should be investigated whether HES-1 is responsible for the reported increased self-renewal obtained by ICN overexpression (151-153) or by stimulation of stem cells with exogenous Delta-like-1 ligand (153). This question could be addressed by inhibiting HES-1 expression in ICN-overexpressing CD34⁺ cells using RNA interference or by using a dominant negative approach. In fact, this approach would reveal which effects of ICN overexpression or Notch ligand binding depend on HES-1 expression.

Inhibition of differentiation is the phenotype that was also observed with HES-1 overexpression in non-hematopoietic cell types. For example, retroviral expression of HES-1 in neural precursors prevents neuronal differentiation (194, 195) by repression of the proneural bHLH transcription factor Mash1 (196). Analogously, HES-1 is believed to inhibit myogenesis by repressing the bHLH transcription factor MyoD (197). HES-1 can repress transcription actively by binding to the N-box and by recruitment of co-repressors such as Groucho/TLE, and passively by forming non-functional heterodimers with positive bHLH factors, preventing them from binding to the E-box (reviewed in (198)).

The identification of genes that are induced or suppressed upon commitment of multipotent stem cells to the lymphoid lineage might also lead to the development of strategies to enhance lymphoid development of stem cells. Our molecular characterization of the CD34⁺CD38⁻CD7⁺ common lymphoid progenitor of human cord blood (see chapter 4) yielded several transcription factors that are differentially expressed compared to multipotent stem cells. Whether one of these represents a real 'master' regulator of lymphopoiesis remains to be determined. Gain-of-function and loss-of-function studies of candidate genes will have to be performed to answer this question. However, some researchers suggest that lymphoid commitment would not occur abruptly, but would be a more gradual and initially reversible process (199). Also in the case of Notch signalling, it has recently been shown that cells that have initiated T-cell specific gene expression in response to Notch signalling are still able to develop into B cells when the Notch activation signal is removed (200). Nonetheless, a considerable number of new or hardly studied genes showed significant differential expression in our microarray analysis and therefore should be worth studying in detail.

A number of differentially expressed genes are related to the Notch signalling pathway. For instance, *DLK1*, which is significantly downregulated in the CD7⁺ population, is a transmembrane protein that binds and inhibits Notch activation. The high expression of

DLK1 in CD7⁻ cells might be a mechanism by which these cells prevent premature activation of their Notch receptor. The downregulation of *DLK1* in CD7⁺ cells might be needed to permit CD7⁺ cells to receive Notch signals and to differentiate into T-cells. On the other hand, *PSEN2*, a component of the γ-secretase complex responsible for the proteolytic cleavage of the intracellular domain of Notch after ligand binding, is also downregulated in the CD7⁺ population, which would correlate with decreased Notch-signalling. The downregulation of *GATA3* in the CD7⁺ population could correlate with lower Notch signalling, as it was shown that *GATA3* is upregulated in response to Notch signalling induced by binding of the Delta-like-1 ligand (200, 201). Also in agreement with decreased Notch signalling in the CD7⁺ population is the upregulation of *NCOR2* (*SMRT*), a co-repressor that is recruited by CBF1 to repress transcription of Notch target genes. Its upregulation in the CD7⁺ population might aid to keep expression of Notch target genes low. Since DLK1 is also expressed on the surface of stromal cell lines that maintain repopulation activity of hematopoietic precursors (202), it could be possible that DLK1 on the surface of CD7⁻ stem cells has a role in stem cell self-renewal. The downregulation of *DLK1* in CD7⁺ cells then correlates with the loss of self-renewal of this cell population. The downregulation of *PSEN2* (and thus Notch signalling) can be reconciled with this hypothesis, for Notch signalling has also been involved in stem cell self-renewal.

Although we confirmed the lymphoid-restricted potential of the human cord blood CD34⁺CD38⁻CD7⁺ common lymphoid progenitor population and additionally showed that they efficiently generate T cells in FTOC, it remains to be determined whether cells with the CD34⁺CD38⁻CD7⁺ phenotype are physiological T-cell precursors. As shown by Porritt et al., artificial *in vitro* culture conditions such as coculture on OP9-DL1 stromal cells can permit T-lymphoid differentiation of cell types that are not conventional T-cell precursors *in vivo* (69). So far, we do not know whether the CD34⁺CD38⁻CD7⁺ subpopulation represents an obligatory intermediate step in the T-lymphoid differentiation cascade. Therefore, before attempts are made to differentiate stem cells *in vitro* into CD34⁺CD38⁻CD7⁺ cells, it is necessary to investigate the thymic homing potential of this cell population in NOD/SCID mouse models.

The power of microarrays is that the expression of tens of thousands of genes is measured simultaneously during a single experiment. However, in order to extract meaningful biological information from this wealth of data, some important issues need to be dealt with. For instance, the reproducibility of microarray experiments is often a problem. First, there is always some normal physiological variation in gene expression in different samples. In

addition, as different samples are hybridized to separate GeneChips, extra variation is introduced, leading to high levels of signal variability. Therefore, proper normalization of gene expression levels across multiple arrays is essential. In order to detect true differentially expressed genes, a microarray experiment is best replicated several times. However, the high cost of Affymetrix oligonucleotide arrays limits the number of replicates that is feasible. In our case, we were also restrained by the rarity of the $CD34^+CD38^-CD7^+$ cord blood population, which required multiple cell sorts for obtaining a sufficient amount of material to hybridize to the chip. Still, the expression values from our two biological repeat experiments showed a high correlation, indicating that the gene expression data we obtained are reliable and reproducible. Validation of the differential gene expression by other methods such as Real-Time PCR is considered 'the gold standard'. However, because of the high number of differentially expressed genes this is impossible to perform for all the differentially expressed genes. We validated the expression of 11 genes by Real-Time PCR on freshly sorted cells, and found that for 10 of these genes, the fold changes obtained by PCR correlated well with those obtained in the microarray analysis. However, in some cases the fold changes detected by microarray analysis are much smaller than those detected by Real-Time PCR and thus not reliable. For instance, while we detected a fold change of 20 for the expression of CD7 between $CD7^+$ and $CD7^-$ cells by Real-Time PCR, the fold change obtained by the microarray analysis was only 2-fold and statistically non-significant. This is one major pitfall of the microarray analysis: in order to reduce the likelihood of obtaining false positives, a very stringent statistical test was used, which has the downside to increase the chance of not detecting genes that are truly differentially expressed. This means that biologically importantly differentially expressed genes might go undetected. Another shortcoming of Affymetrix GeneChip analysis is that probesets on GeneChips are not specific for different splice isoforms of a gene. On the contrary, different isoforms of a gene can be efficiently amplified by PCR using specific primers.

Finally, it is difficult to compare results of microarray analyses conducted by different laboratories, as different algorithms are being used to calculate probeset expression values and different statistical tests are used to determine significant differences.

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Summary

Hematopoiesis is the highly orchestrated process of blood cell formation from a small pool of multipotent hematopoietic stem cells in the bone marrow. The defining properties of hematopoietic stem cells, their self-renewal capacity and multilineage differentiation potential, form the basis for the successful restoration of the hematopoietic system by bone marrow transplantation or hematopoietic stem cell transplantation after chemotherapy or radiation therapy used to treat cancer. However, due to the slow kinetics of the restoration of the T-cell compartment, patients are temporally vulnerable to infection. Therefore, strategies to enhance T-cell development from HSCs could be of great therapeutic value.

Signalling through the Notch-1 transmembrane receptor has been identified as a critical determinant for the lineage choice between B- and T-cell development. Overexpression of the active form of Notch-1 (ICN) in human CD34$^+$ hematopoietic stem cells blocks B-cell development and drives them into T-lymphoid differentiation. In this work we show that physiological stimulation of the Notch pathway by coculturing human CD34$^+$ progenitor cells on a stromal cell layer ectopically expressing the Notch ligand Delta-like-1 also induces T-cell differentiation of human cells. Inversely, by inhibiting physiological Notch signalling during *in vitro* T-cell differentiation in fetal thymus organ culture using γ-secretase inhibitors we show that Notch signalling is essential for human T-cell development. Because constitutive Notch-1 expression ultimately leads to the development of T-cell leukemias, manipulation of stem cells with ICN cannot be applied clinically. Therefore we investigated whether the Notch-1 target gene HES-1 is able to substitute for Notch-1 signalling in inducing T-cell differentiation of human CD34$^+$ hematopoietic stem cells. Our results demonstrate that overexpression of HES-1 alone is not sufficient to impose T-cell differentiation on human hematopoietic stem cells.

The identification of a small lymphoid-committed cell fraction in human umbilical cord blood may also lead to therapeutic applications. We show that CD34$^+$CD38$^-$CD7$^+$ cells have strong T-cell differentiation potential. To identify genes that regulate the lymphoid commitment step we compared the gene expression between CD34$^+$CD38$^-$CD7$^+$ lymphoid-committed progenitors and CD34$^+$CD38$^-$CD7$^-$ multipotent stem cells using Affymetrix oligonucleotide microarrays. Overexpression and silencing studies of selected differentially expressed genes will have to be performed to determine their role in lymphoid development and whether they can be used to instruct lymphoid development.

Samenvatting

Hematopoiese is het sterk georganiseerde proces van bloedcelvorming uitgaande van een kleine verzameling multipotente hematopoietische stamcellen in het beenmerg. De definiërende eigenschappen van hematopoietische stamcellen, namelijk hun vermogen om zichzelf te vernieuwen en te differentiëren tot alle bloedceltypes, vormen de basis voor het herstel van het hematopoietisch systeem met behulp van beenmergtransplantatie of stamceltransplantatie na de behandeling van kanker met chemotherapie of radiotherapie. Echter, door de trage kinetiek waarmee het T-cel compartiment hersteld wordt, zijn patiënten tijdelijk vatbaar voor infecties. Daarom zouden strategieën om de T-cel ontwikkeling van hematopoietische stamcellen te versnellen van grote therapeutische waarde kunnen zijn.

De Notch-1 signaaltransductieweg werd geïdentificeerd als een kritische determinant voor de keuze tussen B- en T-cel ontwikkeling. Overexpressie van de actieve vorm van Notch-1 (ICN) in menselijke $CD34^+$ hematopoietische stamcellen blokkeert B-cel ontwikkeling en stuurt hen in de richting van T-cel ontwikkeling. In dit werk tonen we aan dat fysiologische stimulatie van de Notch signaaltransductieweg in menselijke $CD34^+$ voorlopercellen door ze te kweken op een stromale cellijn die het Notch ligand Delta-like-1 tot expressie brengt, ook T-cel ontwikkeling induceert. Omgekeerd, door fysiologische Notch signalisatie tijdens *in vitro* T-cel ontwikkeling in fetale thymus orgaan cultuur te verhinderen met behulp van γ-secretase inhibitoren, kunnen we aantonen dat Notch signalisatie essentieel is voor menselijke T-cel ontwikkeling. Omdat constitutieve expressie van Notch-1 uiteindelijk leidt tot de ontwikkeling van T-cel tumoren, is manipulatie van stamcellen met ICN niet klinisch toepasbaar. Daarom onderzochten we of het Notch-1 doelgen HES-1 in staat is om Notch-1 signalisatie te vervangen bij het induceren van T-cel differentiatie van menselijke $CD34^+$ hematopoietische stamcellen. Onze resultaten tonen aan dat overexpressie van HES-1 alleen onvoldoende is om T-cel differentiatie op te leggen aan menselijke hematopoietische stamcellen.

De ontdekking van een kleine celfractie in menselijk navelstrengbloed met differentiatiepotentieel beperkt tot lymphoide celtypes, kan ook leiden tot therapeutische toepassingen. We tonen in dit werk aan dat deze $CD34^+CD38^-CD7^+$ cellen sterk T-cel differentiatiepotentieel bezitten. Om genen te identificeren die lymphoide differentiatie reguleren, vergeleken we de genexpressie tussen $CD34^+CD38^-CD7^+$ lymphoide voorlopers en $CD34^+CD38^-CD7^-$ multipotente stamcellen met behulp van Affymetrix oligonucleotide arrays. Overexpressie en uitschakeling van interessante differentieel geëxpresseerde genen zal

uitgevoerd worden om hun rol in lymphoide ontwikkeling te bepalen en om te testen of ze gebruikt kunnen worden om lymphoide ontwikkeling te sturen.

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'Somehow I made it'

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Publications

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Presentations at congresses and meetings

Joint Wintermeeting Belgian Immunological Society – Dutch Society for Immunology; 18-20 December 2002; Veldhoven, The Netherlands; *Oral presentation*

Annual Meeting of the Belgian Immunological Society (BIS); 28 November 2003; Namur, Belgium; *Poster presentation*

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Keystone Symposium 'T-cell Development'; 10-15 February 2004; Banff, Canada; *Poster presentation*